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DNAzyme-regulated CRISPR/Cas12a based fluorescent biosensor for sensitive detection of alkaline phosphatase activity and inhibition

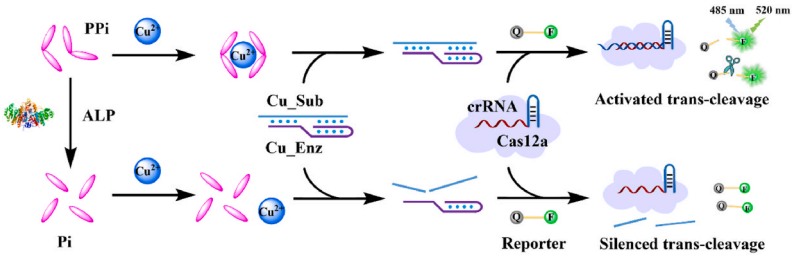
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HIGHLIGHTS

- A sensitive fluorescent strategy is developed based on DNAzyme-regulated CRISPR/Cas12a cascade signal amplification.
- The fluorescent sensing strategy exhibits high sensitivity and selectivity for detection of ALP activity in serum samples.
- The proposed strategy can be applied for evaluating inhibition efficiency and screening ALP inhibitors.
- This work broadens the applications of CRISPR/Cas-based sensing systems in biomedical research.

GRAPHICAL ABSTRACT



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ABSTRACT

Alkaline phosphatase (ALP) is regarded as an emerging biomarker and therapeutic target for various diseases. Herein, we developed a DNAzyme-regulated CRISPR/Cas12a cascade signal amplification strategy for sensitive and selective detection of ALP activity and inhibition. In this assay, the substrate strand of Cu^{2+}-specific DNAzyme was designed as the DNA activator for Cas12a. The substrate strand would be cleaved into two fragments when ALP-catalyzed hydrolysis reaction disabled the complexation between Cu^{2+} and pyrophosphate (PPi). In this case, the cleaved sequences could not further bind to the Cas12a-crRNA complex to activate the *trans*-cleavage activity of CRISPR/Cas12a, which would hamper the cleavage of doubly labeled single-stranded DNA (ssDNA) fluorescence reporter, and thus a turn-off fluorescence signal. The cascade signal amplification strategy greatly improved the detection sensitivity for ALP activity, with a detection limit as low as 0.04 U/L. Moreover, the proposed method was successfully applied for ALP activity detection in serum samples and ALP inhibitory capability evaluation. This method overcomes the shortcoming of conventional methods that show unsatisfactory sensitivity and are susceptible to interfering substances, which might hold great promise in clinical diagnosis and biomedical research.

1. Introduction

Alkaline phosphatase (ALP), a widely distributed membrane-bound enzyme, can catalyze the hydrolysis and dephosphorylation of various monophosphate ester substrates *in vivo* and *in vitro* [1], thus playing a pivotal role in signal transduction and intracellular regulation [2].

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Consequently, ALP has been regarded as a significant biomarker for clinical diagnosis, as the abnormal level of ALP in serum is closely related to various diseases, such as diabetes, bone disease, liver dysfunction, and breast and prostatic cancer [3–5]. Therefore, developing a sensitive and reliable method for detecting ALP activity in clinical diagnosis and treatment is of great concern. Up to date, various methods have been developed for ALP activity detection, such as colorimetry [6,7], fluorometry [8–11], electrochemistry [12–14], chemiluminescence [15,16], and surface-enhanced Raman scattering [17,18]. Among them, fluorescent methods for detecting ALP have attracted significant attention due to their advantages, including high sensitivity, rapid response, and convenient operation [19].

Recently, some fluorescent strategies have been reported for ALP activity assay based on the dephosphorylation of phosphated compounds by ALP [20]. Pyrophosphate (PPi), a natural substrate of ALP, has been frequently applied to detect ALP owing to its unique properties of lower optimum pH compared to other substrates and its strong ability to chelate specific metal ions, such as Zn^{2+}, Cu^{2+}, Hg^{2+} and Fe^{3+} [21,22]. However, traditional methods for evaluating ALP levels using radiolabeled PPi as its natural substrate are laborious and have low sensitivity. Nowadays, various efforts have focused on the development of simple and label-free fluorescent methods for determining ALP with high sensitivity. For example, Liu et al. proposed a turn-on fluorescent method for determining the activity of ALP by using dsDNA-templated copper nanoparticles (CuNPs) coupled with exonuclease-based amplification [23]. In addition, Chen et al. designed a ratio fluorometric technique for ultrasensitive ALP detection based on in situ generation of mercury-doped zinc selenide quantum dots (Hg-doped ZnSe QDs) and quinoxaline derivative (QXD) [24]. However, most of these protocols are limited by complicated nanomaterial synthesis procedure and false-positive signals generated by interfering proteins. Therefore, developing simple and reliable fluorescent strategies for detecting ALP is still highly desirable.

DNAzymes, a kind of catalytically active DNA molecules, have attracted significant attention due to their high metal ion selectivity, excellent programmability, good stability, and ease of modification [25,26]. To date, DNAzymes have been applied to the construction of multifarious sensors for detecting metal ions [27,28], nucleic acids and proteins [29]. More importantly, to further enhance the detection sensitivity, DNAzymes can be incorporated into other signal amplification strategies, such as rolling circle amplification [30], hybridization chain reaction [31], and strand displacement amplification [32]. However, despite significant advancements in these amplification techniques, some limitations still exist that prevent them from being widely used, including being time-consuming, harsh reaction conditions, and sophisticated primer/probe designs. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-based techniques have become popular because of their high sensitivity and simplicity [33–35]. Among them, the CRISPR/Cas12a system has been widely utilized as a triggerable signal amplification tool to construct biosensors for non-DNA targets in ultralow concentrations [36,37].

This study has developed a simple and sensitive fluorescent biosensor for ALP activity detection that utilizes Cu^{2+}-dependent DNAzyme and CRISPR/Cas12a for cascade signal amplification. Inspired by the fact that PPi can strongly chelate with Cu^{2+}, it is speculated that the complexation between PPi and Cu^{2+} will result in effective inhibition of DNAzyme activity. In this case, the intact substrate strands can further bind the crRNA to activate the collateral cleavage activity of Cas12a, breaking up the doubly labeled single-stranded DNA (ssDNA) fluorescence reporter, leading to a significant increase in fluorescence signal. Furthermore, the introduction of ALP can cause hydrolysis of PPi to phosphate (Pi), freeing Cu^{2+} ions in solution and activating the Cu^{2+}-dependent DNAzyme [38], which catalyzes the cleavage of the substrate strands into two fragments. The cleaved sequences cannot recognize and activate the Cas12a-crRNA complex, which prevents the ssDNA fluorescence reporter from being cleaved, resulting in the fluorescence quenching again. Since the decrease in fluorescence signal is related to the concentration of ALP, ALP activity can be estimated by monitoring the change in fluorescence in the system. In addition, the proposed fluorescent strategy has been successfully applied to determine ALP activity in serum samples and evaluate ALP inhibitor efficiency.

2. Experimental section

2.1. Detection of ALP activity

In this assay, 5 μL of ALP with various concentrations, 5 μL of 8 mM PPi, and 5 μL of 2 μM Cu^{2+} were mixed in 50 mM HEPES buffer (pH 7.5, 20 mM NaCl). The mixture was then incubated at 37 °C for 30 min for ALP-catalyzed hydrolysis of PPi. Meanwhile, 5 μL of 800 nM Cu_Enz and 5 μL of 400 nM Cu_Sub were mixed and incubated at 37 °C for 30 min to allow the formation of Cu^{2+}-dependent DNAzyme. Subsequently, 5 μL of 2 mM ascorbic acid (AA) was added to the abovementioned solution and incubated at 37 °C for 80 min to trigger the DNAzyme cleavage reaction. The CRISPR/Cas12a reaction system was prepared by mixing 2 μL of 1.5 μM LbCas12a, 2 μL of 1.5 μM crRNA, 6 μL of 8 μM ssDNA fluorescence reporter, 5 μL of 3 μM dithiothreitol (DTT) and 5 μL of 10 × NEBuffer at 37 °C for 30 min. After that, the DNAzyme cleavage product was mixed with the above Cas12a reaction solution and ultrapure water to 200 μL final volume. Finally, the resulting solution was incubated at 37 °C for 30 min. The fluorescence signal was measured in the wavelength range from 500 to 600 nm with an excitation wavelength of 485 nm using a RF-5301 fluorescence spectrophotometer (Shimadzu, Japan).

2.2. ALP inhibition assay

Different concentrations of theophylline were preincubated with 10 U/L ALP at 37 °C for 30 min to investigate the application of the proposed fluorescent strategy for ALP inhibitor evaluation. The subsequent assay procedure was similar to ALP detection. The inhibition efficiency (IE; %) was calculated based on the following equation:

$$IE\ (\%) = \left[\frac{(F_I - F_0)}{F_B - F_0}\right] \times 100$$

Where F_B, F_0, and F_I respectively represent the fluorescence intensity without ALP, with 10 U/L ALP, and different concentrations of theophylline (TP), an active ingredient of traditional Chinese medicine (TCM).

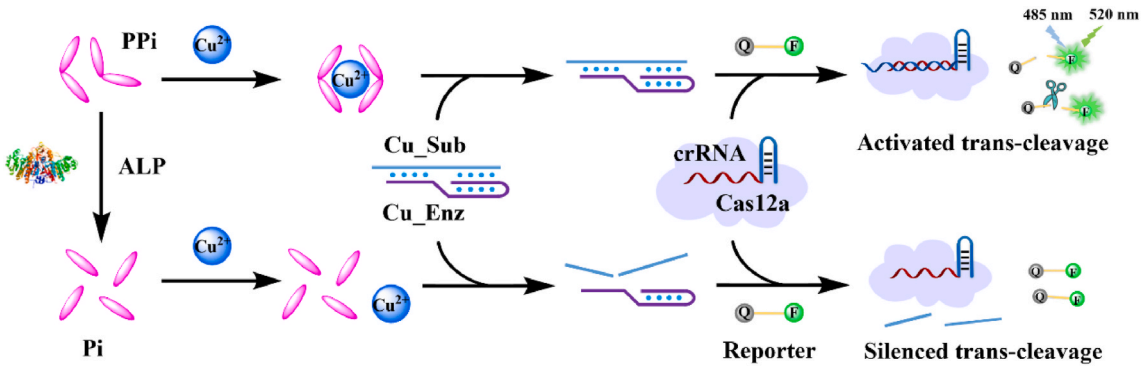
2.3. Human serum sample detection

Human serum samples were obtained from Sun Yat-Sen University Cancer Center (Guangzhou, China). Each of the serum samples was diluted 150 times with HEPES buffer (50 mM, pH 7.5, 20 mM NaCl) and then spiked with different concentrations of ALP (0, 2, 20, 200 U/L). The assay procedure of ALP activity in human serum samples was similar to that in the buffer solution.

3. Results and discussion

3.1. Principle of the fluorescent sensing strategy

The principle of DNAzyme-regulated CRISPR/Cas12a based fluorescent biosensor for ALP activity detection is illustrated in Scheme 1. In the absence of ALP, PPi can strongly chelate with Cu^{2+} ions, leading to effective inhibition of DNAzyme activity. Furthermore, the intact substrate strand (Cu_Sub) binds to the binary complex of Cas12a/crRNA. As a result, the *trans*-cleavage activity of CRISPR/Cas12a is activated, splitting the doubly labeled single-stranded DNA (ssDNA) reporter and producing a strong fluorescent signal. On the other hand, in the presence of ALP, free Cu^{2+} ions are present via ALP-catalyzed hydrolysis of PPi, which activates the Cu^{2+}-specific DNAzyme to cleave Cu_Sub into two fragments. In this case, the cleaved DNA fragments cannot further bind



Scheme 1. Schematic illustration of DNAzyme-regulated CRISPR/Cas12a based fluorescent sensing strategy for ALP activity assay.

to the Cas12a-crRNA complex. Thus, the *trans*-cleavage activity of CRISPR/Cas12a is silenced, so the ssDNA reporter remains intact, which displays a minimal fluorescent signal. Therefore, the detection of ALP activity can be achieved by monitoring the fluorescence change in the system.

3.2. Feasibility of the proposed strategy

The fluorescence experiments were carried out under different conditions to demonstrate the feasibility of the proposed strategy for the fluorescent detection of ALP activity. As shown in Fig. 1, in the absence of Cu_Sub, no apparent fluorescent signal was produced from the ssDNA reporter (curve a). However, a significant increase in fluorescence signal was observed when the Cu_Sub was added to the solution (curve b), and the addition of the enzyme strand (Cu_Enz) had no pronounced effect on the fluorescence signal of the system (curve c). These results confirmed the role of the Cu_Sub in activating the efficient cleavage of the ssDNA reporter by the Cas12a-crRNA complex. With the addition of Cu^{2+}, the fluorescence intensity at 520 nm was dramatically reduced (curve d), indicating that the Cu_Sub was catalytically cleaved by Cu^{2+}-dependent DNAzyme and the Cas12a-catalyzed cleavage of ssDNA reporter was not triggered. With the addition of PPi to the system, fluorescence was recovered due to the strong interaction between PPi and Cu^{2+} (curve e). However, the fluorescence quenching returned after adding ALP to the system (curve f), suggesting that Cu^{2+} was released from the chelate complex via the hydrolysis of PPi by ALP. Furthermore, the fluorescence intensity increased dramatically when ALP activity was effectively inhibited by theophylline (curve g). Therefore, the proposed fluorescent biosensor can be applied for ALP activity and inhibition detection.

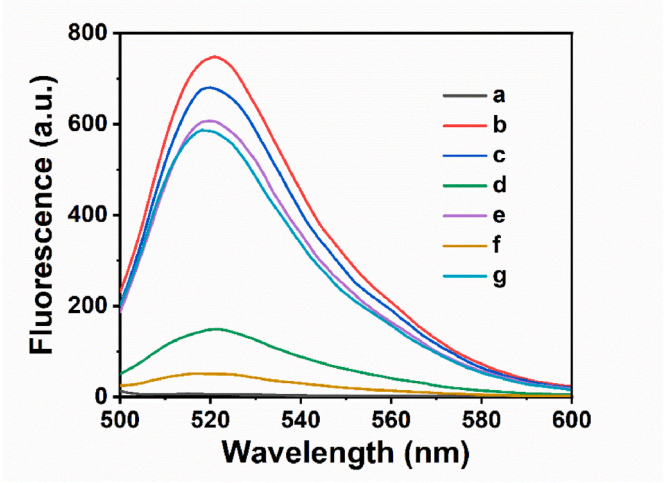


Fig. 1. Fluorescence spectra of the system under different conditions: (a) crRNA + Cas12a + Reporter, (b) a + Cu_Sub, (c) b + Cu_Enz, (d) c + Cu^{2+}, (e) d + PPi, (f) e + ALP, (g) f + TP. Concentrations of crRNA, Cas12a, Reporter, Cu_Sub, Cu_Enz, Cu^{2+}, PPi, ALP and TP were 15 nM, 15 nM, 240 nM, 10 nM, 20 nM, 50 nM, 200 μM, 10 U/L and 500 μM, respectively.

3.3. Optimization of assay conditions

To achieve the best analytical performance of the proposed strategy for ALP detection, some experimental conditions involved in this assay were optimized. The concentration of PPi was optimized first since it serves as an ALP substrate and chelates with Cu^{2+}, preventing the DNAzyme from being active in the absence of ALP. As shown in Fig. S1A, the change in fluorescence intensity ($\Delta F = F_0 - F$) was enhanced significantly with the increase of PPi concentration from 50 to 200 μM, followed by a decrease beyond 200 μM (F_0 and F is the fluorescence intensity of the system without and with ALP, respectively). Thus, 200 μM was chosen as the optimum PPi concentration for the subsequent experiments. Then, the effect of reaction temperature on the *trans*-cleavage activity of CRISPR/Cas12a was investigated. As exhibited in Fig. S1B, the ΔF increased significantly with an increase in reaction temperature from 20 to 37 °C but slightly decreased beyond 37 °C, suggesting that the relatively high temperature contributes to the Cas12a-catalyzed cleavage of the ssDNA reporter. So, 37 °C was chosen as the optimal reaction temperature of CRISPR/Cas12a in the following experiments. The concentration of Cu_Sub was also optimized because it plays a crucial role in activating the efficient cleavage of the ssDNA reporter by the Cas12a-crRNA complex. Fig. S1C shows that the value of ΔF increased with increasing Cu_Sub concentrations from 2.5 to 10 nM and then decreased gradually when the concentration was higher than 10 nM, implying that the Cas12a can be completely activated when the concentration of Cu_Sub is above 10 nM. Therefore, a Cu_Sub concentration of 10 nM was adopted in future experiments. In addition, the effect of DNAzyme reaction time on the cleavage of Cu_Sub was explored. As shown in Fig. S1D, the ΔF was enhanced with the increase of the reaction time and leveled off after 80 min. As a result, 80 min was chosen as the optimized reaction time. Moreover, the amount of Cas12a and the ratio of Cu_Sub to Cu_Enz were optimized. As can be seen in Fig. S1E, the ΔF increased with the increase of the Cas12 concentration, and remained constant when the Cas12a concentration was above 15 nM. Meanwhile, the ΔF value reached maximum when the molar ratio of Cu_Sub to Cu_Enz was 1:2 (Fig. S1F). Hence, 15 nM Cas12a and the molar ratio of 1:2 were selected as the optimal conditions.

3.4. Analytical performance for ALP activity detection

The analytical performance of the DNAzyme-regulated CRISPR/Cas12a sensing system was investigated under optimum conditions. The fluorescence signal of the sensing system decreased gradually with the addition of different concentrations of ALP (0–10 U/L) (Fig. 2A). Furthermore, the results showed an excellent linear relationship between the fluorescence intensity change at 520 nm and the

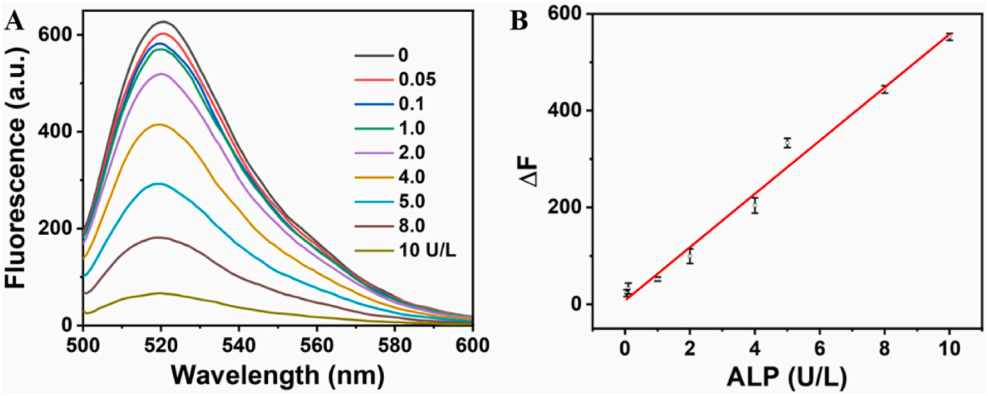


Fig. 2. (A) Fluorescence spectra of the sensing system in the presence of different concentrations of ALP (0, 0.05, 0.1, 1.0, 2.0, 4.0, 5.0, 8.0 and 10 U/L). (B) Linear relationship of the fluorescence intensity change at 520 nm versus the concentration of ALP ranging from 0.05 to 10 U/L. Error bars indicated the standard deviations of three measurements.

concentration of ALP over the range of 0.05–10 U/L (Fig. 2B). The regression equation is $\Delta F = 8.018 + 54.98C$ ($R^2 = 0.991$), where ΔF and C represent the fluorescence intensity change at 520 nm and the ALP concentration (U/L), respectively. The limit of detection (LOD) was estimated to be as low as 0.04 U/L (S/N = 3), which is comparable to or more sensitive than those of some reported fluorescence methods for ALP detection (Table S2). Herein, the high sensitivity of the proposed method is a result of a DNAzyme-regulated CRISPR/Cas12a cascade signal amplification strategy. Moreover, it was necessary to evaluate the selectivity of the ALP activity detection system. To accomplish this, various competitive proteins and enzymes, bovine serum albumin (BSA), glucose oxidase (GOx), glutathione reductase (GR), urease, and glutathione (GSH), were separately added into the sensing system instead of ALP. As illustrated in Fig. 3, the fluorescence intensity of the system containing the nonspecific species was almost the same as that of the blank. However, the fluorescence intensity of the system with ALP dramatically decreased, and there was no significant change when mixed with the above interfering substances. These results revealed that the proposed fluorescent strategy exhibited high specificity for ALP.

3.5. ALP inhibitor efficiency evaluation

The screening of effective ALP inhibitors is an essential pathway of drug development. To evaluate the DNAzyme-regulated CRISPR/Cas12a sensing system for ALP inhibition assay, TP was selected as a model inhibitor. As shown in Fig. 4, the fluorescence signal of the sensing system gradually increased with an increase in the concentration of TP. The half-maximal inhibitory concentration (IC_{50}) of TP was calculated as 86.2 μM for 10 U/L ALP, which is comparable to previous reports [39, 40]. These results indicate that the proposed sensing system can be used for evaluating inhibition efficiency and screening ALP inhibitors.

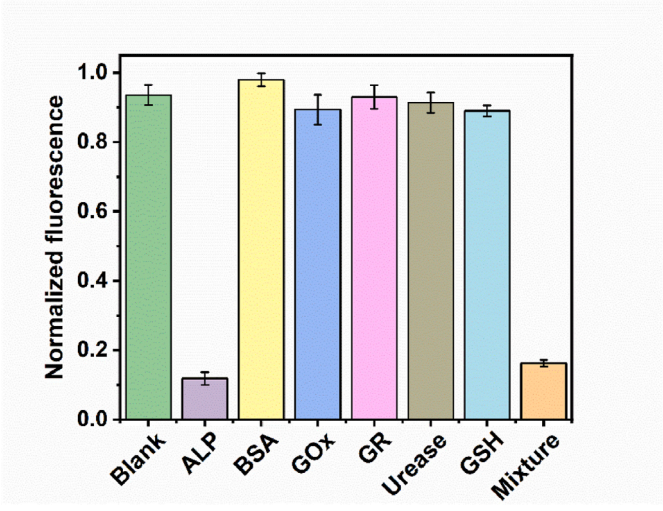


Fig. 3. Selectivity of the ALP activity detection system toward various species: ALP, BSA, GOx, GR, urease and GSH, the concentrations of ALP and GR were 10 U/L and 200 U/L, the others were 20 μg/mL. Error bars were estimated from three replicate measurements.

3.6. Serum samples analysis

In order to evaluate the viability of the fluorescent sensing strategy for clinical analysis, four diluted serum samples containing different concentrations of ALP were detected by the proposed method. The results are shown in Table 1. The concentration of ALP in the undiluted serum from a patient with liver disease was calculated as 337 U/L, which exceeds the normal range of serum ALP in healthy adults (40–190 U/L) and is consistent with the clinical diagnosis. Moreover, the recoveries of the spiked samples were in the range of 94.6–103.9%, indicating that the proposed sensing strategy has significant potential for ALP activity assay in human serum samples.

The proposed fluorescent strategy was further applied to determine the level of ALP in mouse serum. As shown in Table 2, an acceptable recovery of 89.6–111.9% was obtained with a relative standard deviation (RSD) lower than 9.3%. The results demonstrate that the developed method is suitable for detecting ALP activity in mouse serum. In addition, the proposed fluorescent assay was used to explore the protective effect of TP on acetaminophen (APAP)-induced acute liver injury in mice. Fig. 5 illustrates the levels of ALP in the serum of three experimental groups. Compared with the control group, the level of ALP in the APAP group increased significantly, while TP pretreatment showed a significant recovery of the serum ALP level. The effect of protecting liver of TP was further confirmed by the hepatic histological findings (Fig. S2), swelling of liver cells and inflammatory cell infiltration were observed in APAP-treated mice, while these histological changes were significantly suppressed by TP pretreatment.

4. Conclusion

In summary, a novel and sensitive fluorescent biosensor has been developed for ALP activity and inhibition detection based on a DNAzyme-regulated CRISPR/Cas12a signal amplification strategy. The developed biosensor for ALP exhibited a wide linear range from 0.05 to 10 U/L with a LOD of 0.04 U/L. Moreover, the proposed fluorescent strategy was successfully applied for the determination of ALP activity in serum samples and the evaluation of the inhibitory effect of TP on ALP activity. Importantly, this method was further used to investigate the

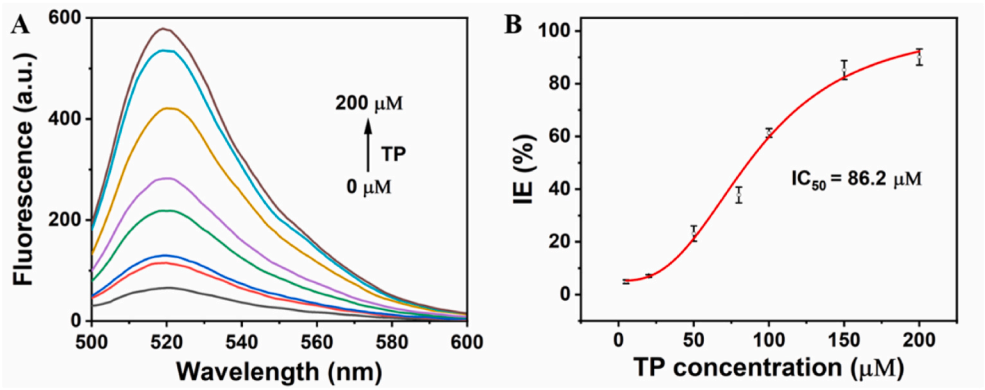


Fig. 4. (A) Fluorescence spectra of the sensing system with addition of different concentrations of TP in the presence of 10 U/L ALP; (B) Effect of TP concentration on ALP inhibition efficiency (IE, %). Error bars represented the standard deviations of three measurements.

Table 1
Detection of ALP activity in human serum samples[a] (mean ± SD[b], n = 3).

Sample	Added (μM)	Found (μM)	Recovery (%)	RSD (%)
1	0	2.25 ± 0.26	–	11.6
2	2.00	4.02 ± 0.40	94.6	10.0
3	20.0	23.1 ± 1.70	103.9	7.4
4	200	197 ± 16.2	97.6	8.2

[a] The serum samples were diluted 150-fold.
[b] The standard deviation of three measurements.

Table 2
Detection of ALP activity in mouse serum samples[a] (mean ± SD[b], n = 3).

Sample	Added (μM)	Found (μM)	Recovery (%)	RSD (%)
1	0	9.44 ± 0.26	–	2.8
2	4.0	12.0 ± 1.13	89.6	9.3
3	8.0	19.5 ± 0.94	111.9	4.8
4	40	48.4 ± 3.64	97.9	7.5

[a] The serum samples were diluted 20-fold.
[b] The standard deviation of three measurements.

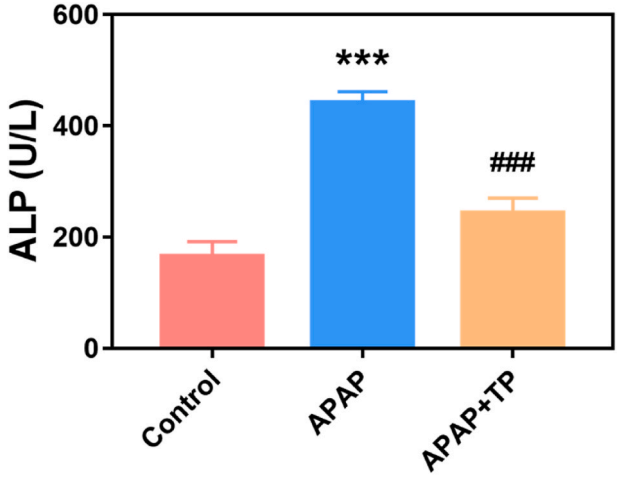


Fig. 5. Serum ALP levels. n = 4/group. The data were expressed as mean ± SD. ***P < 0.001 vs. control group, ###P < 0.001 vs. APAP group.

effect of protecting liver of TP, which will likely open a new direction for the screening of active ingredients from TCM using CRISPR/Cas12-based bioassays. It is envisioned that this method may hold potential applications in clinical diagnostics and drug screening.

CRediT authorship contribution statement

Yufen Lai: Methodology, Investigation, Formal analysis. **Mengyan Li:** Validation, Investigation, Visualization. **Xiaofei Liao:** Investigation, Data curation. **Li Zou:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2022.340518.

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