DNA concatemers-encoded CRISPR/Cas12a fluorescence sensor for sensitive detection of Pb^{2+}based on DNAzyme

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1.Experimental section

1.1. Materials and methods

All oligonucleotide chains used in this study were purchased from Shangya Biotechnology Co., Ltd. (Fuzhou, China). The detailed sequences of oligonucleotide chains are provided in Table S2. Streptavidin-modified magnetic beads were obtained from Xianfeng Nanomaterial Technology Co., Ltd. (Jiangsu, China). EnGen® LbaCas12a (Cpf1) was sourced from New England Biological Laboratories Limited (Beijing, China). Lead nitrate, magnesium chloride, and sodium chloride were procured from Aladdin (Shanghai, China). All chemical reagents were of reagent grade.

Buffers are used in this work as follows:

- Buffer A: 10 mM Tris-HCL, 50 mM NaCl, 10 mM$MgCl_2$, PH=7.2
- Buffer B: 20 mM Tris-HCL, 1 M NaCl, 0.0015% TritonX-100, PH=7.6
- Buffer C: 10 mM Tris-HCL, 50 mM NaCl, PH=7.4

1.2. Preparation of the DNA concatemers

DNA concatemers were designed through the self-assembly of hybrid chain reactions (HCRs). The 10 μM stem-loop structures H1 and H2 were first annealed in Buffer A , respectively (heated in a 95°C metal bath for 10 minutes, then naturally cooled to room temperature over 10 minutes to stabilize the stem-loop structure). In the second step, 200 nM substrate-trigger strand (ST) and 500 nM H1 and H2 were mixed in Buffer A and incubated at 37°C for 60 minutes. The long DNA concatemers containing multiple protospacer adjacent motifs (PAM) and protospacer sequences was formed and stored in 4°C.

1.3. Preparation of the MBs-DNA concatemers (MDD) complexes

First, the DNA concatemers contained biotin-substrate-trigger strand (ST) were mixed with 0.1 mg/mL streptavidin-MBs in Buffer B and incubated for 2 hours at 37°C. Then, the dissociative DNA concatemers were removed by magnetic separation,

and the MBs-DC was purify with ultrapure water for 6-8 times.

1.4. Detection of Pb^{2+} by MDD-Cas12a

First, MBs-DC, DNAzymes, and various concentrations of Pb^{2+} were mixed in Buffer C and incubated at 37°C for 60 minutes. Subsequently, the released DNA concatemers were purified by magnetic separation. Meanwhile, Cas12a (100 nM) was mixed with crRNA (50 nM) in 1×NEBuffer 2.1 and incubate at 37°C for 30 minutes to form the Cas12a/crRNA complexes. Then, 2 μL of DNA concatemers and 1 μL of reporter (20 μM) were added to the preassembled Cas12a/crRNA complexes in a final volume of 20 μL, incubated at 37°C for 60 minutes. The successive fluorescence changes were monitored at a fixed emission wavelength of 520 nm upon a fixed excitation of 490 nm.

1.4. Detection of Real Samples.

1.4.1.The detection of lake water samples

Initially, collect an appropriate volume of the lake water sample and conduct filtration using a 0.45 μm filter membrane to eliminate suspended substances. Subsequently, prepare a series of lead standard solutions with concentrations of 0.05 nM, 0.1 nM, 0.5 nM, 1 nM, 10 nM, 100 nM, and 200 nM respectively. Then, pipette 90 μL of each standard solution and blend it with 10 μL of the MDD - Cas12a sensor. After incubating the mixture at 37°C for 60 minutes, utilize a fluorescence spectrometer to measure the fluorescence intensity of each standard solution, and ultimately, plot the standard curve representing the relationship between the fluorescence intensity and the lead ion concentration.

1.4.1.The detection of milk samples

First, take 1 mL of milk sample, add 2 mL of acetonitrile or methanol, and vortex-mix for 1 minute. Then centrifuge at 10,000 rpm at 4°C for 10 minutes and take the supernatant. Repeat the above steps once to ensure the complete removal of proteins and fats. Next, take the supernatant, add 1 mL of concentrated nitric acid, heat it to 90°C, and digest for 30 minutes. After cooling, make up the volume to 5 mL with deionized water. Then, prepare a series of lead standard solutions (0.05 nM, 0.1 nM,

0.5 nM, 1 nM, 10 nM, 100 nM, 200 nM), and take a certain volume (90 μL) of each standard solution and mix it with the MDD - Cas12a sensor (10 μL). After incubation at 37°C for 60 minutes, measure the fluorescence intensity of each standard solution with a fluorescence spectrometer and plot the standard curve of fluorescence intensity vs. lead ion concentration.

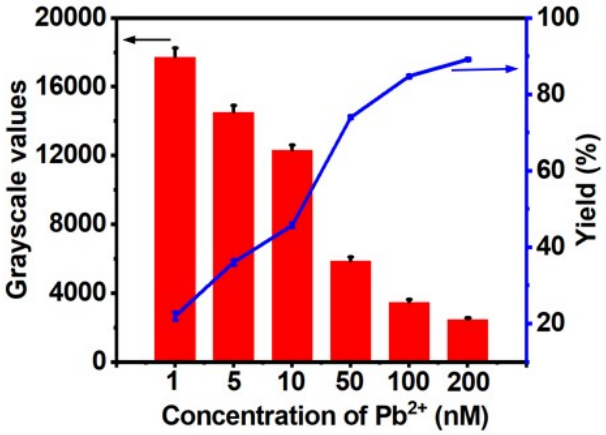

Figure S1. Detailed cleavage yield of DNAzyme for substrate strand at different Pb^{2+} concentrations.

Cleavage yield (%) = $[1 - (I_{ST\text{-}treated}/I_{ST\text{-}initial})] \times 100$

where:

$I_{ST\text{-}initial}$ = grayscale intensity of the intact substrate strand (control).

$I_{ST\text{-}treated}$ = grayscale intensity of the substrate strand after Pb^{2+}-induced cleavage.

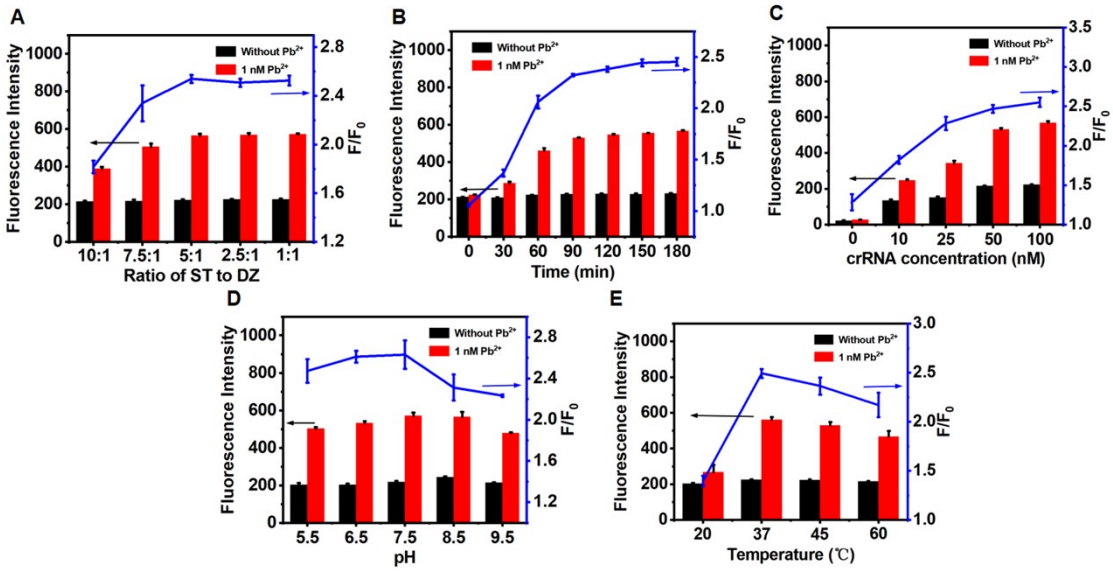

Figure S2. Optimization of experimental conditions. The fluorescence intensity influenced by (A) the ratio of ST to DZ, and (B) the incubation time of Pb^{2+} and DNAzymes, and (C) the crRNA concentration, and (D) the pH of the reaction buffer, and (E) the reaction temperature of Cas12a. F0 is the fluorescence spectrum of the control group (without Pb^{2+}), and F is the fluorescence spectrum of the experimental group (with 1 nM Pb^{2+}). The data are expressed as mean ± SD (n = 3).

Optimizing the experimental conditions is crucial for enhancing the detection performance of the MDD-Cas12a system. We focused on factors such as the ratio of DZ to ST, the incubation time between Pb^{2+} and DNAzymes, the crRNA concentration, the pH of the reaction buffer, and the reaction temperature of Cas12a. To assess parameter performance, we used F/F0 fluorescence response signals, where F represents the fluorescence in the presence of 1 nM Pb^{2+} and F0 represents the fluorescence in the absence of Pb^{2+}. First, in this study, the DNAzyme can hybridize with the substrate strand to catalyze the release of DNA concatemers, so the ST/DZ ratio directly influences the signal activation process of the MDD-Cas12a system. We measured the signal responses at different ST/DZ ratios (10:1, 7.5:1, 5:1, 2.5:1, and 1:1) by fixing the amount of MB-DNA concatemers and varying the amount of DNAzyme (Fig. S2A). The highest signal was obtained when the ST/DZ ratio was 5:1. Further increasing the ST/DZ ratio to 5:1 did not significantly change the detected

signal, likely due to insufficient reaction sites and an excess of substrate strands on the surface of the MB. Moreover, the cleavage time of DNAzymes toward the substrate-trigger (ST) sequences critically governs the sensor's performance. As shown in Figure S2B, extending the incubation time from 0 to 60 minutes resulted in progressively enhanced fluorescence signals. Beyond 60 minutes, no statistically significant improvement in F/F0 ratios was observed, indicating that 60 minutes is the optimal incubation time of Pb^{2+} and DNAzymes. Next, the crRNA concentration was optimized to save costs while ensuring consistent performance. Fig. S2C shows that increasing the crRNA concentration from 0 to 50 nM led to greater Cas12a trans-cleavage activity. Beyond this concentration, the F/F0 ratios did not significantly increase, indicating that 50 nM is the optimal crRNA concentration. Moreover, pH can influence the enzymatic activity of Cas12a. At low pH, Cas12a's activity may be affected due to protonation, while excessively high pH values can disrupt the enzyme's active site, reducing its substrate binding capacity and catalytic efficiency. Therefore, we optimized the pH conditions accordingly. As shown in Fig. S2D, the F/F0 values peak at a pH of 7.5, indicating that pH 7.5 provides the optimal reaction conditions. Additionally, to achieve full trans-cleavage activity of Cas12a, the optimal reaction temperature was investigated. As shown in Fig. S2E, the MDD-Cas12a system achieved an optimal F/F0 ratio at 37°C, indicating that the trans-cleavage reaction has the best effect at this temperature. In conclusion, the optimal reaction conditions for the MDD-Cas12a system have been determined, contributing to improved performance.

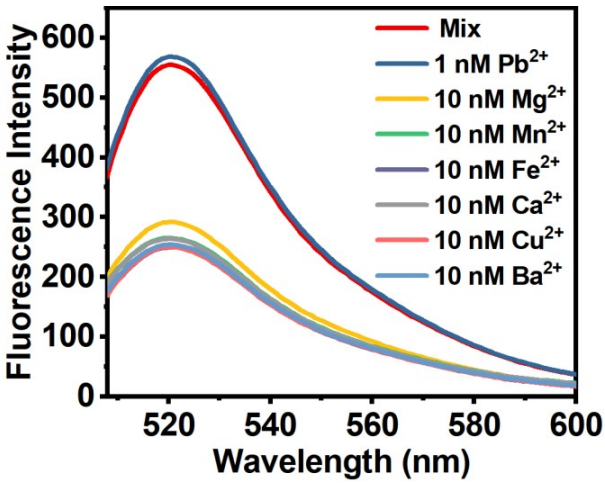

Figure S3. Specificity of MDD-Cas12a for Pb^{2+} detection.

Table S1. Comparison of our work with previously reported lead ion sensors.

Method	Detection technique	Linear range	LOD	Ref.
DNAzyme-based double amplification	Fluorescence	0.1–30 nM	120 pM	(Zhang et al., 2020b)
DNAzyme/MnCoPBAs-PDANCs complex-based biosensor	Fluorescence	3-9 nM	1.6 nM	(Rajaji and Panneerselvam, 2020)
Tetrahedron-based Pb2+-sensitive DNAzyme sensor (TPS)	Fluorescence	0-500 nM	1.0 nM	(Guan et al., 2021)
DNA molecular machine	Fluorescence	0.1-50 nM	0.114 nM	(Tang et al., 2023)
Nanozyme-catalysed CRISPR-Cas12a system	Colorimetry	0.8-2500 nM	0.54 nM	(Xu et al., 2023)
Visual detection based Pb^{2+}-responsive hydrogel	Colorimetry	0–100 nM	5 nM	(Diao et al., 2020)
MDD-Cas12a system	Fluorescence	0-400 nM	112.96 pM	This work

Table S2. Oligonucleotide sequences used in this work.

NAME	Nucleotide Sequence（5’→3’）
DZ	CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT
ST	AGTCTAGGAAACTGCGTTGGTTAAtttACTCACTATrAGGAAGAGATGttttt
H1	TTAACCAACGCAGTTTCCTAGACTCAGTGTAGTCTAGGAAACTGCGTG
H2	AGTCTAGGAAACTGCGTTGGTTAATACGCAGTTTCCTAGACTACACTG
crRNA	UAAUACGACUCACUAUAGGGUAAUUUCUACUAAGUGUAGAUC UAGACUACACUGAGUCUAG
F-Q reporter	FAM-TTATT-BHQ1

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