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Enzyme inhibition-enabled CRISPR/Cas12a biosensing system for heparin-related non-nucleic acid biomarkers

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Different from conventional CRISPR/Cas12a systems that must employ sophisticated functional nucleic acids, protein switch or allosteric transcription factors (aTFs)-based signal conversion for non-nucleic acid analysis, this work realizes more facile non-nucleic acid biomarker quantification through a new heparin-mediated Cas12a inhibition route.

CRISPR/Cas system not only pioneers a new era of genetic engineering,[1,2] but also shows great potential in biosensing applications.[3-6] Particularly, as the discovery of the nucleic acid substrate-actuated collateral cleavage activity of Cas12a/Cas13a proteins[7,8] that can achieve efficient signal amplification, the CRISPR/Cas-based biosensing systems, such as DETECTR,[9] SHERLOCK,[10] HOLMES,[11] and CONAN,[12] have received significant credit in sensitive nucleic acid biosensing. It should be noted that contrary to the plentiful research on nucleic acid detection,[13-15] the detection of non-nucleic acid targets via CRISPR/Cas system is still in its infancy. In recent years, several CRISPR/Cas strategies have been proposed for the analysis of protein targets,[16-19] small molecules,[20] bacterial pathogens,[21] cancer cells,[22] and metal ions[23] with the help of functional nucleic acids such as the aptamer[24] and DNAzyme,[23] aTFs[25,26] or protein switch.[17,18] Nonetheless, most of these strategies must require complicated design and multiple assay steps to convert target recognition to the generation of nucleic acid substrates so as to activate the Cas activity. Therefore, expanding CRISPR/Cas toolbox for non-nucleic acid biomarker analysis is still of great significance.

Quite recently, heparin, a natural anticoagulant drug widely used in the treatment of thrombotic diseases,[27] has been proved to be capable of binding with Cas12a to disable its *trans*-cleavage ability.[28,29] Herein, taking advantage of the inhibition effect of heparin on Cas12a, a new non-nucleic acid biomarker analysis approach is constructed for the quantification of heparinase and heparin binding protein (HBP). Specifically, the inhibitory effect of heparin on Cas12a can be regulated through hydrolyzing high molecule weight-heparin into small fragments by heparinase or removing heparin from the reaction system by specific binding with HBP. As thus, the quantitative information of heparinase and HBP can be facilely reflected by the fluorescent signal produced by Cas12a *trans*-cleavage of fluorophore/quencher co-labeled ssDNA probe (defined as FQ-labeled reporter). This new enzyme inhibition-enabled CRISPR/Cas12a system pioneers a new direction for detecting heparin-related non-nucleic acid targets.

Fig. 1 demonstrates the design principle of the proposed enzyme inhibition-enabled CRISPR/Cas12a system for heparin-related non-nucleic acid biosensing. As is widely acknowledged, the single-stranded DNA (ssDNA) activator can activate the Cas12a *trans*-cleavage activity by hybridizing with the crRNA.[7] Nevertheless, as shown in Fig. 1a, heparin can combine with Cas12a enzyme to prevent the Cas12a/crRNA binding process, which inhibits the *trans*-cleavage ability of the Cas12a system to digest the FQ-labeled reporter, resulting in a negative fluorescence signal.[28,29] Based on this phenomenon, by changing the heparin's length and amount to regulate the inhibitory effect of heparin on Cas12a, the heparin-enabled CRISPR/Cas12a biosensing system can be constructed. On the one hand, as displayed in Fig. 1b, with the introduction of heparinase, which is involved in various medical applications, the high molecular weight heparin can be hydrolyzed into small fragments,[30,31] decreasing the inhibitory effect on Cas12a. On the other hand, target-responsive removal of heparin can also be employed for sensing non-nucleic acid biomarkers such as HBP, whose concentration is positively correlated to the bacterial infection extent.[32] As illustrated in Fig. 1c, the HBP antibody-functionalized MBs can bind with HBP, which can take

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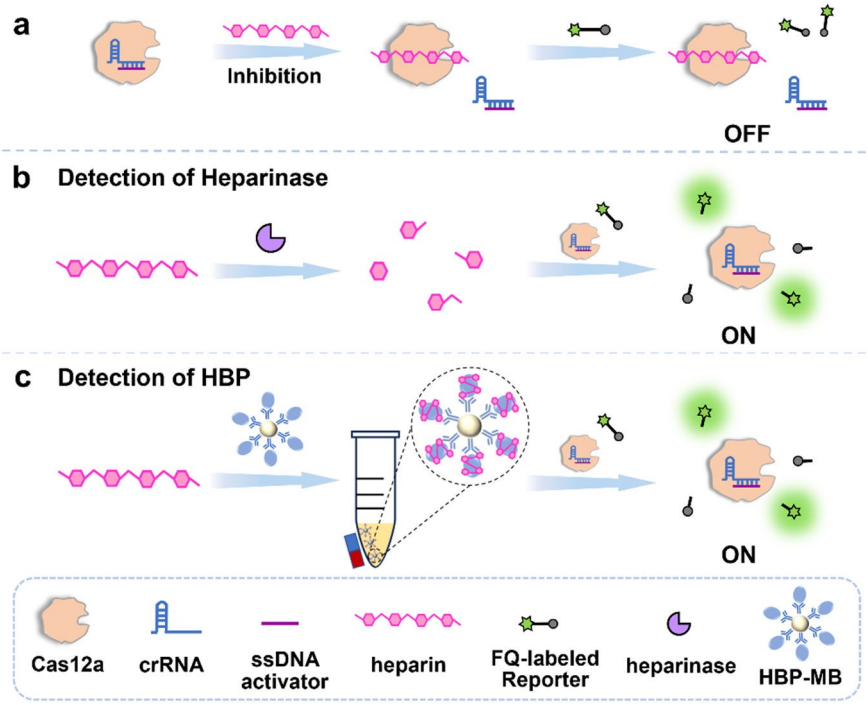

Fig. 1 (a) Heparin can effectively inhibit the *trans*-cleavage ability of Cas12a; (b) Schematic illustration of the enzyme inhibition-enabled CRISPR/Cas12a system for heparinase analysis; (c) Schematic illustration of the enzyme inhibition-enabled CRISPR/Cas12a system for HBP analysis.

away heparin from solution with the facilitation of magnetic separation. Therefore, the inhibitory effect of heparin on Cas12a is decreased with the increased dosage of heparinase and HBP, achieving the recovery of Cas12a *trans*-cleavage activity and positive fluorescence signal.

The heparin's inhibitory effect on the Cas12a system is firstly verified. As displayed in Fig. 2a, the FQ-labeled reporter can be effectively cleaved by the Cas12a/crRNA system to generate a strong fluorescence response (black line) in the presence of ssDNA activator. However, after introducing the heparin (blue line), the fluorescence signal is remarkably reduced to a quite low level that is almost the same as that produced by only Cas12a/crRNA but without ssDNA activator (red line). These results clearly indicate that heparin can effectively inhibit the *trans*-cleavage enzymatic activity of Cas12a. The underlying inhibitory mechanism has been proved to be that heparin competes with crRNA to bind with Cas12a.[28,29]

To obtain the best inhibitory effect, a series of different concentrations of heparin are introduced into the Cas12a *trans*-cleavage system under fixed Cas12a/crRNA/ssDNA activator concentrations (all of 12.5 nM). As can be seen from Fig. 2b, the fluorescence response decreases rapidly with the heparin dosage increasing from 0 to 175 ng/mL, while then keeps almost

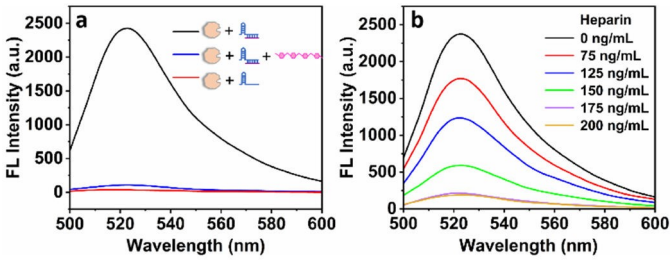

Fig. 2 (a) Investigating the inhibitory effect of heparin on the Cas12a system. 12.5 nM Cas12a, crRNA, and ssDNA activator for black line; 12.5 nM Cas12a, crRNA, and ssDNA activator, 175 ng/mL heparin for blue line; 12.5 nM Cas12a and crRNA for red line. (b) Fluorescence spectra of the Cas12a system under different concentrations of heparin with 12.5 nM Cas12a, crRNA, and ssDNA activator.

stable when the heparin exceeds 175 ng/mL. Therefore, 175 ng/mL heparin is enough to effectively disable the *trans*-cleavage ability of the used Cas12a system, which is selected as the optimal heparin concentration in subsequent heparinase and HBP analysis.

Taking benefit of heparin's effective inhibitory effect on Cas12a, the feasibility and performance of the proposed

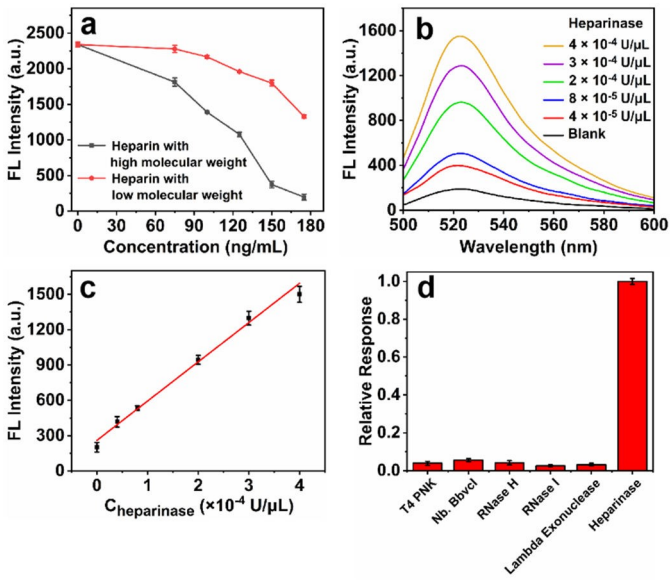

Fig. 3 (a) The inhibitory effect on Cas12a of heparin with different molecular weights. (b) Fluorescence spectra of the proposed assay in the presence of different concentrations of heparinase. (c) Linear relationship between the fluorescence intensity (at 520 nm) and the concentration of heparinase. (d) Relative fluorescence responses aroused by different enzymes (all of 3×10^{-4} U/μL). The signal response of the 3×10^{-4} U/μL heparinase subtracted by the signal response of blank is normalized to be 1. Error bars represent the standard deviation of three replicate tests.

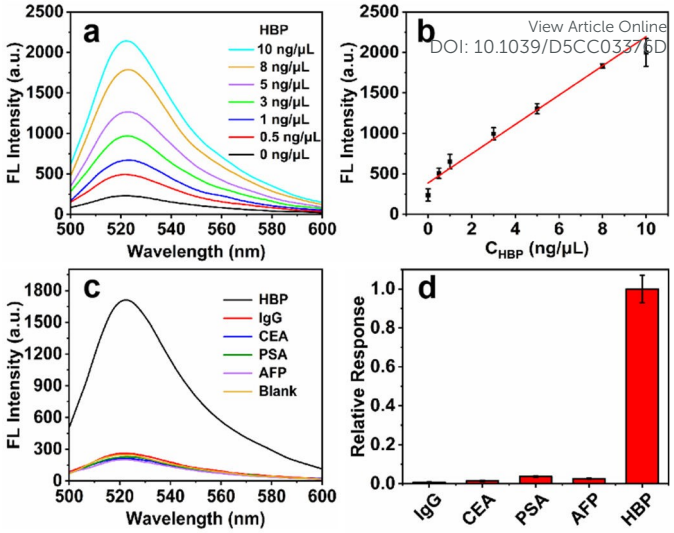

Fig. 4 (a) Fluorescence spectra of the proposed assay system in the presence of different concentrations of HBP. (b) Linear relationship between the fluorescence intensity (at 520 nm) and the concentration of HBP. (c) Fluorescence responses aroused by different proteins. (d) Relative fluorescence responses aroused by different proteins (all of 8 ng/μL). The signal response of the 8 ng/μL HBP subtracted by the signal response of blank is normalized to be 1. Error bars represent the standard deviation of three replicate tests.

enzyme inhibition-based CRISPR/Cas12a system was firstly applied to detect heparinase activity. As the heparinase can hydrolyze the heparin into small fragments, the inhibition effect of heparins with different molecular weights (15000~19000 for the high molecular weight (HMW), and 3800~5000 for the low molecular weight (LMW)) is investigated in the first place. As exhibited in Fig. 3a, the HMW heparin shows a concentration-related rapid inhibition behavior. In contrary, the fluorescence signal decreases more slowly when the LMW heparin of the same concentration is introduced, which verifies that the inhibitory effect can be largely eliminated if the heparin is hydroxylated by heparinase.

With this prerequisite, the heparinase is analyzed under 12.5 nM Cas12a/crRNA/ssDNA activator. As can be seen from Fig. 3b, the fluorescence signal at 520 nm (F) increases gradually with the concentration of heparinase ($C_{Heparinase}$) rising from 0 to 4×10^{-4} U/μL, and the response aroused by as low as 4×10^{-5} U/μL of heparinase can be clearly discriminated from blank control. There is a good linear relationship between F and $C_{Heparinase}$ (Fig. 3c), and the correlation equation is $F = 333.29\ C_{Heparinase}$ (× 10^{-4} U/μL) + 259.93 with a correlation coefficient of $R^2 = 0.9904$. What's more, other types of enzymes, including T4 polynucleotide kinase (T4 PNK), nicking endonuclease Nb. BbvcI, RNase H, RNase I, and Lambda Exonuclease, are applied to evaluate the specificity of the proposed assay for heparinase analysis. It can be seen from Fig. 3d that a significant signal can be monitored only when heparinase is introduced, suggesting the good specificity of the proposed assay. It should be noted that the detection sensitivity of this method is superior to or at least comparable to many of the currently reported heparinase detection assays (Table S2). To further investigate the potential feasibility of the proposed assay in complex samples, heparinase is spiked into 1% fetal bovine serum (FBS) to conduct the proposed assay. As shown in Fig. S1, the fluorescence signals are coincident with those in pure buffer, suggesting the potential usefulness of this assay for clinical diagnosis. These results clearly demonstrate the high feasibility of the proposed enzyme inhibition-enabled CRISPR/Cas12a system for heparinase analysis.

Consequently, based on the specific binding capacity of HBP to heparin, we conducted the HBP analysis. According to the principle illustrated in Fig. 1c, the more HBP is introduced, the more heparin is taken away from the solution by the HBP antibody-functionalized magnetic MBs. Therefore, the *trans*-cleavage activity of Cas12a as well as the resulted fluorescence signal will be positively related to the HBP concentration. As can be seen from Fig. 4a, under 12.5 nM Cas12a/crRNA/ssDNA activator, the fluorescence intensity (F) increases with the HBP concentration (C_{HBP}) ascending from 0 to 10 ng/μL. The linear relationship between the F and C_{HBP} is displayed in Fig. 4b, and the linear correlation equation is $F = 180.80\ C_{HBP}$ (ng/μL) + 387.44 with the correlation coefficient R^2 of 0.9916. It is worth noting that the direct binding of HBP with heparin can also effectively suppress the inhibition effect of heparin on the Cas12a system, which allows for the homogeneous HBP sensing. As illustrated in Fig. S2a, when HBP combines with heparin in the homogeneous solution, the level of unbound heparin in the solution decreases in an HBP dosage-responsive way. Therefore, the introduction of HBP to the heparin-inhibited Cas12a system will result in an increased fluorescence signal. As can be seen from Fig. S2b and c, the fluorescence signal at 520 nm increases gradually with the ascending concentration of HBP with a good

linear relationship. Although the direct HBP-heparin binding in homogeneous solution is also feasible to regulate the inhibitory effect on Cas12a, to cater to the specific and precise HBP analysis in real-world clinical applications, magnetic beads are required to capture and separate HBP from complex matrices. Therefore, the magnetic separation-based HBP detection well complements the homogeneous system in different application scenarios to meet actual needs. Furthermore, different kinds of proteins are randomly selected and tested to evaluate the specificity of the proposed assay for HBP analysis. As can be seen from Fig. 4c and d, only HBP can arouse a strong fluorescence response, whereas the fluorescence signals aroused by other non-specific proteins are almost the same as that of the blank control, indicating that the proposed assay possesses an excellent selectivity for HBP.

In summary, based on the heparin's inhibitory effect on Cas12a, a new enzyme inhibition-enabled CRISPR/Cas12a biosensing system is proposed for the analysis of heparinase and HBP. This new principle realizes the simple and facile CRISPR/Cas-based non-nucleic acid biomarker analysis, which well complements to the existing functional nucleic acids, protein switch or aTFs-assisted sensing protocols, and expands the toolbox of CRISPR/Cas-based biosensing systems.

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Data availability

All data supporting the findings of this study are available within the article and ESI.

Conflicts of interest

There are no conflicts of interest to declare.

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Data availability statements

All data supporting the findings of this study are available within the article and ESI.