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Fluorescence Regulation of Poly(thymine)-templated Copper Nanoparticles *via* an Enzyme-triggered Reaction towards Sensitive and Selective Detection of Alkaline Phosphatase

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ABSTRACT: The activity of alkaline phosphatase (ALP) is a crucial index of blood routine examinations, since the concentration of ALP is highly associated with various human diseases. To address the demands of clinical tests, efforts should be made to develop more approaches that can sense ALP in real samples. Recently, we find that fluorescence of poly(30T)-templated copper nanoparticles (CuNPs) can be directly and effectively quenched by pyrophosphate ion (PPi), providing new perspective in designing sensitive biosensors based on DNA-templated CuNPs. In addition, it has been confirmed that phosphate ion (Pi), product of PPi hydrolysis, does not affect the intense fluorescence of CuNPs. Since ALP can specifically hydrolyze PPi into Pi, fluorescence of CuNPs is thus regulated by an ALP-triggered reaction, and a novel ALP biosensor is successfully developed. As a results, ALP is sensitively and selectively quantified with a wide linear range of 6.0×10^{-2} U/L to 6.0×10^{2} U/L and a low detection limit of 3.5×10^{-2} U/L. Besides, two typical inhibitors of ALP are evaluated by this analytical method, and different inhibitory effects are indicated. More importantly, by challenging this biosensor with real human serums, the obtained results get a fine match with the data from clinical tests, and the serum sample from a patient with liver disease is clearly distinguished, suggesting promising applications of this biosensor in clinical diagnosis.

INTRODUCTION

Biomaterials including protein, peptide and DNA, can serve as templates to synthesize numerous nanomaterials.[1] For example, diverse nanoparticles can be synthesized using bovine albumin as building blocks.[2] GSH, a tripeptide, can involve in the formation of the silver nanoparticles and plays an indispensable role in the process as stabilizing agent.[3] In comparison with protein and peptide, DNA is a versatile programmable template that facilitate assemble functionalized nanomaterials, due to its inherent molecular recognition, designable sequence and simple conformation. Accordingly, various DNA-templated nanomaterials reveal prominent properties and are applied in catalyst field,[4] molecular assembly and release,[5,6] logic and sensing devices.[7,8] Owing to its low cost, ease of preparation and strong fluorescence, DNA-templated copper nanoparticles (CuNPs) draw great attention in the realm of DNA-templated nanomaterials. As reported, both single-stranded and double-stranded DNA can be used as templates for copper nanoparticles synthesis.[9,10] Considering that the formation of double-stranded DNA needs extra hybridization event and the efficiency of hybridization is far from satisfactory, single-stranded DNA-templated CuNPs are considered to be more promising in fabricating functional molecular devices, especially biosensors.

Currently, there are two main strategies of designing biosensors based on DNA-templated metal nanomaterials. One is manipulation of raw materials, including DNA template and metal ions,[11,12] and the other is property operation of prepared nanomaterials, such as fluorescence regulation of DNA-templated nanoparticles.[13,14] Manipulation of raw materials, DNA in particular, is an ever changing but indirect manner. To acquire sufficient signals of biosensors, DNA has to suffer from complicated processes to provide suitable templates, while metal ions have to be excessive in order to meet the requirements of nanomaterials synthesis. As a result, it is difficult to ensure the sensitivity of analytical systems by manipulating raw materials. On the other hand, DNA-templated nanomaterials possess salient properties that can be effectively and facilely operated by other molecules. Obviously, operating properties of DNA-templated nanomaterials is a simple and direct manner in developing sensitive approaches, but cannot avoid the drawback of poor selectivity. For example, both sulfide and dopamine can quench the fluorescence of CuNPs,[15,16] thus these two molecules may serve as interferential components for each other in testing. Accordingly, more efforts should be exerted to reconcile the sensitivity and selectivity of DNA-templated nanomaterials-based biosensors.

Alkaline phosphatase (ALP), a biomarker that can dephosphorylate corresponding substrates into inorganic

phosphates,[17] is an important indicator of the health. High activity of ALP is associated with liver and bone-related diseases,[18] while low activity of ALP is the sign of hypophosphatasia and some other diseases.[19,20] As a consequence, up to now, myriads of methodologies in ALP detection have been developed, such as colorimetry,[21,22] fluorescence,[23,24] surface enhanced resonance Raman scattering,[25,26] electrochemistry,[27,28] chemiluminescence,[29] and electrochemiluminescence.[30] Even though the aforementioned approaches have their own superiority, they still encounter inevitable issues. For example, colorimetry is probably not to meet the detective demands of sensitivity. Electrochemiluminescence requires sophisticated operation and long analysis time in spite of its high sensitivity. Considering that activity of ALP is a clinical index, ALP quantitative approaches that are simple, reliable and applicable to serum, become increasingly urgent to us.

We recently find that the fluorescence intensity of CuNPs can be evidently quenched by pyrophosphate ion (PPi), but some of its analogues, such as phosphate ion (Pi), do not have the same ability. This phenomenon provides new insight into direct and effective fluorescence regulation of CuNPs by small molecules, which is very valuable in designing advanced biosensors. Unfortunately, fluorescence quenching of CuNPs by small molecules is not exclusive. Besides PPi, sulfide and dopamine are also competent to this task. To address this issue, we promote the selectivity of an analytical approach by introducing an enzyme-triggered reaction. On a basis of these principles, a novel ALP biosensor is developed using poly(30T)-templated CuNPs as a fluorescent indicator. Experimental results reveal that this biosensor can quantify the activity of ALP sensitively and selectively, evaluate the inhibitors of ALP in detail, and test ALP in human serum samples accurately.

EXPERIMENTAL SECTION

Chemicals and Materials. ALP and bovine serum albumin were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China). Potassium pyrophosphate ($K_4P_2O_7$), dipotassium phosphate (K_2HPO_4), monopotassium phosphate (KH_2PO_4), potassium phosphate (K_3PO_4), 3-(N-Morpholino)propanesulfonic acid (MOPS), copper sulfate ($CuSO_4$), ascorbic acid (Vc), ethylenediaminetetraacetic acid disodium salt (EDTA • 2Na), sodium orthovanadate (Na_3VO_4), cytochrome C and glucose oxidase were purchased from Sigma-Aldrich Co., Ltd (China). 15 human serum samples were obtained from Nanjing Chest Hospital, and used with the approval of the ethical committee of Nanjing Normal University. All other chemicals used in this work were of analytical grade and directly used without additional purification.

Oligonucleotides (poly(30T): 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3') were obtained and purified with HPLC by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China).

Poly(30T) was dissolved in ultrapure water. ALP and human serum samples were diluted with ultrapure water. $CuSO_4$ was dissolved in 10 mM MOPS (pH 7.5) as a stock solution. Other solutions were prepared with ultrapure water (18.2 MΩ cm) from a Milli-Q purification system (Bedford, MA).

Apparatus. The fluorescence measurements were carried out on an Fluoromax-4 spectrometer (Horiba, France). The fluorescence emission spectra of CuNPs were recorded from 500 nm to 800 nm at room temperature with a 340 nm excitation wavelength by a 400 nm optical filter. The slits for excitation and emission were set at 5 nm and 5 nm, respectively. The transmission electron microscopy (TEM) images were obtained on a JEM-200CX instrument (Japan) using an accelerating voltage of 200 kV. The high-resolution transmission electron microscopy (HRTEM) images were recorded on a JEOL-2100F apparatus at an accelerating voltage of 200 kV.

Formation of CuNPs. 30 μL 10 μM poly(30T), 10 μL 1 mM $CuSO_4$, 15 μL 20 mM Vc and 245 μL MOPS (10 mM, pH 7.5) were mixed and allowed to react for about 15 min in the dark at room temperature.

Investigation of Quenching Effect of PPi on CuNPs. 300 μL as-prepared CuNPs reacted with $K_4P_2O_7$, K_2HPO_4, KH_2PO_4 and K_3PO_4 for 15 min in the dark, respectively. Afterwards, fluorescence spectra of samples were recorded.

Construction of ALP-responsive Fluorescent System. First, 2 μL ALP of different final concentrations were incubated with 20 μL 3.5 mM $K_4P_2O_7$ at 37 °C for 90 min. After that, 300 μL as-prepared CuNPs was added into the mixture. Finally, the fluorescence spectrum was recorded at a fixed reaction time of 15 min in the dark. To testify the selectivity of this biosensor, different proteins (ALP, EDTA-treated ALP, heat-treated ALP, bovine serum albumin, cytochrome C and glucose oxidase) with the same concentration were employed. The concentration of purchased ALP is 25.8 mg/mL (6.6×10^5 U/L), and diluted into (258 μg/mL) before usage. Because 2 μL diluted ALP is mixed with 20 μL other solution, the final concentration of ALP was 23.5 μg/mL. Accordingly, other proteins were also decided to be 23.5 μg/mL to keep the same concentration. To obtain EDTA-treated ALP, 1 μL 5 mM EDTA·2Na were mixed with 1 μL 516 μg/mL ALP at 37 °C for 20 min. To obtain heat-treated ALP, ALP was incubated at 100 °C for 10 min.

Evaluation of Inhibitory Effect of Na_3VO_4 and EDTA towards ALP. To evaluate the effect of ALP inhibitor, 1 μL Na_3VO_4 and EDTA·2Na with different concentrations were mixed with 1 μL 1.33×10^4 U/L ALP at 37 °C for 20 min, respectively. Then, 20 μL 3.5 mM $K_4P_2O_7$ was added into the mixture at 37 °C for 90 min. The following detection process was the same as the procedure of ALP detection.

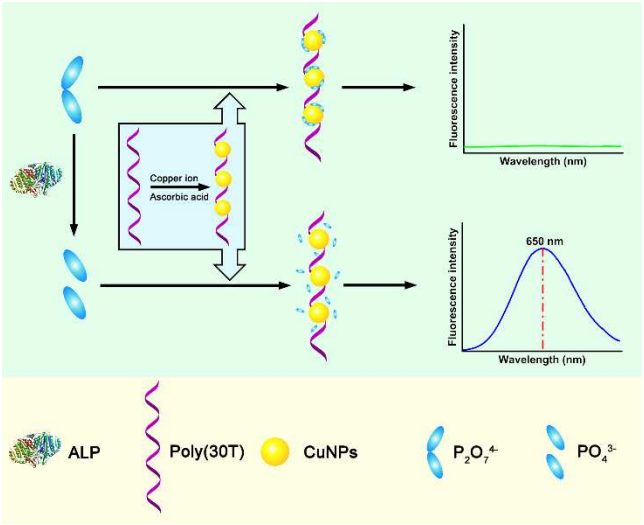
Analysis of ALP in Real Serum Samples. Human blood samples were first coagulated to obtain supernate (serum samples). Then, each serum sample was divided into two parts. One was tested by the clinical method (colorimetric assay), while the other was assayed by our proposed biosensor. Specifically, 2 μL diluted 1% human serum was added with 20 μL 3.5 mM $K_4P_2O_7$ at 37 °C for 90 min. Then, quantitation of ALP was carried out according to the aforementioned procedure.

RESULTS AND DISCUSSION

Principles of a Novel Fluorescent ALP Biosensor. Some molecules can directly react with metal nanoparticles, and thus affect the fluorescence response of metal nanoparticles.[15,16] Inspired by these, we try to explore new molecules that can operate the fluorescence of metal nanoparticles. By accident, we find PPi can quench the fluorescence of CuNPs,

while Pi does not have the similar capacity. Accordingly, by integrating ALP-catalyzed reaction, a novel ALP biosensor is fabricated. Detailed detection procedure has been illustrated in Scheme 1. Using poly(30T) as template, CuNPs with strong fluorescence is conveniently formed. In the absence of ALP, PPi will react with CuNPs and quench the fluorescence of CuNPs. However, in the presence of ALP, PPi will be hydrolyzed by ALP, securing the fluorescence of CuNPs. Therefore, ALP can be quantified by the fluorescence intensity of poly(30T)-templated CuNPs. Remarkably, Zhang and coworkers reported an excellent fluorescent PPi biosensor based on the interaction between PPi and copper ions.[24] Different from that work, this work is designed to sense PPi from the perspective of direct interaction between PPi and CuNPs.

Scheme 1. Schematic illustration of fluorescent ALP biosensor based on poly(30T)-templated CuNPs and an enzyme-triggered reaction



Effects of PPi and its Analogues on the Fluorescence Property of Poly(30T)-templated CuNPs. Fluorescence property of poly(30T)-templated CuNPs is first investigated. Poly(30T)-templated CuNPs possess a typical fluorescence spectrum with a maximum excitation wavelength at 340 nm (Figure 1A). It is reported that the maximum emission wavelengths of double-stranded and single-stranded DNA-templated CuNPs are 570 nm and 615 nm, respectively.[31] According to our results, the maximum emission wavelength of poly(30T)-templated CuNPs is 650 nm, which should be ascribed to the modification of preparation procedure and the difference of DNA template. Having identified the fluorescence behavior of poly(30T)-templated CuNPs, we attempt to operate its fluorescence intensity by PPi and its analogues. As shown in Figure 1B, fluorescence of poly(30T)-templated CuNPs is not affected by HPO_4^{2-}, $H_2PO_4^-$ and Pi, even if the concentrations reach 10 mM. However, distinct effect is observed if PPi is mixed with CuNPs. It can be found that PPi can significantly quench the fluorescence of poly(30T)-templated CuNPs. As the increase of PPi concentration, the fluorescence intensity of CuNPs at 650 nm decreases rapidly and then approaches a plateau. When the concentration of PPi is higher than 220 μM, the fluorescence of CuNPs can be totally quenched. Morphological changes of poly(30T)-templated CuNPs caused by PPi are further characterized by TEM and HRTEM. In the absence of PPi, CuNPs are monodispersed (Figure 1C), and the mean diameter of CuNPs is 2.41 nm (inset of Figure 1C), while aggregation of CuNPs is clearly observed in the presence of PPi (Figure 1D). The phenomenon demonstrates that CuNPs could interact with PPi, rendering the aggregation of CuNPs, which induces the fluorescence quenching of CuNPs.

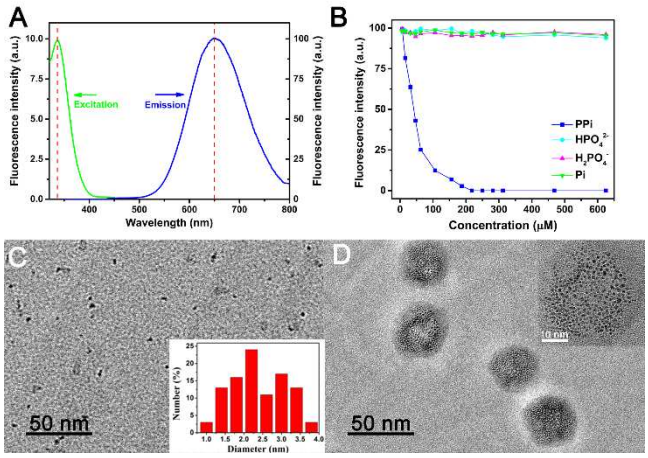


Figure 1. (A) Fluorescence excitation and emission spectra of the poly(30T)-templated CuNPs. (B) Fluorescence intensity of poly(30T)-templated CuNPs at 650 nm with various concentrations of PPi, HPO_4^{2-}, $H_2PO_4^-$ and Pi. (C) TEM image of poly(30T)-templated CuNPs. Inset: Diameter distribution of poly(30T)-templated CuNPs. (D) TEM image of poly(30T)-templated CuNPs in the presence of 220 μM PPi. Inset: HRTEM image of CuNPs in the presence of 220 μM PPi.

Fabrication of ALP Biosensor by Fluorescence Regulation of CuNPs *via* an Enzyme-triggered Reaction. Since PPi and Pi have distinguished effects on the fluorescence response of poly(30T)-templated CuNPs, and ALP can specifically catalyze the hydrolysis of PPi to Pi at physiological pH,[32] it becomes possible to regulate the fluorescence intensity of CuNPs by an ALP-triggered reaction. As shown in Figure 2A, in the presence of PPi, fluorescence intensity of CuNPs is negligible, owing to the quenching effect of PPi on CuNPs. However, if PPi is pre-treated with ALP, intense fluorescence of CuNPs is maintained. Accordingly, taking CuNPs as an indicator and employing ALP-triggered reaction, a fluorescent ALP biosensor is developed by us.

Incubation time of ALP and PPi reaction is optimized to improve performance of this biosensor. The fluorescence intensity of CuNPs increases in the first 90 min, and then remains unchanged, when the incubation time is prolonged up to 150 min (Figure S1). Therefore, 90 min is chosen as the optimal incubation time for the following studies. Under the optimized conditions, the dependence of emission intensity at 650 nm ($F_{650\ nm}$) on the concentration of ALP (C_{ALP}) is further investigated. As the concentration of ALP increases from 6.0×10^{-2} U/L to 3000 U/L, more PPi is consumed, avoiding the quenching effect of PPi and thus preserving fluorescence of CuNPs (Figure 2B). A linear dependence between $F_{650\ nm}$ and C_{ALP} in the range of 6.0×10^{-2} U/L to 6.0×10^{2} U/L is obtained (Figure 2C). The linear regression equation is $F_{650\ nm} = 1.99 + 0.157C_{ALP}$ (U/L) with a correlation coefficient of 0.996. The calculated detection limit is 3.5×10^{-2} U/L (S/N = 3). In comparison with previously reported ALP biosensor (Table S1), our biosensor reveals superior analytical performance

because of its wide linear range and low detection limit. Considering that the concentration of ALP in human serum is normally in the range of 20 U/L to 140 U/L, this biosensor is suitable for routine tests.

To verify the selectivity of this analytical approach, the proposed fluorescent ALP biosensor is challenged with some interferential proteins including EDTA-treated ALP, heat-treated ALP, bovine serum albumin, cytochrome C, and glucose oxidase. As shown in Figure 2D, only ALP can significantly increase fluorescence of CuNPs, and the fluorescence intensity of ALP is at least 50 times larger than those of interferential proteins. The outstanding selectivity of this biosensor should be attributed to high efficiency and specificity of an enzyme-catalyzed reaction. That is, except ALP, other interferential proteins cannot catalyze the hydrolysis of PPi, and thus have no impact on the quenching of PPi to CuNPs. As there are complex proteins in human serum, improved selectivity by employing an enzyme-triggered reaction is very valuable in clinical applications.

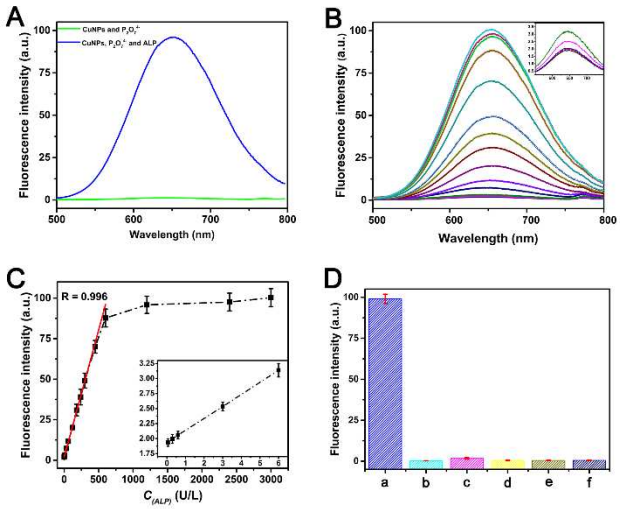


Figure 2. (A) Fluorescence spectra of poly(30T)-templated CuNPs added into 3.2 mM PPi with and without 600 U/L ALP. (B) Fluorescence spectra of poly(30T)-templated CuNPs added with 3.2 mM PPi in the presence of various concentrations of ALP (from 6.0×10^{-2} to 3000 U/L). Inset: Regionally enlarged drawing with the concentrations of ALP from 6.0×10^{-2} to 6.0 U/L. (C) The dependence of fluorescence intensity on the concentration of ALP. Inset: The calibration curve with the concentrations of ALP from 6.0×10^{-2} to 6.0 U/L. (D) Fluorescence responses of poly(30T)-templated CuNPs added with 3.2 mM PPi in the presence of (a) ALP, (b) EDTA-treated ALP, (c) heat-treated ALP, (d) bovine serum albumin, (e) cytochrome C, and (f) glucose oxidase. The concentration of different proteins is 23.5 μg/mL.

Inhibition Assay. Investigations on the inhibitor of enzyme are of great importance in drug design.[33] Besides the ability of ALP detection, this biosensor can also be used to evaluate inhibitors of ALP. It is reported that ALP has two types of inhibitors.[34] In particular, the inhibition of Na_3VO_4 to ALP is reversible and competitive, while the inhibition of EDTA is irreversible and noncompetitive. Therefore, Na_3VO_4 and EDTA are chosen as two typical inhibitors to verify the applicability of inhibition assay. According to the results presented in Figure 3, it can be found that both Na_3VO_4 and EDTA can remarkably inhibit the activity of ALP, and Na_3VO_4 is more effective than EDTA in inhibiting the activity of ALP. Also, in comparison with fluorescence intensity without inhibitor, slight inhibition caused by low concentration of inhibitors (500 pM) can be precisely sensed by this biosensor. In addition, different inhibition curves of Na_3VO_4 and EDTA reflect different inhibition mechanisms, which is in accordance with previous studies.[34] These experimental results demonstrate that the approach is capable to depict the inhibitory effect on ALP activity in detail.

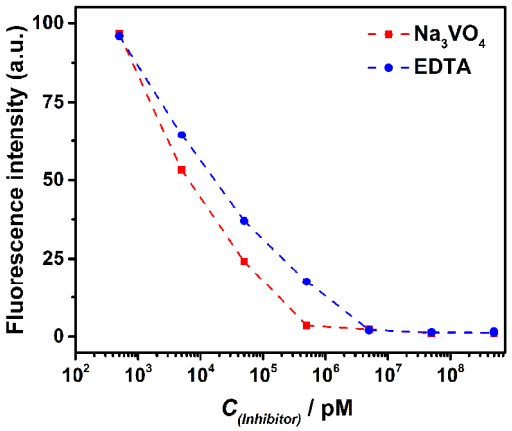


Figure 3. The dependence of fluorescence intensity of ALP-responsive system on the concentration of Na_3VO_4 and EDTA.

Quantitation of ALP Activity in Human Serum Samples. Since ALP is an essential parameter to indicate the condition of our body, various assays have been established to measure the ALP activity in serum. As far as we know, colorimetric ALP assay is currently considered as a "gold" method that has been used in most hospitals. Having identified its satisfactory analytical performances, we attempt to challenge the proposed biosensor with human serum samples. In order to ensure the accuracy and reliability of the results, 15 human serum samples are selected independently and randomly. Then, these samples are detected by our biosensors and clinical test. As shown in Figure 4, the activity of ALP scatters in a wide range from less than 50 U/L to more than 400 U/L. It is noteworthy that "serum 13" is donated by a patient with liver disease, which can be accurately distinguished by our biosensor with high value of detected ALP activity. More importantly, the results obtained from this fluorescent biosensor are highly consistent with the values from clinical tests. To our knowledge, 50 μL serum is necessary in clinical ALP test. However, the amount of serum required by this biosensor is much less (2 μL). Therefore, we believe this biosensor might be expediently developed to be a practical analytical platform.

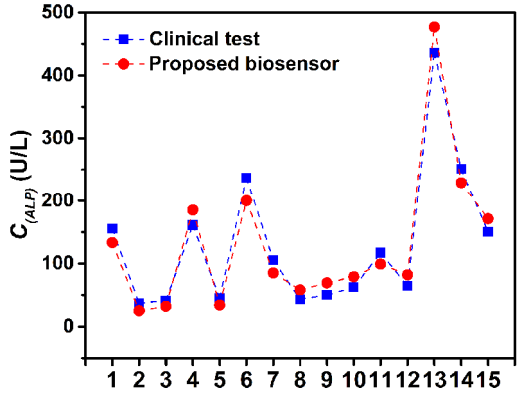


Figure 4. The correlation between the data from clinical tests and the results obtained by the proposed biosensor.

CONCLUSION

In summary, on a basis of the finding that PPi can quench the fluorescence of poly(30T)-templated CuNPs, a novel ALP biosensor has been fabricated. Since the preparation procedure of poly(30T)-templated CuNPs is simple, and PPi can directly react with as-prepared CuNPs, the designed biosensor is very facile and sensitive. As a result, ALP in a wide range can be accurately sensed by the proposed biosensor. Relying on the integration of an enzyme-triggered reaction, multivariate effects on fluorescence of CuNPs is solved, thus significantly improving the selectivity of this method. Besides, two types of ALP inhibitors have been evaluated by this quantifiable approach, and different efficiencies are precisely displayed by the obtained results. Moreover, results of detected ALP concentration in 15 human serum samples are highly consistent with the data from clinical tests, which is rare in pertinent biosensors. Therefore, the developed biosensor reveals broad potential applications in clinical medicine. More importantly, we believe this study may arouse more passion for searching new molecules that can directly affect the properties of nanomaterials, and improving the analytical performance of nanomaterials-based biosensors by introducing enzyme-triggered reactions.

ASSOCIATED CONTENT

Supporting Information

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

Effect of incubation time on the fluorescence of CuNPs (Figure S1); Comparison of different ALP biosensors (Table S1).

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Guo, Y.; Cao, F.; Lei, X.; Mang, L.; Cheng, S.; Song, J. *Nanoscale* **2016**, *8*, 4852-4863.
(2) Huang, P.; Bao, L.; Yang, D.; Gao, G.; Lin, J.; Li, Z.; Zhang, C.; Cui, D. *Chem. Asian J.* **2011**, *6*, 1156-1162.
(3) Kumar, S.; Bolan, M. D.; Bigioni, T. P. *J. Am. Chem. Soc.* **2010**, *132*, 13141-13143.
(4) Chakraborty, S.; Babanova, S.; Rocha, R. C.; Desireddy, A.; Artyushkova, K.; Boncella, A. E.; Atanassov, P.; Martinez, J. S. *J. Am. Chem. Soc.* **2015**, *137*, 11678-11687.
(5) Becerril, H. A.; Ludtke, P.; Willardson, B. M.; Woolley, A. T. *Langmuir* **2006**, *22*, 10140-10144.
(6) Liu, C.; Qing, Z.; Zheng, J.; Deng, L.; Ma, C.; Li, J.; Li, Y.; Yang, S.; Yang, J.; Wang, J.; Tan, W.; Yang, R. *Chem. Commun.* **2015**, *51*, 6544-6547.
(7) Fan, D.; Zhu, J.; Liu, Y.; Wang, E.; Dong, S. *Nanoscale* **2016**, *8*, 3834-3840.
(8) Zhang, L.; Zhu, J.; Guo, S.; Li, T.; Li, J.; Wang, E. *J. Am. Chem. Soc.* **2013**, *135*, 2403-2406.
(9) Qing, Z.; He, X.; He, D.; Wang, K.; Xu, F.; Qing, T.; Yang, X. *Angew. Chem., Int. Ed.* **2013**, *52*, 9719-9722.
(10) Rotaru, A.; Dutta, S.; Jentzsch, E.; Gothelf, K.; Mokhir, A. *Angew. Chem., Int. Ed.* **2010**, *49*, 5665-5667.
(11) Ma, J.-L.; Yin, B.-C.; Wu, X.; Ye, B.-C. *Anal. Chem.* **2016**, *88*, 9219-9225.
(12) Teng, Y.; Jia, X.; Zhang, S.; Zhu, J.; Wang, E. *Chem. Commun.* **2016**, *52*, 1721-1724.
(13) Wang, Y.; Sun, Q.; Zhu, L.; Zhang, J.; Wang, F.; Lu, L.; Yu, H.; Xu, Z.; Zhang, W. *Chem. Commun.* **2015**, *51*, 7958-7961.
(14) Zhang, K.; Wang, K.; Zhu, X.; Gao, Y.; Xie, M. *Chem. Commun.* **2014**, *50*, 14221-14224.
(15) Liu, J.; Chen, J.; Fang, Z.; Zeng, L. *Analyst* **2012**, *137*, 5502-5505.
(16) Wang, H.-B.; Zhang, H.-D.; Chen, Y.; Huang, K.-J.; Liu, Y.-M. *Sens. Actuators, B* **2015**, *220*, 146-153.
(17) Zheng, F.; Guo, S.; Zeng, F.; Li, J.; Wu, S. *Anal. Chem.* **2014**, *86*, 9873-9879.
(18) Dong, L.; Miao, Q.; Hai, Z.; Yuan, Y.; Liang, G. *Anal. Chem.* **2015**, *87*, 6475-6478.
(19) Ooi, K.; Shiraki, K.; Morishita, Y.; Nobori, T. *J. Clin. Lab. Anal.* **2007**, *21*, 133-139.
(20) Whyte, M. P.; Greenberg, C. R.; Salman, N. J.; Bober, M. B.; McAlister, W. H.; Wenkert, D.; Van Sickle, B. J.; Simmons, J. H.; Edgar, T. S.; Bauer, M. L.; Hamdan, M. A.; Bishop, N.; Lutz, R. E.; McGinn, M.; Craig, S.; Moore, J. N.; Taylor, J. W.; Cleveland, R. H.; Cranley, W. R.; Lim, R.; Thacher, T. D.; Mayhew, J. E.; Downs, M.; Millan, J. L.; Skrinar, A. M.; Crine, P.; Landy, H. *N. Engl. J. Med.* **2012**, *366*, 904-913.
(21) Whyte, M. P. *Nat. Rev. Endocrinol.* **2016**, *12*, 233-246.
(22) Jiao, H.; Chen, J.; Li, W.; Wang, F.; Zhou, H.; Li, Y.; Yu, C. *ACS Appl. Mater. Interfaces* **2014**, *6*, 1979-1985.
(23) Gao, Z.; Deng, K.; Wang, X.-D.; Miro, M.; Tang, D. *ACS Appl. Mater. Interfaces* **2014**, *6*, 18243-18250.
(24) Zhang, L.; Zhao, J.; Duan, M.; Zhang, H.; Jiang, J.; Yu, R. *Anal. Chem.* **2013**, *85*, 3797-3801.
(25) Li, G.; Fu, H.; Chen, X.; Gong, P.; Chen, G.; Xia, L.; Wang, H.; You, J.; Wu, Y. *Anal. Chem.* **2016**, *88*, 2720-2726.
(26) Ruan, C.; Wang, W.; Gu, B. *Anal. Chem.* **2006**, *78*, 3379-3384.
(27) Wu, Z.; Zhou, C.-H.; Pan, L.-J.; Zeng, T.; Zhu, L.; Pang, D.-W.; Zhang, Z.-L. *Anal. Chem.* **2016**, *88*, 9166-9172.
(28) Hayat, A.; Andreescu, S. *Anal. Chem.* **2013**, *85*, 10028-10032.
(29) Park, L.; Bae, H.; Kim, Y.-T.; Lee, J. H. *Anal. Methods* **2011**, *3*,

156-160.
(30) Jiang, H.; Wang, X. *Anal. Chem.* **2012**, *84*, 6986-6993.
(31) Xu, F.; Shi, H.; He, X.; Wang, K.; He, D.; Guo, Q.; Qing, Z.; Yan, L.; Ye, X.; Li, D.; Tang, J. *Anal. Chem.* **2014**, *86*, 6976-6982.
(32) Zhao, X.; Liu, Y.; Schanze, K. S. *Chem. Commun.* **2007**, 2914-2916.
(33) Kazantsev, A. G.; Thompson, L. M. *Nat. Rev. Drug Discovery* **2008**, *7*, 854-868.
(34) Whisnant, A. R.; Gilman, S. D. *Anal. Biochem.* **2002**, *307*, 226-234.

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