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Heparin Specifically Inhibits CRISPR/Cas12 Activation, Enabling Ultrasensitive Heparin Detection and Gene Editing Regulation

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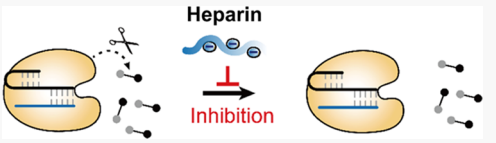
ABSTRACT: Heparin is a highly sulfated linear glycosaminoglycan that is used as an anticoagulant to prevent and treat thrombotic diseases. Herein, we find that heparin specifically inhibits the activation of the Cas12 protein through the competitive binding of heparin and crRNA to Cas12. Studies illustrate that heparin's high molecular weight and strong negative charge are critical parameters for its inhibitory effect. This unexpected finding was engineered for the detection of heparin, affording a low detection limit of 0.36 ng/mL for fluorometric quantification. We further developed a rapid lateral flow-based system named HepaStrip (heparin strip), allowing heparin monitoring in clinical samples within 20 min. Finally, *in vivo* investigations revealed that heparin can regulate gene editing with the clusters of the regularly spaced short palindromic repeat (CRISPR)/Cas12 system in *Escherichia coli*. Heparin blocks the formation of Cas12-crRNA ribonucleoprotein, allowing the application of CRISPR for rapid and field-deployable detection of heparin and unleashing the potential use of heparin in future anti-CRISPR applications.



INTRODUCTION

CRISPR/Cas is composed of clusters of regularly spaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins, which can resist invasive foreign genetic elements.[1,2] The CRISPR/Cas12a system (Cpf1) is a single RNA-guided endonuclease showing extraordinary characteristics in gene editing.[3] Cas12a and crRNA form a Cas12a−crRNA ribonucleoprotein (RNP), allowing the identification and cutting of target genes. Cas12a can be guided to cut almost any sequence of interest by altering the crRNA sequences.[4−6] After RNP binds to the target DNA, Cas12a sequentially cuts each strand of DNA through the RuvC nuclease domain by *cis* cleavage and, meanwhile, nonspecifically cuts nearby ssDNA through *trans* cleavage.[7] The target DNA-induced cleavage of Cas12a provides a flexible and convenient way for both genome editing and bioanalysis.[8,9] In particular, CRISPR/Cas12-based analytical systems possess high specificity, sensitivity, and programmability, revolutionizing the field of diagnostics and biosensing.[10−12]

Recently, the CRISPR/Cas system has been widely adopted for diagnostic and therapeutic applications.[13,14] Nevertheless, the means to control the CRISPR process are limited, posing security risks in the CRISPR/Cas technology. The longer the CRISPR/Cas system remains active in the cell, the more likely it is to cause unnecessary editing and dangerous side effects; therefore, it is critical to inactivate CRISPR/Cas after finishing its job.[15] For example, the sustained CRISPR/Cas9 editing of the cell genome will inevitably lead to p53 dysfunction, which is a critical tumor-suppressor gene, significantly increasing the cancer risk.[16,17] Existing anti-CRISPR technologies mainly include anti-CRISPR (Acr) proteins, drugs, or modified Cas enzymes to control the CRISPR system.[18,19] Although several strategies have been proposed, all current anti-CRISPR agents are heterologous proteins or newly developed chemicals that have unclear safety in humans and require significant efforts to reveal their clinical validity and safety. The application of existing drugs to control the CRISPR/Cas system would represent a significant discovery.

Heparin, a highly sulfated glycosaminoglycan, interacts with cationic proteins due to its negative charge and structure, intervening in various physiological processes.[20] Heparin is a clinically used anticoagulant and can effectively prevent thrombosis by inactivating the coagulation factors.[21] In addition to its well-known anticoagulant effects, heparin also has potential pharmaceutical applications,[22] including anti-inflammatory activity and cancer progression control. Nevertheless, its narrow therapeutic range and potential complications necessitate careful monitoring.[23−25] However, clinical heparin assays, such as activated clotting time (ACT) and activated partial thromboplastin time (aPTT), have limitations like high cost and impracticality at high heparin doses.[26] In cardiac surgery, conventional monitoring methods are influenced by anticoagulant factors, leading to challenges in

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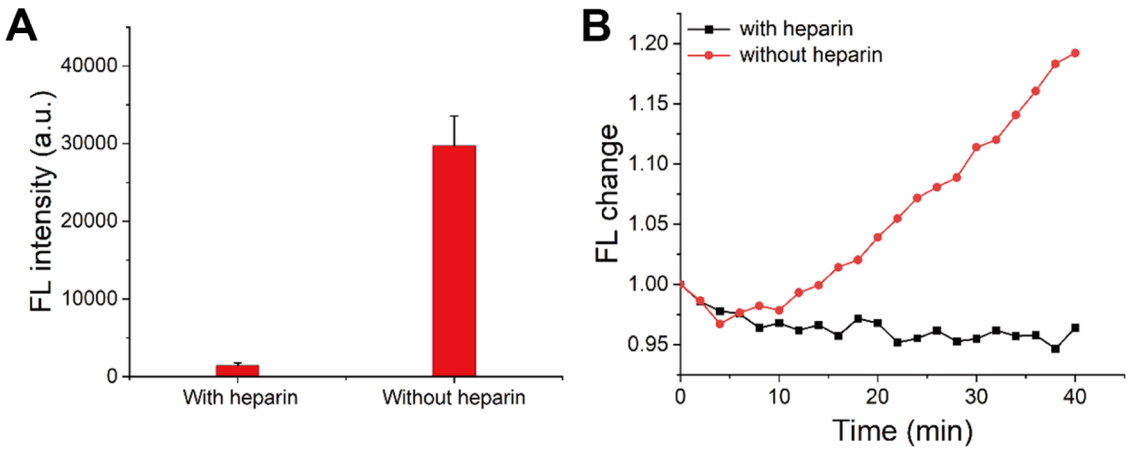

Figure 1. Inhibitory effect of heparin on Cas12a. (A) Fluorescence intensity of the reaction system in the presence and absence of heparin. (B) Fluorescence changes of the CRISPR/Cas12a reporting system affected by heparin. Fluorescence change: F/F_o (F and F_o are fluorescence intensities at times t and 0, respectively).

quantifying circulating heparin levels.[27,28] Fluorescence detection, despite its advantages of sensitivity and simplicity, faces difficulty distinguishing heparin from chondroitin sulfate (CS), hyaluronic acid (HA), and low-molecular-weight heparin (LMWH).[29,30] Therefore, there is still a need to develop methods for heparin analysis with an improved performance.

Surprisingly, in this study, we found that heparin, a conventional anticoagulant drug, can inhibit the CRISPR/Cas12a system. Heparin noncovalently interacts with Cas12a, rigidifying the conformation of Cas12a and preventing the Cas12a−crRNA RNP formation. The newly discovered inhibitory effect was repurposed for the sensitive and precise detection of heparin, enabling the distinction of heparin from most other glycosaminoglycans using portable test strips. Furthermore, utilizing a green fluorescent protein (GFP)-reported CRISPR/Cas12a gene editing system, we confirmed that the inhibitory effect of heparin on Cas12a could be extended to the *in vivo* environment. This research reveals, for the first time, that heparin can inhibit Cas12a both *in vitro* and *in vivo*, demonstrating a highly specific assay to monitor heparin and broadening the diversity of anti-CRISPR strategies.

EXPERIMENTAL SECTION

Detection of Heparin Based on the CRISPR/Cas12a System. The CRISPR/Cas12a reporting mix was prepared by mixing 6 μL of CRISPR-derived RNA (crRNA, 100 nM), 3 μL of 10× buffers (pH 7.9), 1.5 μL of FQ reporter (10 μM), and 1.5 μL of Cas12a (200 nM) at 25 °C for 10 min. Different concentrations of heparin were added to the Cas12a−crRNA−FQ reporter complex at 37 °C for 5 min to allow the binding. A total of 24 μL of the reaction solution described above was mixed with 6 μL of DNA probe. The resulting reaction mixture was incubated at 37 °C for 30 min and then inactivated at 65 °C for 10 min. The fluorescence was measured by a microplate reader (λ_{ex} = 484 nm, λ_{em} = 530 nm, fluorescence measured from the top). The same protocol was followed for the real samples.

Lateral Flow Detection. To detect heparin with lateral flow test strips (Genenode, China), the assay was modified as follows: (1) the concentration of Cas12a protein was increased to 500 nM; and (2) the FAM-ssDNA-biotin reporter (FB reporter, 10 μM) was used to replace the FQ reporter. The reaction mixture was incubated at 37 °C for 30 min and inactivated at 65 °C for 10 min; then, 50 μL of reaction droplets was added to the sampling area and allowed to incubate for 2−5 min. Finally, the strips were photographed with a smartphone camera and analyzed with Adobe Illustrator (AI).

RESULTS AND DISCUSSION

Inhibitory Effect of Heparin on Cas12a. To verify the inhibitory activity of heparin, a rapid fluorescence evaluation system was constructed *in vitro*. When the activator (ssDNA), Cas12a−crRNA, and ssDNA labeled with FITC and BHQ1 (FQ reporter) were mixed (sequences in Table S1), strong fluorescence was observed (Figure 1A). This result indicates that the activator initiated the DNase activity of Cas12a, causing the shredding of the FQ reporter due to *trans* cleavage. Nonetheless, there was a negligible enhancement of fluorescence when heparin was added to the system, suggesting that heparin inhibited the *trans*-cleavage activity of Cas12a for the FQ reporter. Additionally, similar inhibitory effects were observed in dsDNA-triggered Cas12a activation systems (Figure S1). Further analysis, evident from the opposing fluorescence in the sample with and without heparin, confirmed that heparin potently inhibited the cleavage activity of Cas12a (Figure 1B). Therefore, we confirmed that heparin could intervene in Cas12a activation and cleavage, an unprecedented result that had not been previously reported.

Inhibition Mechanism of Heparin toward Cas12a. To understand the underlying principle of the inhibitory effect (Figure 2A), we put forward two hypotheses based on the mechanism of Cas12a activation and the conformational changes required for complete R-loop formation (crRNA−DNA heteroduplex formation): (1) heparin impacts the formation of Cas12a−crRNA RNP (see Figure 2B) and (2) heparin impacts the binding of the Cas12a−crRNA complex to the activator, thereby affecting the cleavage activity of Cas12a (see Figure 2C). Based on the hypotheses, we conducted the corresponding fluorescence experiments. Curve 1 (heparin preincubated) showed lower fluorescence increases than curve 3 (without heparin), indicating that the preincubation between Cas12a and heparin resulted in the activation of Cas12a, suggesting that heparin affected the subsequent formation of Cas12a−crRNA RNP. There was little difference between

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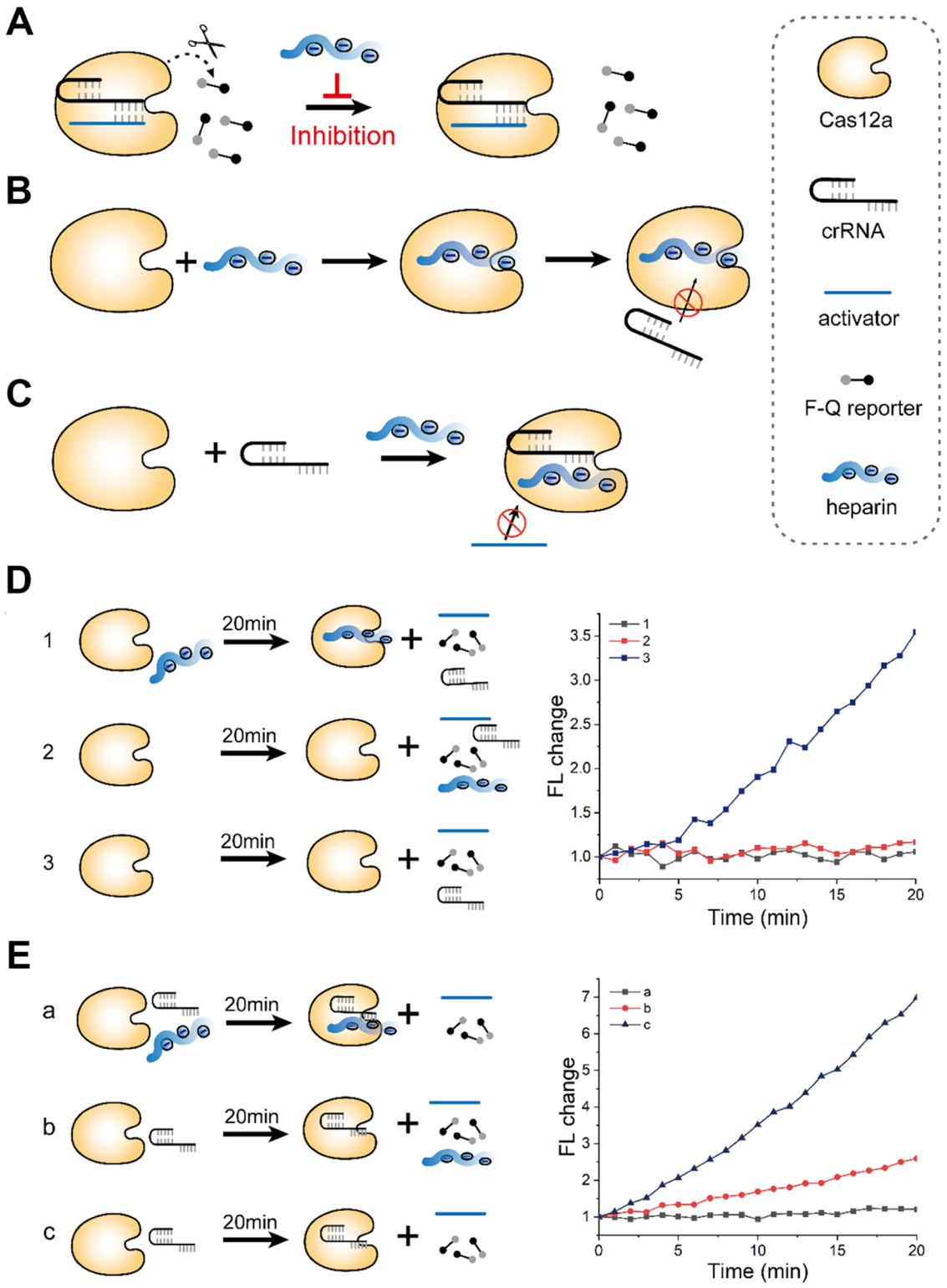

Figure 2. (A–C) The possible ways that heparin inhibits Cas12a activation: (A) heparin inhibits the CRISPR/Cas12a system, (B) heparin binds to Cas12a to inhibit the formation of Cas12a–crRNA, and (C) heparin intervenes in the recognition between Cas12a–crRNA and the activator. (D, E) Readout of the fluorescence intensity after adding heparin in a different order: (D) Cas12a reacts with heparin for 20 min before the addition of the activator–crRNA–FQ reporter (curve 1), Cas12a was incubated under the same conditions for 20 min before the addition of heparin and the activator–crRNA–FQ reporter (curve 2), and Cas12a reacts with PBS for 20 min before the addition of the activator–crRNA–FQ reporter (curve 3); and (E) Cas12a–crRNA reacts with heparin for 20 min before the addition of the activator–FQ reporter (curve *a*), Cas12a–crRNA was incubated under the same conditions for 20 min before the addition of heparin and the activator–FQ reporter (curve *b*), and Cas12a–crRNA reacts with PBS for 20 min before the addition of the activator–FQ reporter (curve *c*).

curves 2 and 1, suggesting that even if Cas12a and heparin were not preincubated, heparin could rapidly bind to Cas12a upon addition, inhibiting the activity of Cas12a (Figure 2D). The fluorescence increase in curve *a* (Cas12a–crRNA

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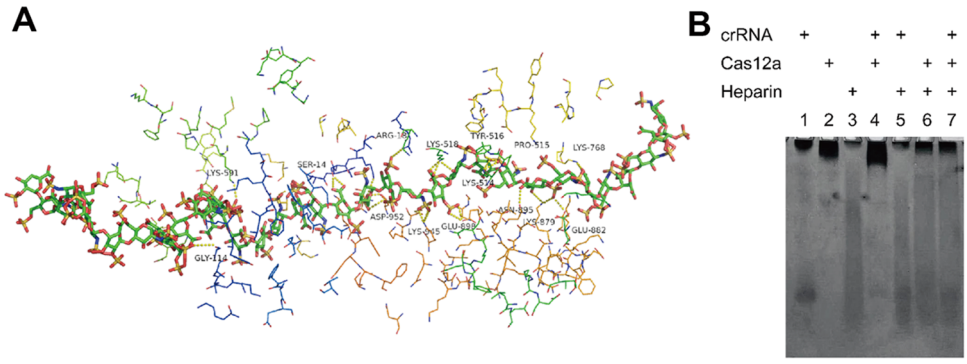

Figure 3. Mechanism of heparin toward Cas12a. (A) Schematic diagram of the amino acid site of the heparin ligand-binding Cas12a protein. (B) Combination of heparin and Cas12a was verified by silver staining with native polyacrylamide gel electrophoresis (native-PAGE). The final concentrations of crRNA, heparin, and Cas12a were 1.2 μM, 0.6 mg/mL, and 0.6 μM, respectively.

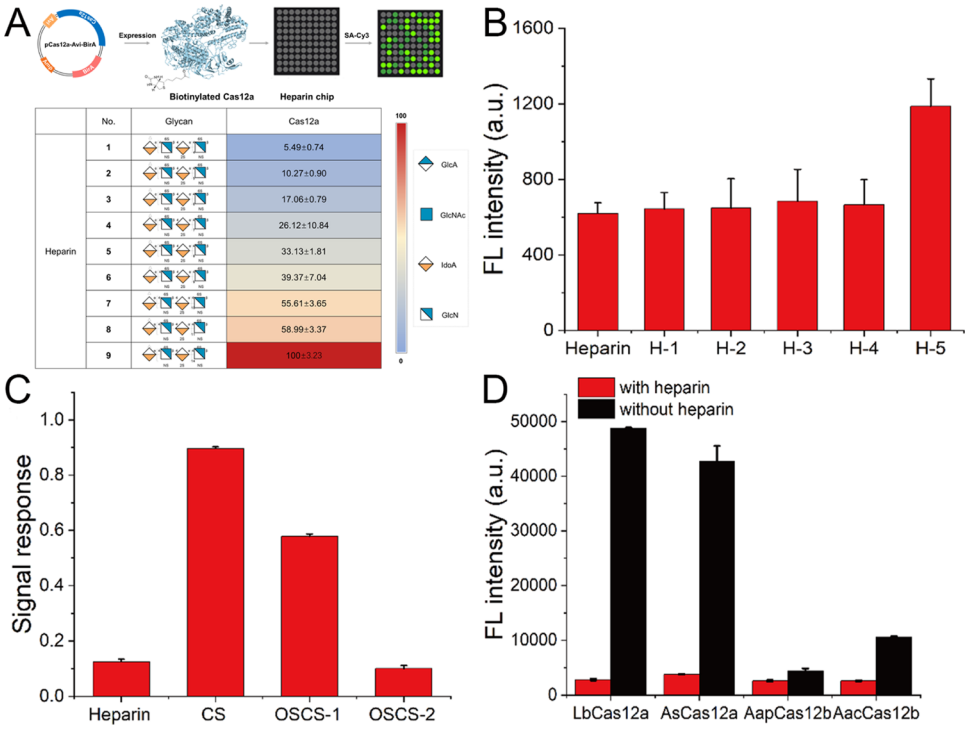

Figure 4. Interaction between heparin and Cas12a. (A) Schematic representation of the heparin chip assay (top). Heatmap of the relative binding of biotinylated Cas12a to each member of the heparin library. Binding signals were normalized with respect to the compound with the greatest binding. The errors represent ±SD across technical replicates (n = 4) (below). (B) Fluorescence intensities of heparin with different molecular weights (H-1 > H-2 > H-3 > H-4 > H-5). (C) Comparison of heparin with CS and two forms of oversulfated chondroitin sulfate (OSCS). Their concentration is 500 ng/mL. Signal response: the ratio of the fluorescence intensity between the sample and heparin and the blank group. (D) Fluorescence intensities of various Cas12 proteins in the presence of heparin.

preincubated with heparin) was much lower than that in curve *c* (Cas12a−crRNA preincubated without heparin), indicating that the *trans*-cleavage activity of Cas12a was inhibited, suggesting that heparin−Cas12a binding was stronger than the crRNA−Cas12a binding. Notably, the Cas12a−crRNA RNP in curve *b* was formed before the addition of heparin, and the fluorescence increase was significantly faster than that in curve *a*, indicating that part of Cas12a−crRNA could activate enzyme activity after binding with the activator (Figure 2E). These results suggest that heparin directly interacts with Cas12a, affecting the formation of Cas12a−crRNA RNP by disabling Cas12a, supporting the hypothesis that heparin impacts the formation of Cas12a−crRNA RNP.

Cas12a adopts a bilobed structure with a central, highly positively charged groove, which is the likely region where a highly electronegative heparin chain directly interacts. Hence, we conducted computational studies to investigate the binding characteristics of Cas12a to heparin. The simulation showed that the Cas12a protein is composed of more than 40 α helices, more than 20 β-sheets, and random coils (Figure S2) and the hydrophobic and positively charged groove of Cas12a is located on the protein surface. Moreover, the heparin molecule runs through the entire active site, interacting in a noncovalent manner (Figures S3 and 3A). Heparin enters the active site of Cas12a mainly through hydrophobic and van der Waals forces, and the results show that heparin can form numerous hydrogen bonds with Cas12a (Table S2), stabilizing

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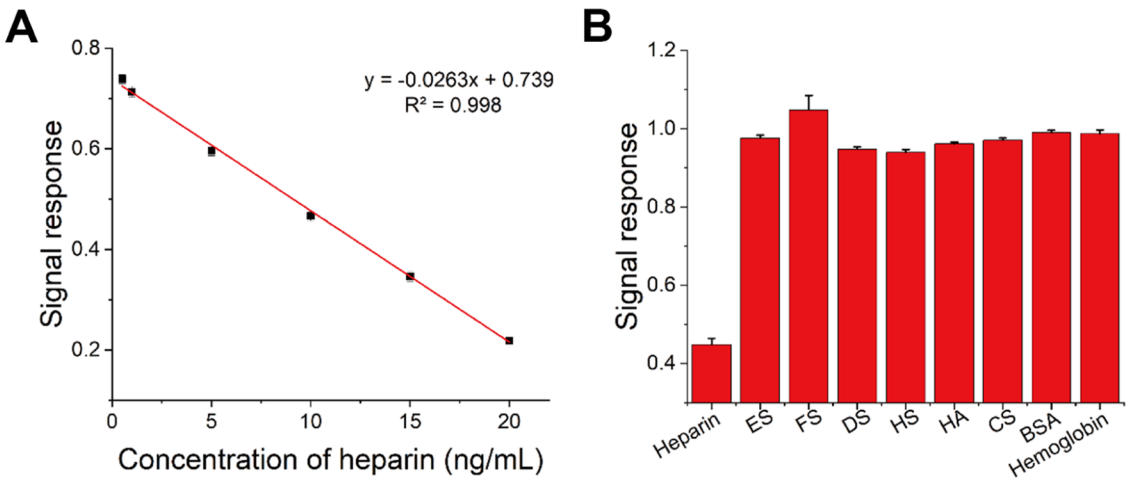

Figure 5. Sensitive and selective analyses of heparin. (A) Linear relationship between the fluorescence signal and the concentration of heparin using the FQ reporter for quantification. (B) Selectivity of the method for heparin. The concentrations of interferon and heparin were 100 and 10 ng/mL, respectively. Signal response: the ratio of the fluorescence intensity of the heparin-containing sample to that of the blank reaction mixture. Here, DAS, ES, FS, CS, HA, HS, and BSA correspond to dalteparin sodium, enoxaparin sodium, fondaparinux sodium, chondroitin sulfate, hyaluronic acid, heparan sulfate, and bovine serum albumin, respectively.

Table 1. Detection of Heparin in the Plasma by the Cas12a-Based Method

sample	content (ng/mL)	spiked (ng/mL)	detected (ng/mL)	recovery (%)	RSD (n = 6, %)
plasma	not detected	5	4.6	93.5	1.1
		10	9.6	96.5	2.3
		15	14.9	99.7	1.7

heparin−Cas12a binding. When the groove is tightly occupied by heparin, crRNA will not easily interact with Cas12a, preventing the formation of the R-loop structure. Therefore, the molecular docking results support our hypothesis that heparin directly interacts with Cas12a, inhibiting the activation of Cas12a and delaying the formation of Cas12a−crRNA RNP.

Native polyacrylamide gel electrophoresis (native-PAGE) with silver staining was conducted to visualize the nucleic acids, proteins, and polysaccharides to further reveal the binding between heparin and Cas12a (Figure 3B). Lanes 1, 2, and 3 represent crRNA, Cas12a, and heparin, respectively. Heparin is a heteropolysaccharide with molecular weight ranging from 6000 to 20,000; thus, it shows dispersed bands in lane 3. In lane 4, the band of crRNA became shallower, and the slowly immigrated bands located on the top of the lane were stronger than in lane 2, which explains the formation of Cas12a−crRNA RNP. Lane 5 was the sample mixed with crRNA and heparin and showed no observable interaction after native-PAGE resolution. Moreover, the band intensities of crRNA in lanes 5 and 7 were similar and higher than those in lane 4, indicating that the binding between heparin and Cas12a prevented the crRNA−Cas12a binding, consistent with the observed experimental results. Notably, lanes 6 and 7, in the presence of heparin and Cas12a, slowly immigrated into heparin bands, causing the large heparin fractions to become weaker, indicating that large heparin chains were bound to Cas12a.

Interaction between Heparin and Cas12a. A chip comprising heparin with a backbone IdoA2S-GlcNS6S including (dp4−dp14) was used to explore the relationship between the molecular weight of heparin and its inhibitory effect on Cas12a. Biotinylated Cas12a was expressed, purified, and incubated on the chip. The binding affinity of these compounds to Cas12a was represented as a heatmap (Figures S4and 4A). Among compounds **1**−**9**, compound **9** exhibited the highest binding affinity and compound **1** exhibited the lowest binding affinity. This suggests that an increased chain length generally leads to a higher binding affinity. To validate the pattern, unfractionated heparin was separated using size exclusion chromatography (SEC) to explore the relationship between the molecular weight of heparin and its inhibitory effect on Cas12a (see Figure S5). The fluorescence intensity results indicated that high-molecular-weight heparin fractions were more efficient in inhibiting Cas12a activity (Figure 4B). In contrast, the low-molecular-weight heparin fraction failed to potently inhibit Cas12a activation, as observed in the higher fluorescence of the fifth sample. The fluorescence results are consistent with the results of native-PAGE, further demonstrating that high-molecular-weight heparin fractions could bind to Cas12a, thereby inhibiting the activity of Cas12a. This size-dependent binding is reasonable based on the size of the positively charged groove of Cas12a.

Oversulfated chondroitin sulfate (OSCS), an unnatural synthetic glycosaminoglycan that shares a similar sulfation level to heparin, was used to demonstrate that the heparin−Cas12a complex formation relies on electrostatic interactions. The synthesized OSCS (OSCS-1 and OSCS-2) is more electronegative than CS due to strongly acidic sulfo group introduction, and its electronegativity can be controlled by altering its degree of sulfation during chemical synthesis.[31] The ^{1}H NMR results (Figure S6) confirmed that OSCS-2 has a higher degree of sulfation and stronger electronegativity than OSCS-1. CS had a negligible influence on fluorescence, while heparin, OSCS-1, and OSCS-2 caused a significant fluorescence decrease, indicating that Cas12a activities were affected by heparin, OSCS-1, and OSCS-2 but were not affected by CS (Figure 4C). The fluorescence changes in the sample added with OSCS-2 were much higher than those in the sample added with OSCS-1, indicating that OCSC-2 was more effective in inhibiting the activation of Cas12a. Since CS, OSCS-1, and OSCS-2 share similar molecular weights, these

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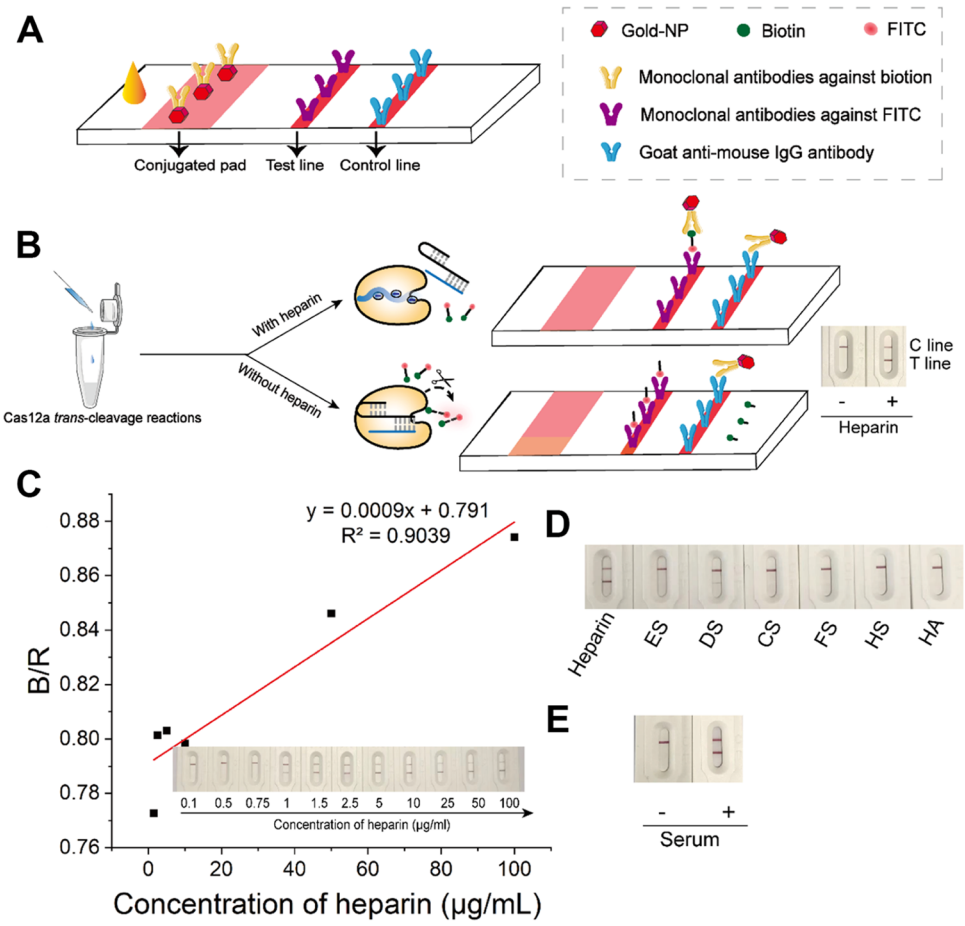

Figure 6. HepaStrip for heparin detection. (A) Schematic diagram of immunochromatographic test strips. (B) CRISPR-based lateral flow for heparin detection. (C) Corresponding CRISPR/Cas12a-based lateral flow assay was performed under different concentrations of heparin. (D) Selectivity of lateral flow-based experiments. The concentrations of the interferents and heparin were 1.5 μg/mL. (E) Detection of heparin in serum by lateral flow-based experiments.

results demonstrate that electronegativity is a critical factor influencing the binding of heparin to Cas12a.

Considering the presence of hydrophobic and positively charged grooves in Cas12 proteins that function as active sites, we wondered if heparin could inhibit other Cas12 proteins.[14,32,33] As a demonstration, the responses of four Cas12 proteins, including LbCas12a, AsCas12a, AapCas12b, and AacCas12b (hereafter collectively referred to as Cas12), to heparin were investigated, and Cas12 showed no evidence of *trans* cleavage after heparin addition (see Figure 4D). The results demonstrated that heparin could inhibit various Cas12 proteins with different origins, including LbCas12a, AsCas12a, AapCas12b, and AacCas12b

Sensitive and Selective Analysis of Heparin. An overdose of heparin can be fatal, and there are genetic differences in heparin metabolism, making the monitoring of circulating heparin levels critical. However, many current heparin assays adopt the competitive binding mechanism requiring the use of an intermediate, such as protamine and AuNPs,[34,35] which are complex mixtures that can give variable results. Therefore, we explored a novel approach for heparin detection utilizing the CRISPR/Cas12a system, focusing on heparin's direct inhibition of Cas12a activation.

After optimization of the analytical conditions for Cas12a-enabled heparin analysis (Figures S7 and S8), we observed that the response signal, calculated by comparing the fluorescence of heparin-containing samples with that of blank samples with minimal systematic errors, decreased with increasing heparin concentrations (Figure 5A). This method exhibited a linear range of 0.5−20 ng/mL ($y = -0.0263x + 0.739$, $R^2 = 0.998$) and a limit of detection (LOD) of 0.36 ng/mL (signal-to-noise ratio = 3), showcasing outstanding performance compared with previously reported heparin assays (Table S3). In terms of molarity, the linear range was 0.03−1.2 μM, with an LOD of 0.02 nM. Considering the therapeutic heparin level is 1.7−10 μM,[36] this method may allow the clinical monitoring of heparin with less volume of the blood sample, which is highly desirable.

To examine the selectivity of this method in heparin detection, the influence of interference, including the impact of low-molecular-weight heparin (ES, FS, DS), glycosaminoglycans (HS, HA, CS), and biomolecules (BSA, hemoglobin), on CRISPR/Cas12a, was investigated. Only the sample containing heparin showed low fluorescence, indicating that heparin specifically inactivated Cas12a (Figure 5B). Notably, the concentrations of all interfering substances were ten times higher than that of heparin in this selectivity test. Discriminating heparin from its analogues, especially other glycosaminoglycans like CS and HA, is rarely realized in the existing point-of-care testing (POCT) heparin assays (Table S4), and accurate discrimination typically relies on sophisticated methods, such as liquid chromatography−mass spec-

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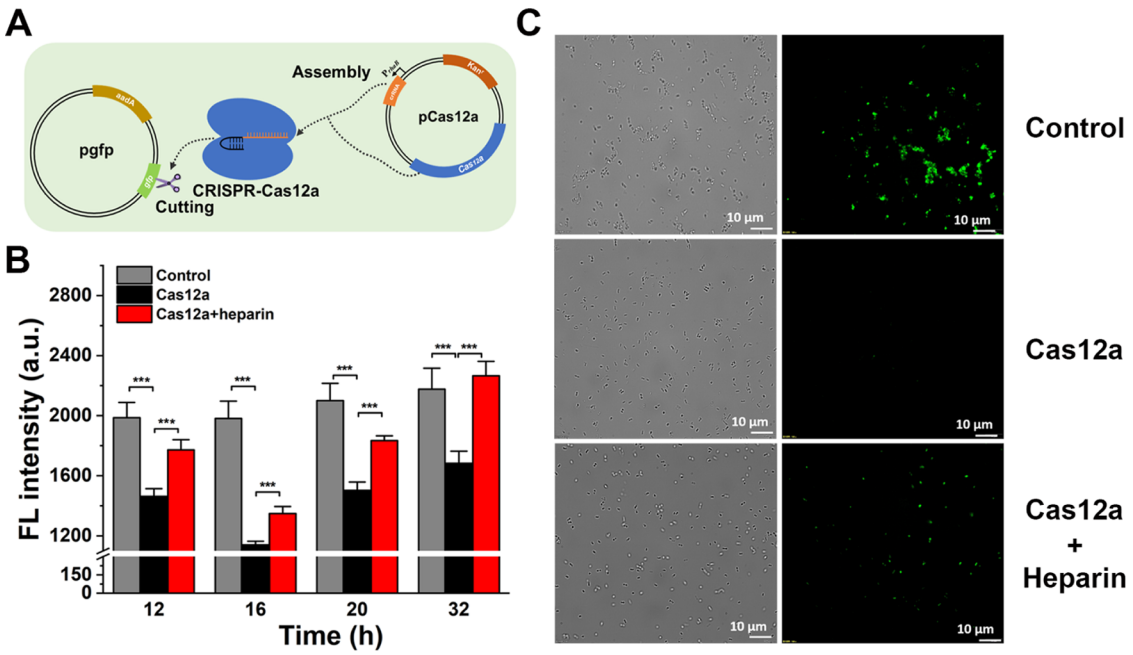

Figure 7. *In vivo* inhibition of heparin toward CRISPR/Cas12a. (A) Double-plasmid-based CRISPR/Cas12a system for gene editing. (B) Fluorescence intensity of the reaction system in the presence of rhamnose and rhamnose/heparin with respect to time. Data are presented as mean ± SEM derived from triplicate samples (n = 3). Statistical significance is determined using a two-tailed Student's t-test: ***p < 0.001. (C) Fluorescence microscopy (FM) images of *E. coli* BL21(DE3) with two plasmids and various treatments. FM bright-field (top) and fluorescence (below) images are also shown (scale bar 10 μm).

trometry (LC−MS). Mechanistic studies show that heparin inhibition of Cas12a depends on two factors: the high molecular weight and high negative charge of heparin. Although CS and HA have molecular weights similar to heparin, their electronegativities are much lower. The electronegativity of LMWH is equivalent to that of heparin, but the molecular weight is much lower than that of heparin. Hence, electronegativity and a high-molecular-weight heparin chain are needed to inhibit Cas12a strongly. The CRISPR/Cas12a-based method can detect heparin with outstanding selectivity rooted in its analytical mechanism.

We performed heparin detection in human plasma samples to study the feasibility of this method in real sample analysis. Plasma was obtained after centrifugation of fresh blood (ethylenediaminetetraacetic acid anticoagulated) from healthy volunteers at the Nanjing Hospital of Chinese Medicine. According to the calibration curve presented in Figure 5A, the spiked heparin levels were analyzed, and the corresponding recovery was calculated. As shown in Table 1, a recovery from 93.5 to 99.7% was obtained, and the highest relative standard deviation (RSD) was 2.3%. The results indicate that this method affords high accuracy and precision, demonstrating its potential for clinical applications.

Detection of Heparin on Lateral Flow Test Strips. We developed HepaStrip (short for heparin strip), a CRISPR/Cas12a-based lateral flow assay, under optimized conditions for the rapid detection of heparin with a visual readout. The composition of HepaStrip is illustrated in Figure 6A, featuring a sample pad, a conjugate pad, a test line, a control line, and an absorbent pad. Streptavidin-modified gold nanoparticles (AuNP-SA) were preassembled into the conjugate pad. The control (C) and test (T) lines on the test paper were immobilized with antimouse IgG and anti-FAM antibodies, respectively. In the presence of heparin, the *trans*-cleavage activity of Cas12a was inhibited (Figure 6B), preventing the cleavage of the FAM/Biotin reporter (FB reporter) modified with FAM and biotin at both ends. On this occasion, the intact FB reporter combined with streptavidin-modified gold nanoparticles (AuNP-SA) using streptavidin−biotin interaction in the conjugated pad, forming a AuNP-SA−FB reporter conjugate. As this conjugate flowed to the test (T) line, it was captured by the anti-FAM antibody. Excessive AuNP-SA, higher than the FB reporter amount, flowed over to the control (C) line and was captured by the anti-IgG antibody. In the presence of heparin, red bands appeared on both the T and C lines of the HepaStrip. In the absence of heparin, Cas12a was activated, and the FB reporter was cleaved, generating two ssDNA fractions having FAM and biotin, respectively. In the conjugation pad, the biotin-ended ssDNA conjugated with AuNP-SA, while the FAM-ended ssDNA directly flowed through the pad without conjugation. The FAM-ended ssDNA was captured by the anti-FAM antibody on the T line, showing no red color on the T line, while the biotin-ended ssDNA conjugated with AuNP-SA was captured at the C line, affording a red line. Different concentrations of heparin were determined, as shown in Figure 6C. The presence of heparin could be detected by the naked eye at 1.5 μg/mL. Moreover, the color change of the T line was analyzed by RGB to digitalize the readout. A linear range of 1−100 μg/mL (y = 0.0009x + 0.791, R^2 = 0.9039) and a LOD of 0.45 μg/mL (signal-to-noise ratio = 3) were obtained. Compared to established methods, HepaStrip is a simpler analytical setting with improved performance for heparin analysis (Table S3).

The selectivity of HepaStrip was next evaluated. Only heparin was able to block the activity of Cas12a, showing T and C lines on the test strip, confirming the high selectivity of the lateral flow assay for heparin (Figure 6D). Expanding the application to test sensitivity for the lowest serum heparin

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concentrations, serum spiked with heparin (25 μg/mL) and diluted ten times showed clear T and C lines on the strip, while serum samples without heparin displayed only a C line due to FB reporter cleavage (Figure 6E). Hence, HepaStrip facilitates the visual detection of heparin in clinical samples. Although the sensitivity of HepaStrip is lower than that of the fluorescent assay, it is sufficiently sensitive to identify and detect heparin in human serum at standard treatment dosages.

Inhibition of CRISPR/Cas12a Gene Editing *In Vivo*. We next sought to determine the inhibitory potency of heparin in the regulation of endogenous gene editing in *Escherichia coli*. A selection strain was generated to screen Cas12a activity by targeting GFP. The percentage of GFP-negative cells directly correlates to Cas12a activity (Figure 7A). In the absence of Cas12a inhibitors, the percentage of GFP-negative strains increased, leading to low fluorescence. However, in the presence of a Cas12a inhibitor, the percentage of GFP-negative strains decreases, leading to an increase in fluorescence intensity. The impact of heparin on GFP expression in BL21(DE3) was negligible within the range of 0.1−200 μg/mL, consistent with the low toxicity of heparin.[37] Thus, a concentration of 200 μg/mL was selected, leading to enhanced fluorescence observed at different incubation times (12, 16, 20, and 32 h). Although the fluorescence increments varied at different time points, a discernible overall upward trend emerged when compared to the Cas12a group (Figure 7B), suggesting a diminished Cas12a cutting efficiency in the presence of heparin. Furthermore, fluorescence microscopy illustrated nearly complete quenching of fluorescence in the Cas12a group compared to the control, while fluorescence enhancement was recorded when heparin was supplemented in the system in the field of view with similar bacterial density (Figure 7C). These results demonstrate that heparin had an inhibitory effect on Cas12a *in vivo*.

CONCLUSIONS

In summary, we found that heparin can inhibit CRISPR/Cas12a and suggest the underlying mechanisms. Heparin hindered the loading of crRNA by directly binding to Cas12a, thereby preventing the formation of the Cas12a−crRNA complex and blocking the DNA cleavage reaction. During the heparin−Cas12a binding, both high molecular weight and high negative charge play critical roles. Considering these findings, a heparin assay with high sensitivity and selectivity was established based on CRISPR/Cas12a. Additionally, signal transduction was achieved by a lateral flow assay, affording a POCT for rapid detection of heparin. Finally, we explored whether heparin could control CRISPR/Cas12a gene editing *in vivo* and found that heparin efficiently inhibited CRISPR/Cas12a activation. This study provides a powerful analytical tool for the direct detection of heparin and also broadens the application of CRISPR/Cas in the field of analysis. We put forward the anti-CRISPR concept for gene editing using heparin as anti-Cas12a, providing ideas for regulating the process of CRISPR/Cas12a technology in the future.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.4c00403.

Experimental section, fluorescence intensities of ssDNA and dsDNA in the reaction system with and without heparin, heparin samples with decreasing molecular weight separated by size exclusion chromatography (SEC), optimization of reaction time for the CRISPR−Cas12a system, hydrogen bond distance statistics, comparison of representative methods for the analysis of heparin, results and discussion, and references (PDF)

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Notes

The authors declare no competing financial interest.

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