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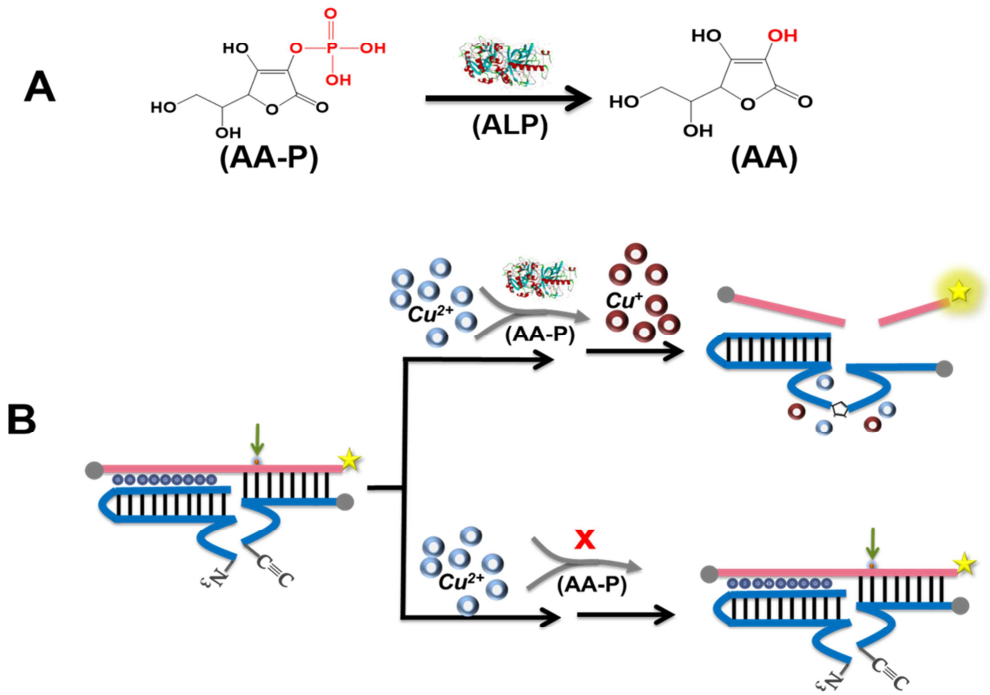
GRAPHICAL ABSTRACT



A sensitive fluorescence biosensor for alkaline phosphatase activity based on the Cu(II)-dependent DNAzyme

Mengmeng Zhao[a], Yajuan Guo[a], Lixu Wang[a], Fang Luo[b]*, Cuiying Lin[a]*, Zhenyu Lin[a], Guonan Chen[a]

[a] College of Chemistry, Fuzhou University, Fuzhou, Fujian 350116, China. E-mail: lcuiying@fzu.edu.cn (C.Lin); Tel&Fax:0086-591-22866135

[b] College of Biological Science and Technology, Fuzhou University, Fuzhou, Fujian 350116, China. E-mail: luofang0812@163.com (F.Luo)

ABSTRACT

Alkaline phosphatase (ALP) plays an important role in phosphate metabolism processes; deviation from its normal level may indicate different kinds of diseases, so it is highly necessary to develop some simple and sensitive methods to monitor the ALP level. In this study, a simple, high selective, and sensitive fluorescent biosensor has been proposed for ALP activity determination. The Cu(II)-dependent DNAzyme (Cu-Enzyme) are divided into two parts: Cu-Enzyme 1 and Cu-Enzyme 2, and labelled with alkyne and azido groups, respectively. The Cu-substrate (Cu-Sub) is labelled with a FAM fluorophore (6-carboxyfluorescein) at the 3'-end and an additional quencher (BHQ1) at the 5'-end. The 5'-end of Cu-Enzyme 1 is labelled with BHQ1 as well. The hybridization of the Cu-Enzyme 1 and Cu-Enzyme 2 with Cu-Sub strand results in the low background fluorescence signal because the fluorescence from FAM is quenched. The addition of ALP can hydrolyze AA-P into AA, which can

reduce Cu(II) into Cu(I) and in turn catalyze the cycloaddition of Cu-Enzyme 1 and Cu-Enzyme 2 to form a modified Cu-Enzyme. Then the modified Cu-Enzyme catalyzes the cleavage of the Cu-Sub strands into two pieces. One piece containing FAM fluorophore can easily diffuse into solution and give off a strong fluorescence signal. The enhanced fluorescent intensity has a linear relationship with the ALP concentration in the range of 0.36—54.55 U L^{-1} with the detection limit of 0.14 U L^{-1} (S/N = 3). The proposed biosensor has been successfully applied to detect ALP in serum samples with satisfied results.

Key words: alkaline phosphatase; Cu(II)-dependent DNAzyme; click chemistry; fluorescence.

1. Introduction

Alkaline phosphatase (ALP), an essential enzyme in phosphate metabolism [1,2], can catalyze the dephosphorylation process of nucleic acids, proteins, and some small molecules [1,3]. The deviation of ALP from its normal level may indicate different kinds of diseases such as hepatobiliary disease, bone diseases, breast and prostatic cancer, and diabetes [4-6]. Thus, it is highly necessary to develop some simple and sensitive methods for ALP level determination. Till now, many different techniques, such as electrochemistry [7], fluorescence [8-10], colorimetric [11], chemiluminescence [12], surface enhanced resonance raman scattering (SERRS) [13] and capillary electrophoresis [14], have been frequently applied to detect ALP. Among these methods, the fluorescent assays of ALP have attracted much attention for their reliability, convenience, high sensitivity, and accessible instrument requirement. [15,16] For example, Qian et al. reported a sensitive fluorometric assay for ALP on the basis of the aggregation and disaggregation of carbon quantum dots (CQDs) [17]. Xiang et al. proposed a turn-off fluorescent biosensor for ALP determination utilizing highly fluorescent g-C_3N_4 nanosheet [8].

Click chemistry was firstly proposed by Sharpless in 2001 [18]. The remarkable advantages of the click chemistry reactions are mild reaction conditions, simple operation, easier purification, high reaction speed, good yields, and selectivity [19,20]. The Cu(I)-catalyzed azides and alkynes cycloaddition (CuAAC) reaction, a typical example of click reaction, has attracted considerable attraction [21-23]. It has been reported that a highly fluorescent triazole complex could be produced through ligating

the weak fluorescent 3-azidocoumarins and terminal alkynes [24]. This reaction has been applied early to develop fluorescence biosensor for Cu(II), histidine, pyrophosphatase (PPase) activity [25-27]. However the compound which takes part in the reaction can be oxidized easily and hence causes a high background signal, which may cause negative affect to the stability and sensitive of the developed biosensors. So, it is necessary to search some ways to address this problem.

Cu(II)-dependent DNAzyme (Cu-Enzyme), which can irreversibly catalyze the oxidative cleavage of its corresponding DNA substrates upon the introduction of the Cu(II) as cofactors [28-30]. If the Cu(II)-dependent DNAzyme sequence are divided into two parts (Cu-Enzyme 1 and Cu-Enzyme 2) and labelled with alkyne and azido groups, respectively. The modified Cu-Enzyme can be formed through the CuAAC reaction. Early reports showed that ALP can hydrolyze ascorbic acid 2-phosphate (AA-P) into ascorbic acid (AA) through dephosphorylating [31,32]. And the produced AA can reduce Cu(II) into Cu(I) to trigger the click reaction. Inspired by this fact, a simple and sensitive fluorescent biosensor for ALP detection based on the modified Cu-Enzyme and the strategy of click chemistry has been proposed in this study, which combines the advantages of the high specificity of the modified Cu-Enzyme, high selectivity of CuAAC, and high sensitivity of fluorescence detection technique. Compared with the previously reported click chemistry methods based on the fluorescent triazole complex, the strategy of click chemistry in this context showed easier operation process and lower background signals [26]. And such proposed biosensor was eventually employed for the detection of ALP in human serum samples.

2. Experimental section

2.1. Reagents

The oligonucleotides were synthesized by Sangon Inc. (Shanghai, China). Their sequences are shown below:

Cu-Sub: 5'-quencher-AGCTTCTTTCTAATACGGCTTACC-FAM-3'

Cu-Enzyme 1: 5'-quencher-GGTAAGCCTGGGCCTC-CΞC-3'

Cu-Enzyme 2: 5'-N_3-TTTCTTTTTAAGAAAGAAC-3'

(Where the FAM fluorophore corresponds to 6-carboxyfluorescein, the quencher is BHQ1). NaH_2PO_4, Na_2HPO_4 and L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (AA-P) were bought from Shanghai Chemical Reagent Company (Shanghai, China). AA, $CuSO_4$, $MgCl_2$, NaCl and other reagents were obtained from Alfa Aesar China Co. Ltd (Tianjin, China). All reagents and solvents were of analytical grade or better and were used directly without further purification. All solutions were prepared with double distilled water (Milli-Q, Millipore, resistance 18.2 MΩ·cm). DNA buffer solutions were prepared by dissolving DNA into PBS buffer solution (50 mM, pH 7.4). The human serum samples were provided by the Hospital of Fuzhou University (Fujian, China) from three volunteers.

2.2. Fluorescence spectroscopic measurements

Cu-Enzyme 1 (0.25 μM) was firstly hybridized with Cu-Enzyme 2 (0.25 μM) and Cu-Sub (0.25 μM) at 37 °C in 100 μL of 50 mM PBS buffer (50 mM PB, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl) for 1 h. Then 4 μL of 1mM Cu(II), 100 μL of 0.1 M AA-P and different concentrations of ALP were added to the abovementioned solution to

trigger CuAAC and DNA-cleaving reaction. The mixed solution was subsequently incubated at 37 ºC for 3 h, and the fluorescence emission spectra were collected from 490 to 600 nm with the excitation wavelength of 480 nm at room temperature by a Fluorescence spectrophotometer (F4600 Hitachi, Inc.). The excitation and emission slit widths were all set as 5.0 nm and the fluorescence intensity at 520 nm was used for quantitative analysis.

3. Results and discussion

3.1. The principle of the fluorescence biosensor for ALP

Scheme 1 shows the principle of the proposed fluorescence biosensor for ALP. Cu-Enzyme 1 was modified with an alkyne groups at its 3'-end and labeled with a quencher (BHQ1) at the 5'-termini; Cu-Enzyme 2 contained a 5'-azido; Cu-Sub was labeled with a FAM fluorophore (6-carboxyfluorescein) at the 3'-end and an additional quencher was attached on the 5'-end. After hybridization of the Cu-Enzyme 1 and Cu-Enzyme 2 with Cu-Sub strands to form a DNA triplex, the background fluorescence signal was low owing to the dual-quencher labeling method [33]. The presence of AA-P has no effect to the fluorescence signal of the system. But the further addition of ALP to the system can cause hydrolysis of AA-P into AA; the produced AA can reduce Cu(II) into Cu(I), which in turn links Cu-Enzyme 1 and Cu-Enzyme 2 through the CuAAC reaction between alkyne and azido groups to form a modified Cu-Enzyme. With Cu(II) as cofactor, the resulting modified Cu-Enzyme can catalyze the cleavage of the Cu-Sub strands into two pieces. One piece containing FAM fluorophore has short single stranded DNA and low unwinding temperature

125 (Tm = -7.8 °C), it can easily diffuse into solution and give off a strong fluorescence
126 signal. The enhanced fluorescence intensity has a relationship with the amount of
127 ALP, so a simple, sensitive and selective fluorescence biosensor for ALP
128 determination can be developed.

129 A simple assistant assay was performed to verify our presumption firstly. As shown
130 in Fig. 1A, the fluorescent intensity from the DNA triplex was negligible (curve a),
131 suggesting the fluorescence from the FAM is quenched efficiently. If Cu(II) or AA
132 was added to the system which contains DNA triplex complex separately, the
133 fluorescence signal nearly has no change (curve b and curve c), these results indicate
134 that these two compounds cause no effect to the fluorescence of the system. But upon
135 the simultaneous addition of Cu(II) and AA to the abovementioned solution, obvious
136 fluorescent enhancement was observed (curve d), indicating that Cu-Enzyme 1 and
137 Cu-Enzyme 2 can be linked through CuAAC to form a modified Cu-Enzyme to
138 catalyse the cleavage of the Cu-Sub strands. Then a further control study was
139 performed to test the feasibility of our presumption. As shown in Fig. 1B, without
140 ALP, the fluorescent intensity was weak when the AA-P and Cu(II) existed in the
141 reaction solution (curve e). But the fluorescence intensity increased dramatically after
142 the addition of ALP (curve f). This is probably because ALP can hydrolyze AA-P into
143 AA, and the produced AA can reduce Cu(II) into Cu(I), which in turn triggers the
144 CuAAC reaction and produces the Cu-Enzyme; the formed Cu-Enzyme induces the
145 cleavage of the Cu-sub strands into two fragments under the catalysis of Cu(II). One
146 fragment containing the FAM fluorophore-labelled short-oligonucleotide could easily

diffuse into the solution, resulting in the enhancement of fluorescence signal. The aforementioned results confirm the feasibility of the proposed biosensor.

The preferred position for Scheme 1

The preferred position for Fig.1

3.2. Optimization of the reaction conditions

Some experimental conditions were optimized to acquire the best performance of the proposed biosensor. The pH value of the buffer solution was firstly optimized because it affects the activity of ALP greatly. As shown in Fig. 2A, the fluorescence intensity of the system increased with the increase of the pH value in the range of 5.0 ~ 8.5 and then decreased gradually when the pH was higher than 8.5. The possible reason is that the ALP can become inactive at high pH value. So, pH 8.5 was chosen in this study. The concentration of AA-P was also optimized because it plays an important role in the formation of AA. As shown in Fig. 2B, the fluorescence intensity of the system increased firstly with the increase of AA-P concentration and then reached a plateau after 50 mM. So 50 mM of AA-P was chosen as the optimized concentration for the following study. The reaction time after the addition of ALP affects the formation of modified Cu-Enzyme and the cleavage of Cu-Sub greatly, so it was optimized too. Fig. 2C illustrates the fluorescence intensity of the system enhanced with the increase of the reaction time and reached a plateau after 3 h. So 3h was chosen as the best reaction condition.

The preferred position for Fig.2

3.3. Calibration curve of ALP detection

The fluorescent emission spectra with different concentrations of ALP were tested under the optimized conditions. Fig. 3 reveals that the fluorescent intensity gradually increased with the increase of ALP concentration. There is a linear relationship between the fluorescent intensity and ALP concentration in the range of 0.36－54.55 U L^{-1} (see inset in Fig. 3). The correlation coefficient (R^2) is 0.995; the limit of detection (LOD) is estimated to be 0.14 U L^{-1} (S/N = 3), which is lower than those of some existing fluorescence methods for ALP detection (shown in Table 1) [9,17,34,35]. In addition, to verify the reproducibility of the proposed biosensor, five replicated experiments were performed at 5 U L^{-1} ALP and the relative standard deviation (RSD) was calculated to be 5.5%, this result indicates that the proposed biosensor possesses good repeatability. Furthermore, if the hybridization product of Cu-Enzyme 1, Cu-Enzyme 2 and Cu-Sub was stored at 4 °C for four weeks, the background signal did not change. This outcome demonstrates that, unlike the 3-azidocoumarins used in our early studies [25-27], the hybridization product has good stability and therefore guarantees the good reproducibility of the proposed biosensor.

The preferred position for Fig.3

3.4. The selectivity and interference study of the proposed biosensor

The specificity and tolerance of the proposed biosensor for ALP detection were studied as well. Several proteins such as urease, horseradish peroxidase (HRP), albumin human (HSA), pyrophosphatase (PPase), thrombin, glucose oxidase (GOx), trypsin, protein phosphatase 1 (PP1) and glucose-6-phosphatase (G-6-Pase) were chosen as representative interferents. As show in Fig. 4A, distinct enhanced fluorescence signal was detected via the addition of ALP. However, the fluorescent intensity was nearly the same as that of the blank when other interferences were added. These results indicate that the proposed biosensor possesses good specificity for ALP.

Additionally, to demonstrate the anti-interference property of this sensor toward ALP, the competition study was also carried out through the addition of ALP to the solution in the presence of other proteins respectively. Fig. 4B shows that other proteins did not significantly affect the fluorescent response of ALP. These results also indicate that the proposed biosensor has great application potential for the detection of ALP in complicated biological samples.

The preferred position for Fig.4

3.5. Detection of ALP in serum samples

The ALP in human serum samples was detected to demonstrate the practical application of the proposed biosensor. The reducing substances in serum such as AA, uric acid (UA), and glutathione may cause the reduction of Cu(II) into Cu(I) and hence have influence on the performance of the sensor. When Cu(II) and the serum samples were added to the hybridization product of Cu-Enzyme 1, Cu-Enzyme 2 and Cu-Sub, a significant fluorescence signal was detected. This result suggests the component in the serum samples can cause some interference. But thanks to the high sensitivity of the proposed biosensor, this interference can be avoided through the dilution of the samples. When the serum samples were diluted by the buffer solution for 100 times, the fluorescent intensity of the system was nearly the same as that of the blank (no serum sample was added to the hybridization product), demonstrating that nearly no interference exists at this condition. Then the concentrations of ALP in the diluted serum samples (diluted 100 times) were determined by the proposed method, results show that the ALP of the tested samples were determined to be 78, 112, and 85 U L^{-1}, respectively. These results are in accordance with other methods [36,37]. Standard addition recovery rates were obtained to further verify the validity of the proposed biosensor. As shown in Table 2, the recovery rates of the ALP ranged from 93.3－110%. These analysis results suggest that the proposed fluorescent biosensor is available for effectively detecting ALP in real samples.

The preferred position for Table 1

4. Conclusion

In summary, a simple, high selective, and sensitive fluorescent biosensor has been successfully developed for ALP based on the modified Cu-Enzyme and the strategy of click chemistry. The Cu-Enzyme which has been divided into two fragments cannot be assembled into a complete one without ALP and does not possess the catalytic property accordingly. With the addition of ALP, the two fragments can assemble to form a modified Cu-Enzyme through CuAAC; the modified Cu-Enzyme possesses the catalytic activity and hence results in the enhancement of the fluorescence intensity. The proposed biosensor has high selectivity and has been applied to detect ALP in human serum samples with satisfied results. Hence, our current strategy opens up a new way for further application of the metal ions-dependent DNAzymes.

Acknowledgements

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Figures and Captions

Scheme 1 The principle of the proposed fluorescence biosensor for ALP activity.

Fig. 1 (A) Fluorescence of the system under different conditions: (a) hybridization product the three DNA strands; (b) Cu(II) was added into the solution alone; (c) AA

was added into the solution alone; (d) Cu(II) and AA were added into the solution simultaneously; **(B)** Fluorescence of the system: (e) without ALP (20 μM Cu(II) + 50 mM AA-P) and (f) after the mixture with ALP (20 μM Cu(II) + 50 mM AA-P + 18.18 U L^{-1} ALP)

Fig. 2 Effect of **(A)** media pH; **(B)** AA-P concentration and **(C)** the reaction time on the fluorescence intensity.

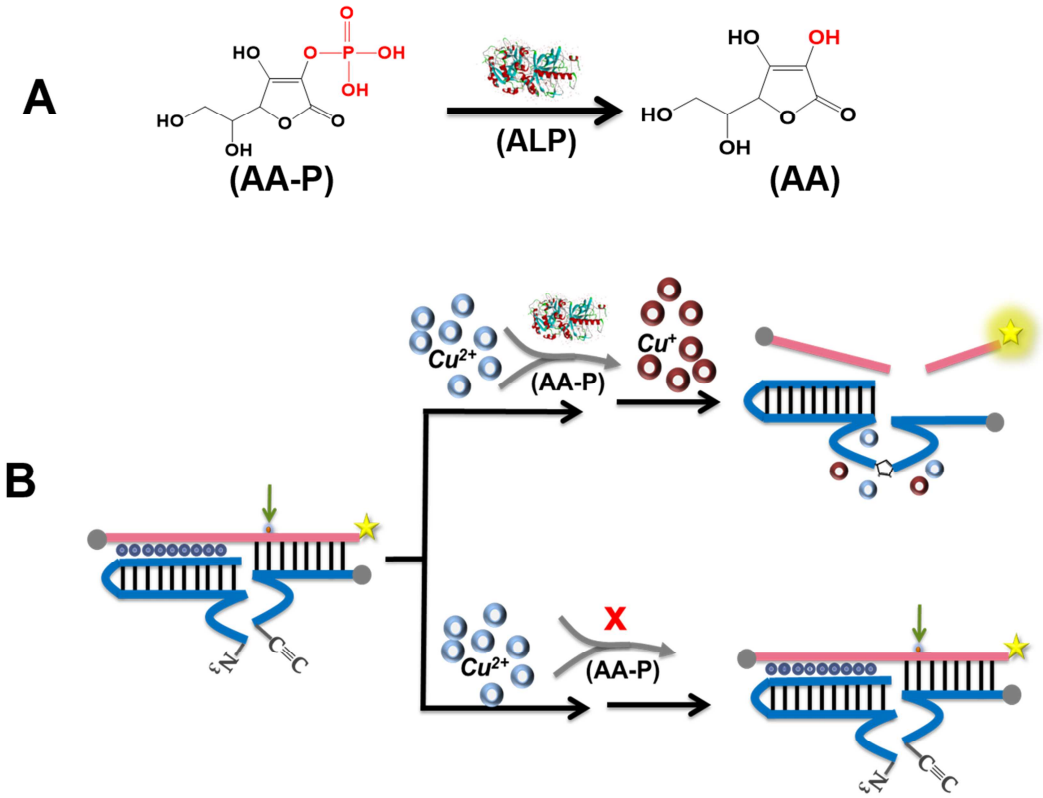
Fig. 3 Fluorescence spectra in the presence of different concentrations of ALP: (a)0 U L^{-1}; (b) 0.36 U L^{-1}; (c) 2.27 U L^{-1}; (d) 5.68 U L^{-1}; (e) 11.36 U L^{-1}; (f) 18.18 U L^{-1}; (g) 27.27 U L^{-1}; (h) 31.82 U L^{-1}; (i) 45.45 U L^{-1}; (j) 54.55 U L^{-1}. And calibration curve between fluorescence intensity and ALP concentrations.

Fig. 4 (A) Selectivity of the proposed sensor; **(B)** Anti-interference property of the proposed sensor for ALP in the presence of other interferences. (ALP (10 mU), urease (100 mU), HRP (100 mU), HAS (20 μg mL^{-1}), PPase (100 mU), thrombin (20 μg mL^{-1}), GOx (100 mU), trypsin (20 μg mL^{-1}), PP1 (100 mU) and G-6-Pase (20 μg mL^{-1}))

Table 1 Comparison of the proposed method with other fluorescent approaches developed for ALP detection.

Table 2 Detection results of ALP in human serum samples using the proposed sensor(n = 3)[a]

392 **Scheme 1**



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394 **Figure 1**

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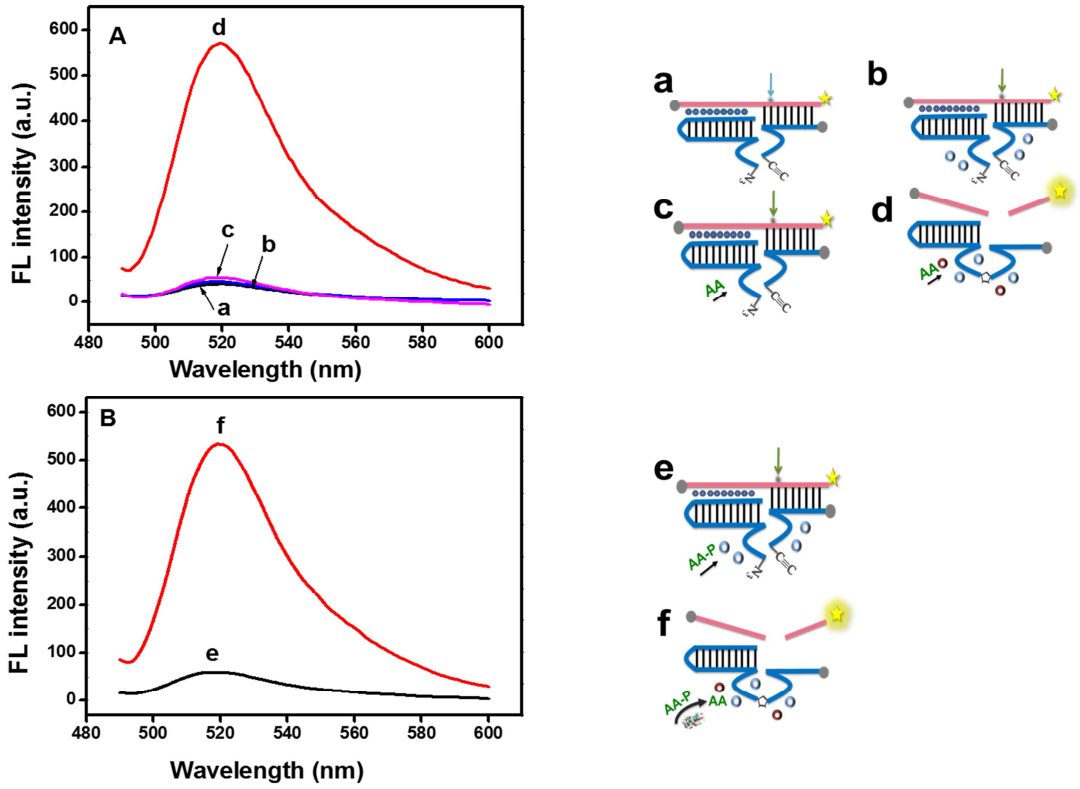

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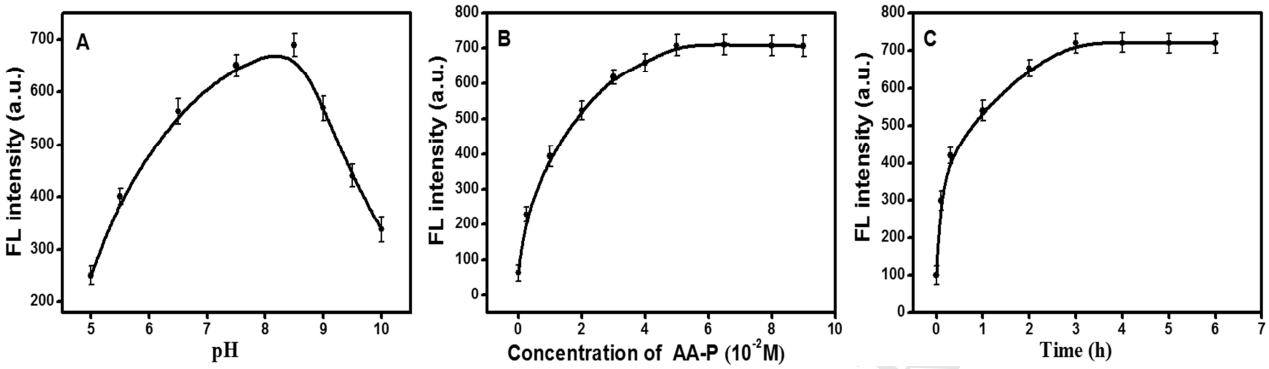
Figure 2



423 **Figure 3**

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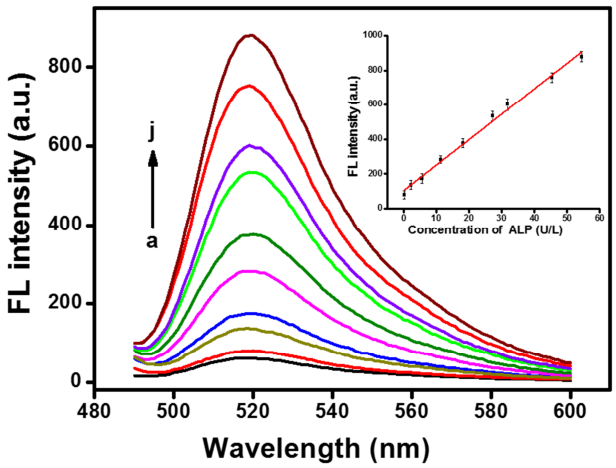

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426 **Figure 4**

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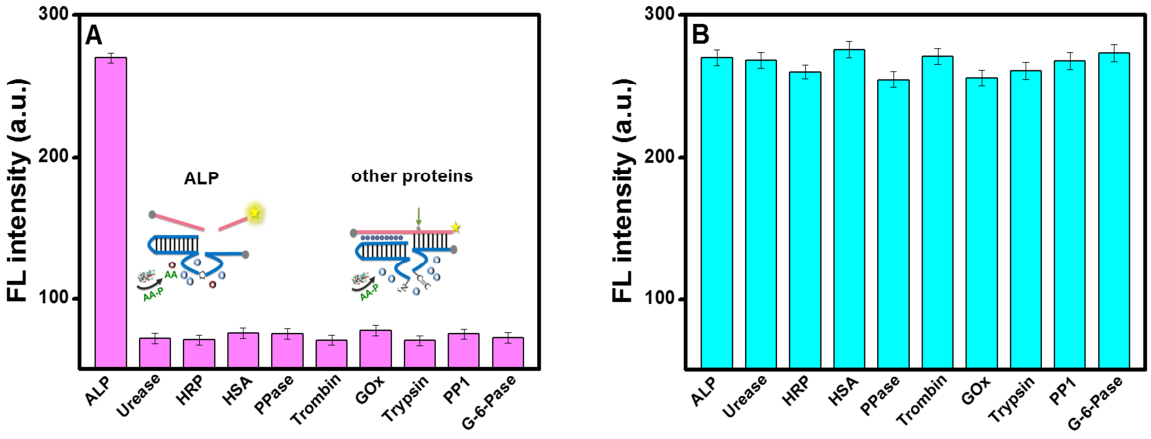

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429

430 **Table 1**

Ref.	LOD of ALP (U/L)	Dynamic range (U/L)
ref.9	10	25 – 200
ref.17	1.4	4.6 – 383.3
ref.34	60	0 – 2800
ref.35	1.1	16.7 – 782.6
This work	0.14	0.36 – 54.55

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448 **Table2**

Sample number	Found in sample (U/L)	Spiked (U/L)	Total found (U/L)	Recovery (%)	RSD (%)
		10.0	89	110	4.7
1	78	30.0	106	93.3	6.1
		50.0	131	106	6.4
		10.0	123	110	4.9
2	112	30.0	143	103	6.6
		50.0	165	106	6.3
		10.0	95.5	105	5.8
3	85	30.0	114	96.7	4.2
		50.0	138	106.0	6.3

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450 [a] The samples had been diluted for 100 times before determination.

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HIGHLIGHTS

1. We have proposed a simple, high selective and sensitive fluorescent biosensor for ALP.
2. The proposed biosensor combines the high selectiviey and efficiency of the click reaction and the high sensitivity of the fluorescence detection.
3. The proposed biosensor has been successfully applied to detect ALP in serum samples with satisfied results.