Electronic Supplementary Information

Universal electrochemical biosensor based on CRISPR/Cas12a and DNA tetrahedron for ultrasensitive nucleic acid detection

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1. Chemicals and apparatus

Lba Cas12a (Cpf1) was purchased from Guangzhou Meige Biological Technology Co., Ltd. Nt.BsmAI, T4 ligase, Phi29 DNA Polymerase were obtained from NEB Co., Ltd (Beijing, China). dNTP mixture solution, PAGE related reagents (Acryl/Bis 30% Solution (29:1), nucleic acid dyes, TBE buffers, TE buffers, DEPC-treated water, loading buffers and DNA marker were purchased from Sangon Biotechnology Co. Ltd. Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) and TCEP (tris(2-carboxyethyl) phosphine hydrochloride) were purchased from Sigma-Aldrich. The normal human serum was acquired from Solarbio Tech Co., Ltd. (Beijing, China).

The HPLC-purified oligonucleotides were synthesized and purified from Sangon Biotechnology Co. (Shanghai, China). The sequences used in this study were shown in Table S1. All other chemicals were analytical reagent grade and can be used without further purification. All aqueous solutions were freshly prepared with ultrapure water (18.2 MΩ cm) with a Millipore system. All electrochemical measurements were performed on a CHI 760E electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd, China).

2. Experimental section

2.1 Self-assembly of TDN

First, an equimolar amount of four single-stranded DNAs (a, b, c, d) used to form the TDN structure was mixed in TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0) containing 3 mM TCEP, creating a mixed solution of TDN with a final concentration of 1 μM. Subsequently, the prepared mixture was annealed in a PCR machine at 95 °C for 10 min, rapidly cooled to 4 °C, and maintained for at least 10 min. Finally, the prepared TDN was stored at 4 °C for further use.

2.2 Endonuclease-mediated rolling circle amplification (E-RCA) and activation of the CRISPR/Cas12a system

Initially, 2 μL of 200 nM padlock probe and an equal volume of different concentrations of target underwent annealing at 90 °C for 5 min. Subsequently, 1 μL of 10×T4 DNA ligase buffer, 10 U T4 DNA ligase, and ddH_2O were added, followed by incubation for 20 min at 37 °C. After the padlock probe circularization, the reaction was halted by heating for 10 min at 65 °C. Next, 10 μL of a solution containing 1×CutSmart buffer, 1×Phi29 DNA polymerase buffer, Phi29 DNA polymerase (10 U), Nt.BsmAI (5 U), and dNTPs was introduced, and the reaction proceeded for 80 min at 37 °C.

For the activation of the CRISPR/Cas12a system, the specific steps were as follows. Typically, 5 μL of E-RCA product, 2 μL of buffer 1, 2 μL of 1 μM Cas12a, 2 μL of 1 μM crRNA, and 1 μL of 10 μM FQ fluorescent probe were combined in an EP tube, and the volume was adjusted to 20 μL with ddH_2O. After reacting at 37 ℃ for 30 min, the liquid was transferred to a quartz cuvette and made up to 100 μL with ddH_2O. Finally, the activation of the CRISPR/Cas12a system was confirmed through fluorescence recording at 490 nm excitation.

2.3 Electrode pretreatment and modification

Initially, the bare glassy carbon electrode (GCE) was pretreated based on our previous work [1]. Subsequently, the pretreated electrode was immersed in a 5 mM $HAuCl_4$ solution and electrodeposited at -0.2 V for 200 s. After rinsing the electrode with ddH_2O, 6 μL of TDN solution was drop-cast onto the electrode surface and left to incubate overnight at 4 °C. Following this, the electrode underwent a gentle rinse with 0.01 M PBS to remove any unbound TDN solution and blocked with MCH (1 mM) to occupy unbound sites. Finally, the obtained electrode was preserved at 4 °C for subsequent experimental procedures.

2.4 Construction of CRISPR/Cas12a-driven TDN framework-based sensing platform

Refer to Part 2.2 for activation of Cas12a system. Subsequently, 10 μL of the solution was cast onto the surface of the modified electrode and allowed to incubate at 37 °C for 60 min. Following the reaction, the electrode underwent a gentle wash with PBS buffer. Finally, the electrode was submerged in 5 mL of 0.01 M PBS buffer for differential pulse voltammetry (DPV) signal measurement.

2.5 Polyacrylamide gel electrophoresis (PAGE)

10 % PAGE gel electrophoresis analysis was run in 1 × TBE buffer at a constant voltage at 100 V for 60 min. Finally, the electrophoretic gel was reacted in a nucleic acid dye for 15 min and images are collected by a JENA UVsolo Imager.

2.6 Cell culture and nucleic acid extraction

Human breast cancer cells (MCF-7), human endothelial cells (Ec) and human lung carcinoma cells (A549 cells), which were from ATCC cell bank, were selected as real samples. A549 and MCF-7 cells were cultured in DMEM medium with 1% non-essential amino acids, 10% fetal bovine serum (FBS) and 1% penicillin streptomycin/double at 37 °C with a humidified atmosphere (95% air and 5% CO_2). Ec cells were cultured in 90% RPMI 1640 medium (Hyclone, USA) with 10% FBS (Solarbio, China) at 37 °C with a humidified atmosphere (95% air and 5% CO_2). After 24 h of culture, cells in exponential were collected and washed twice with sterile PBS. Then, nucleic acids in cells samples were extracted by Ezup Column Animal Genomic DNA Purification Kit (Sangon, Inc., Shanghai, China) according to the manufacturer's protocol. Finally, the obtained samples were diluted and stored at -20 °C for further use.

For RNA extraction, the total RNA extraction was obtained using the Trizol reagent according to the instruction. Finally, the obtained cellular extracts were frozen at -80 °C for further use.

2.7 Recovery experiment.

The detection of miRNA-155 in normal human serum was performed by the standard addition method. Firstly, the normal human serum was a commercial synthetic sample, which was purchased from Beijing Solarbio Technology Co., Ltd. Before the recovery experiments, the normal human serum was diluted 20-fold by 0.01M (PH=7.4) PBS. Then, different concentrations of miRNA-155 (10 pM, 1 pM, 100 fM) were added to the diluted normal human serum. Following that, the subsequent experimental operations were carried out according to the part 2.2 and 2.4 of Electronic

Supplementary Information. Thereafter, differential pulse voltammetry (DPV) was carried out to detect the concentration of miRNA-155 in spiked serum samples and recorded the current response values. The ΔI value was finally converted to the molar concentration of miRNA-155 by comparing with the standard linear calibration curve to calculate the recovery.

Table S1. All DNA and miRNA sequences used in this work.

Name	Sequence (5'-3')
MiRNA-155	UUAAUGCUAAUCGUGAUAGGGGU
ctDNA KRAS	GTTGGAGCTAGTGGCGTAG
Padlock probe	Phosphate′-GATTAGCATTAAAAGAGAC GGG CGG GCCAAACTGCTGGG AAGAGACACCCCTATCAC
Padlock probe (P_1)	Phosphate′-TAG CTC CAA CAAGAGAC GGG CGG GCC AAA CTG CTG GG AAGAGACCTA CGC CAC
Tetrahedron a	MBCATCTTACCTTACCTTTACATTCCTAAGTCT GAAACATTACAGCTTGCTACACGAGAAGAGCC GCCATAGTA
Tetrahedron b	SHTATCACCAGGCAGTTGACAGTGTAGCAAGC TGTAATAGATGCGAGGGTCCAATAC
Tetrahedron c	SHTCAACTGCCTGGTGATAAAACGACACTACG TGGGAATCTACTATGGCGGCTCTTC
Tetrahedron d	SHTTCAGACTTAGGAATGTGCTTCCCACGTAG TGTCGTTTGTATTGGACCCTCGCAT
Let-7a	UGAGGUAGUAGGUUGUAUAGUU
MiRNA-21	UAGCUUAUCAGACUGAUGUUGA
MiRNA-141	UAA CAC UGU CUG GUA AAG AUGG
$M1_{155}$	UCUGAAGGACCACCGCAUCUCUAC
$M3_{155}$	UCUGAAGGACAACCGCAUCGCUAC
Random RNA	UUGUACUACACAAAAGUACUG
$M1_{ctDNA}$	GTT GGA GCT AGT GGC CTA
$M2_{ctDNA}$	GTT GGA GCT AGT GTA GTA G
$M3_{ctDNA}$	GTT GGA GCT AGT GGT AGA G
Random DNA	CGGCAGAGGCATCTTCAACG
crRNA	UAAUUUCUACUAAGUGUAGAUGGGCGGGCCA AACUGCUGGG
FQ-reporter	HEX-TATTATT-BHQ1

3. Results and discussion

3.1 10%PAGE Characterization

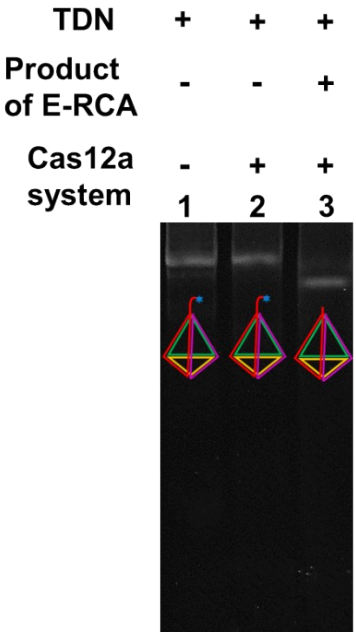

Fig. S1 10% PAGE assay for the feasibility of TDN cleavage mediated by CRISPR/Cas12a.

3.2 Feasibility analysis

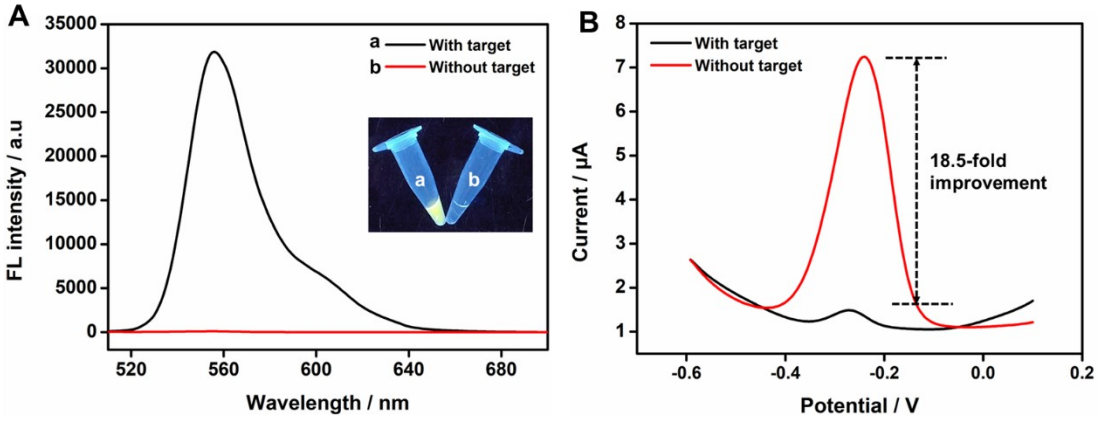

Fig. S2 Fluorescence (A) and electrochemical response (B) in the presence or absence of target

3.3 Optimization of experimental parameters

To enhance the sensor's performance in analyzing and detecting the target, various key experimental parameters were optimized. Firstly, we studied the cyclization time of padlock probe. As depicted in Fig. S3A, the fluorescence signal steadily rose with increasing T4 DNA ligase action time, and then reached a stable level after 20 min, indicating that the cyclization of the padlock probe had reached saturation. Therefore, 20 min was chosen as the optimal cyclization time. Then, we optimized the amplification time of E-RCA strategy. In Fig. S3B, the fluorescence intensity showed a significant rise between 40 and 80 min of amplification time before stabilizing. Therefore, 80 min was the appropriate time for E-RCA amplification. Subsequently, the influence of electrodeposition time of AuNPs on the performance of sensing electrode was evaluated. In Fig. S3C, we could clearly observe that the CV peak current signal first increased (100 s to 200 s) and then decreased (200 s to 300 s) with the increase of electrodeposition time. This was because the electrodeposition of AuNPs on the electrode had reached saturation at 200 s, and too long time was not conducive to electron transfer. Therefore, the electrodeposition time of 200 s was applied to the subsequent experimental operation. Finally, we optimized the cutting time of TDN pendant probe on the surface of sensing electrode mediated by CRISPR/Cas12a. As displayed in Fig. S3D, the DPV response decreased sharply within 15 to 60 min, and then stabilized, suggesting that MB signal molecules on TDN pendant probe were basically completely sheared. Therefore, 60 min was the best cutting time for CRISPR/Cas12a on the electrode.

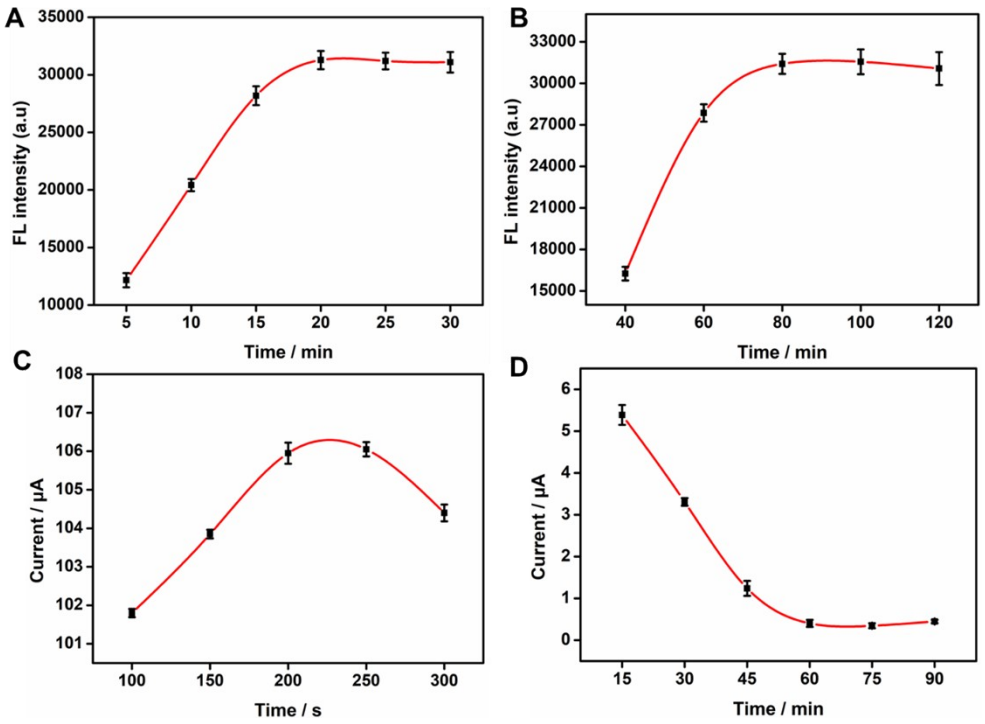

Fig. S3. Optimization of cyclization time of padlock probe (A), amplification time of E-RCA strategy (B), electrodeposition time of AuNPs (C), and cutting time (D).

3.4 Universality of the proposed biosensor

To validate the universality of the proposed sensing platform, circulating tumor DNA (ctDNA), an effective non-invasive biomarker, was employed as the detection object. Initially, the target recognition region on the padlock probe (P1) was adjusted according to the sequence of ctDNA KRAS, and then the performance of the sensor was assessed under optimized conditions. As illustrated in Fig. S4A-4B, the current response decreased progressively with rising concentrations of target ctDNA KRAS, and the current change showed a strong linear correlation with the logarithm of the target concentration within the range of 10 fM to 1 nM. The corresponding regression equation was $\Delta I = -0.915\ IgC_{ctDNA\ KRAS}$ / pM-2.881 (R^2=0.991), and the LOD was 0.2 fM. Compared to other strategies for ctDNA detection (Table S3), this method showed outstanding advantages in sensitivity and linear range.

Furthermore, the specificity of the sensor platform for ctDNA KRAS detection was evaluated. Different DNA samples with 100 pM concentration was used as interferents, including single base mismatch (M1), double base mismatch (M2), triple base mismatch (M3) and random DNA. Fig. S4C showed that the current intensity decreased only in the presence of target ctDNA KRAS, indicating that the prepared sensor exhibited high specificity for ctDNA KRAS determination. Additionally, the constructed biosensor was applied to assess the expression levels of ctDNA KRAS in different cell samples, highlighting its practical application potential. From Fig. S4D, a significant fluctuation in current change was observed only in A549 cell samples, indicating a higher expression level of ctDNA KRAS in A549 cells, consistent with previous studies [2].

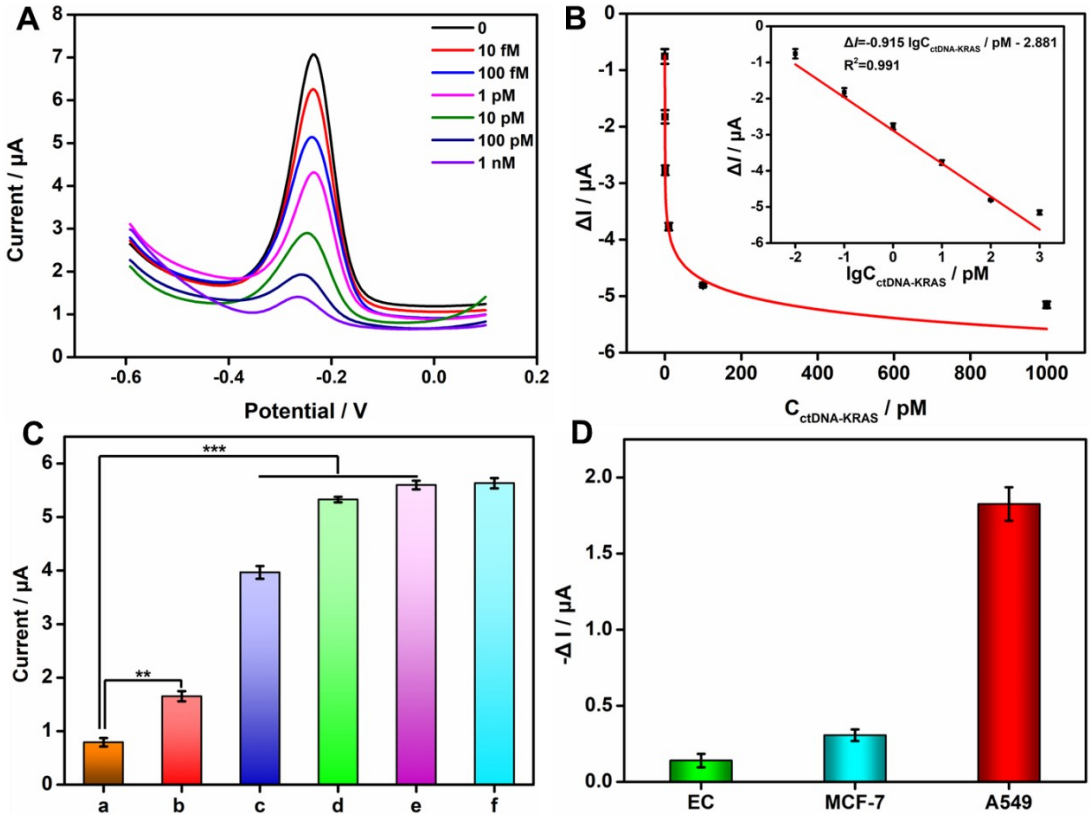

Fig. S4 (A) DPV responses of the biosensor across varying ctDNA KRAS

concentrations. (B) The calibration curve for the ΔI versus the logarithm of ctDNA. (C) Specificity investigation of the sensor (a→f, ctDNA KRAS, M1, M2, M3, random DNA, and blank). (F) Validation of ctDNA KRAS levels from different cells by our strategy.

Table S2. The comparison of the proposed strategy with other methods for miRNA detection

Method	Linear range	Detection limit	Reference
Fluorescence	20 fM-5 nM	10.02 fM	[3]
Fluorescence	100 pM-13.33 nM	381.78 pM	[4]
ECL	10 fM-10 nM	2.9 fM	[5]
SERS	10 fM-1 nM	5.13 fM	[6]
Fluorescence	5 fM-20 pM	2.89 fM	[7]
PEC	1 pM-100 nM	54 fM	[8]
Electrochemistry	100 fM to 100 pM	82 fM	[9]
FRET	10 fM-10 nM	3.5 fM	[10]
Electrochemistry	10 fM to 1 nM	0.5 fM	This work

Table S3. The comparison of the proposed strategy with other methods for ctDNA detection

Method	Linear range	Detection limit	Reference
SERS	10 fM-1 nM	100 fM	[11]
Electrochemistry	10 fM-1 nM	2.86 fM	[12]
Fluorescence	100 pM-1 μM	39 pM	[13]
Colorimetric	1 fM-1 pM	0.65 fM	[14]
Fluorescence	1 pM-400 pM	316 fM	[15]
Fluorescence	10 fM–100 pM	5.6 fM	[16]
Electrochemistry	1 fM to 100 pM	0.66 fM	[17]
Electrochemistry	10 fM-10 nM	1.6 fM	[18]
Electrochemistry	10 fM to 1 nM	0.2 fM	This work

Table S4. Determination of miRNA-155 in human serum by using the proposed biosensor (n=3). The normal human serum was diluted 20-fold by 0.01M PBS.

Sample	Add (pM)	Found (pM)	Recovery (%)	RSD (%)
1	0.1	0.0997	99.70	0.93
2	1	1.0965	109.65	3.16
3	10	11.9333	119.33	2.01

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