Supporting Information for Publication

Twice-walking strategy based on three-dimensional DNA walking machine driven by duplex-specific nuclease

Sheng Lei,[a, e] Zi Liu,[b] Qidong Zhang,[a] Zezhi Li,[a, f] Gaiping Li,[c] Lina Zou,[c] Guobi Chai,[a] Jian Mao,[a, d] Baoxian Ye,[*b] Jianxun Zhang,[*a]

[a]Zhengzhou Tobacco Research Institute of China National Tobacco Company, Zhengzhou 450001, P.R. China.

[b]Henan Key Laboratory of Biomolecular Recognition and Sensing, Henan Joint International Research Laboratory of Chemo/Biosensing and Early Diagnosis of Major Diseases, College of Chemistry and Chemical Engineering, Shangqiu Normal University, Shangqiu 476000, PR China.

[c]College of Chemistry, Zhengzhou University, Zhengzhou 450001, P.R. China.

[d]Beijing Life Science Academy, Beijing, 102299, P.R. China.

[e]College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, P.R. China.

[f]School of Light Industry, Beijing Technology and Business University, Beijing 100048, P.R. China.

Table of contents

1. Chemicals and materials

Duplex-specific nuclease (DSN), DSN Master Buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, pH 8.0) and DSN stop solution (10 mM EDTA) were obtained from Evrogen Joint Stock Company (Moscow, Russia). Trisodium citrate dihydrate, 6-mercapto-1-hexanol (MCH), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), gold (III) chloride trihydrate (HAuCl4·3H2O) and Tris-HCl were acquired from Sigma Chemical Co. (St. Louis, USA). Ultrapure water (specific resistance of 18.2 MΩ·cm) obtained from Thermo Scientific Barnstead GenPure water purification system (Thermo Fisher Scientific Co., Ltd, Shanghai, China) was used in the experimental processes. All DNA oligonucleotides (shown in Table S1) were produced by Takara Biomedical Technology Co. (Beijing, China).

2. Experimental section

2.1 Preparation of 3D cleat DNA walking machine.

AuNPs were synthetized according to literature, reducing $HAuCl_4$ with sodium citrate (Fig. S1).[1] H1 were incubated with AuNPs in a molar ratio (H1&AuNPs) of 200. After blending in the shaker for 12 h (100 rpm), buffer 1 (100 mM Na_2HPO_4, 100 mM KH_2PO_4, 1 M NaCl, pH 7.4) was added to the mixture to achieve a final concentration of 10 mM PB, and then shaken at 37 °C for 40 h. Finally, the compound was centrifuged (12000 rpm, 15 min), washed three times, and then dispersed in buffer 2 (10 mM Na_2HPO_4, 10 mM KH_2PO_4, 0.1 M NaCl, pH 7.4) to obtain H1&AuNPs. The concentration of H1&AuNPs was calculated by the formula (Lambert-Beer).

2.2 Procedure of the Twice-walking Strategy for Electrochemical Detection of MiRNA

Fabrication of the electrochemical biosensor: The Au electrode (AuE, 3 mm in diameter) was successively polished with 0.05 and 0.3 μm alumina and then sonicated for 4 min in water and ethanol by turns. They were then subjected to a series of electrochemical cleaning steps as described previously.[2] Afterwards, 5 μL H2 (0.8 μM) in TNaKT buffer (20 mM Tris, 140 mM NaCl, 5 mM KCl, 10 mM TCEP, pH 7.5) was dropped on AuE and incubated at 25 °C for 8 h. Then, the resulting AuE was allowed to incubate with 5 μL of 1 mM MCH for 1 h to obtain the sensing interface. At each step above, the electrodes were slowly and carefully washed with clean buffer (20 mM Tris, 0.05 % Tween, pH 7.5) and Tris-HCl buffer (20 mM Tris, pH 7.5), respectively.

The 3D cleat DNA walking reaction: Different concentrations of target miRNA-

141 and DSN (0.01 U/μL) were added to the H1&AuNPs (1 μM) and completely reacted at 37°C for 2 h in DSN Master Buffer.

The multi-legged walking reaction: 5 μL of the mixed solution from previous step was introduced onto the modfied electrode (MCH/H2/AuE) and incubated for 70 min at 37 °C.

3. Sequences of the Oligonucleotides Employed in This Work

Table S1 Sequences of the Oligonucleotides Employed in This Work

Oligo Name	Sequence (5'→3')
H1*	SH-TTTTTTCGAGUGUCAGUCCAUCUUUACCAGACAGTGTTACAGATGGAC-FAM
H1-D	SH-TTTTTTCGAGTGTCAGTCCATCTTTACCAGACAGTGTTACAGATGGAC-FAM
H1	SH-TTTTTTCGAGUGUCAGUCCAUCUTTACCAGACAGTGTTACAGATGGAC
W (5)	GUAACACUGUCUGGU GAUGG
W (6)	GUAACACUGUCUGGU AGAUGG
W (7)	GUAACACUGUCUGGU AAGAUGG
W (8)	GUAACACUGUCUGGU AAAGAUGG
H2	SH-TTTTAGATGGACTGACACTCGCTATCAGACAGTGTTACGAGTGTCA-Fc
mi-RNA-141	UAACACUGUCUGGUAAAGAUGG
One-base	UAACACUGUCAGGUAAAGAUGG
Two-base	UAACAGUGUCUGGUAUAGAUGG
mi-RNA 21	UAGCUUAUCAGACUGAUGUUGA

4. Instrumentation and measurement

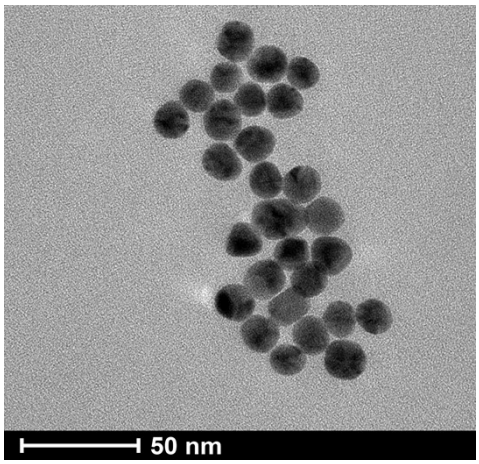
All the electrochemical analytical experiments were carried out on A CHI 650A electrochemical workstation (CH Instruments, Shanghai, China). The traditional three-electrode system included the Gold disk electrodes (AuE, 3 mm in diameter) as working electrode, platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. CV experiments were conducted in PBS (100 mM PB, 100 mM KCl, pH 7.4) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ by scanning the potential between −0.2 and 0.6 V with scan rate of 50 $mV \cdot s^{-1}$. DPV measurements were performed in PBS buffer (100 mM Na_2HPO_4, 100 mM KH_2PO_4, 100 mM KCl, 100 mM $NaClO_4$, pH 7.4) with potential from -0.05 to 0.5 V and pulse parameters of the pulse amplitude of 50mV, the sample width of 16.7 ms and the pulse width of 50 ms. Transmission electron microscopic (TEM) images were performed on a M3000 transmission electron microscope (FEI, USA). UV-Vis absorption spectra were carried out on a Lambda 35 UV-Vis spectrometer (Beijing, China).

5. Cell Culture, Total RNA Extraction, cDNA Synthesis and Quantitative Real-time PCR

Human breast cancer cell line (MCF-7 cells) was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium (Thermo Scientific Co., Ltd, Shanghai, China) supplemented with 15% fetal bovine serum (FBS), penicillin 100 IU/ml, and streptomycin 100 μg/ml, at 37 °C in a 95% humidified and 5% CO2 cell incubator. Total RNA samples were extracted from the cells by using the Total RNA Isolation Kit (Beibei Biotechnology Co., Ltd, Henan, China) according to the manufacturer's procedures. The Mir-X miRNA First-Strand Synthesis Kit and the Mir-X miRNA qRT-PCR TB Green Kit (Takara Biomedical Technology Co., Ltd, Beijing, China) is used for converting miRNAs into cDNA and quantifying target miRNAs.

164

165 6. TEM image of Au NPs.



166 Fig. S1 TEM image of Au NPs.

167

168

169

170

171

172

173

174

175

176

177

178

179

7. Polyacrylamide gel electrophoresis

Polyacrylamide gel (PAGE) was prepared by adding 5 × Tris-boric acid (TBE) buffer, acrylamide (30%), tetramethylenediamine (TEMED) and ammonium persulfate (10%) solution in proportion to ultra-pure water. The DNA samples (1 μM) were annealed at 95 °C for 5 min and cooled to room temperature, then mixed well with 2 μL of loading buffer and 2 μL of CYBER Gold, respectively, and then injected slowly into the 12% polyacrylamide gel through micro-syringe. The electrophoresis experiment was proceeded at 80 V constant voltage for 120 min and the gel image was visualized under UV light.

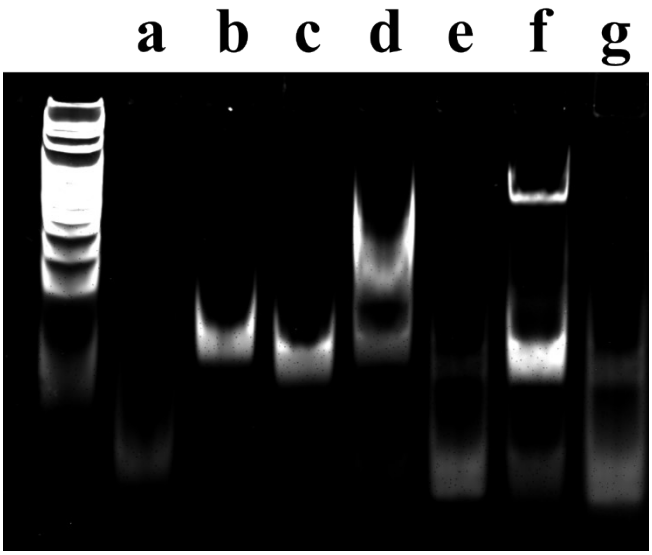
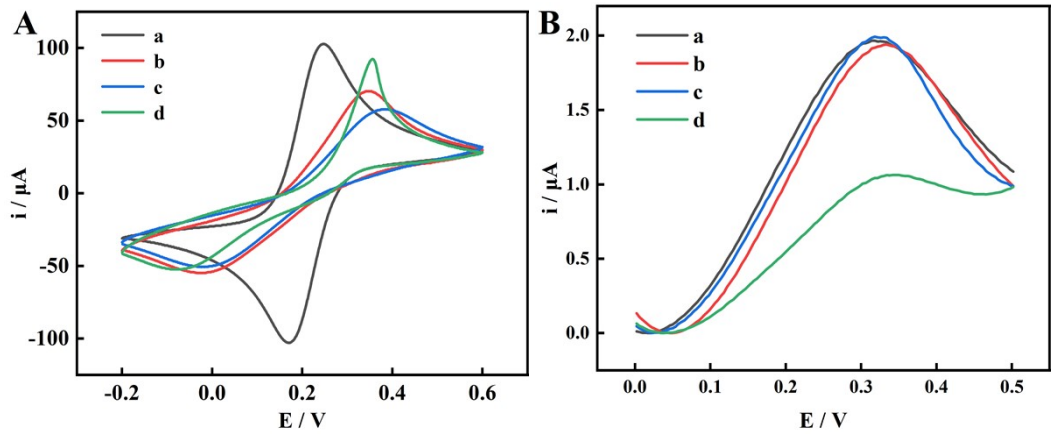


Figure S2. PAGE image of (a) miRNA, (b) H1, (c) H2, (d) miRNA + H1, (e) miRNA, + H1 + DSN, (f) solution (e) with DSN stop solution + H2, (g) solution (e) + H2.



8. Electrochemical characterizations of the proposed biosensor

Fig. S3 (A) CV of different electrode in 0.1 M KCl solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$: (a) bare AuE, (b) H2/AuE, (c) MCH/H2/AuE, (d) after cleavage reaction of DSN; (B) DPV responses of the MCH/H2/AuE after reaction with (a) no miRNA-141 and DSN, (b) no DSN, (c) no miRNA and (d)100 fM target and 0.01 U/μL DSN.

9. Optimization of Experimental Conditions

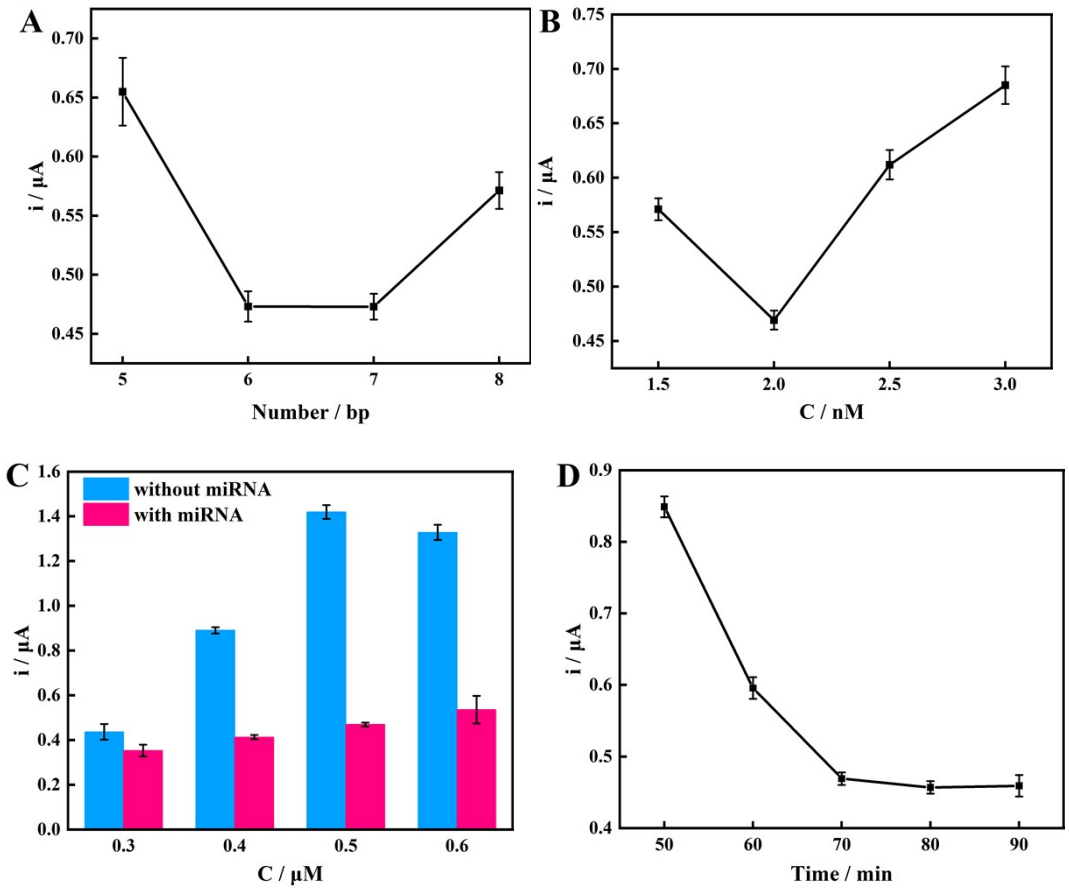
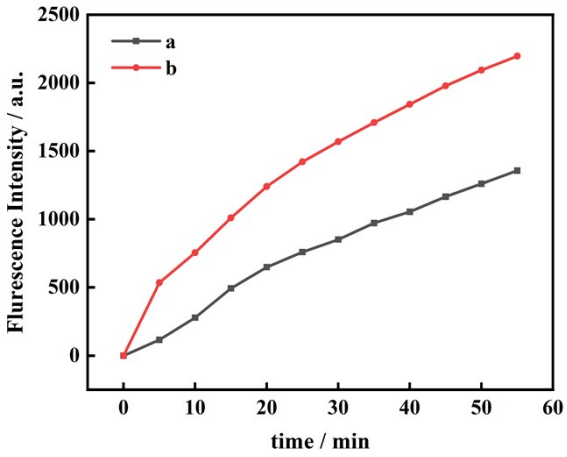


Fig. S4 (A) Effects of length of cleat structure in H1; (B) Effects of concentration of H1&AuNPs; (C) Effects of assembly concentration of H2; (D) Effects of multi-legged DNA walking reaction time, error bar, SD, n = 3.



10. Comparison of sensing strategy without cleat

Fig. S5 Fluorescence analysis of (a) H1-D&AuNPs (without cleat) and (b) H1*&AuNPs (with cleat) after reaction with 50 fM W (6) and 0.01 U/μL DSN.

11. Comparison of this Strategy with Other Studies

Table S2. Comparison of this Strategy with Other Studies for Detection of miRNA.

analytical strategy	step count	linear range	LOD	refs
Cleat-equipped molecular walking machine	1	0.5 fM to 5 pM	0.26 fM	3
Molecular machine based on toehold-mediated strand displacement reactions	1	5 fM to 500 pM	1.4 fM	4
Bipedal DNA walker	1	1 fM to 100 fM	0.22 fM	5
DNA rolling machine	1	1 fM to 100 pM	0.28 fM	6
Controllable 3D DNA nanomachine	2	100 aM to 1 nM	**33.1 aM**	7
Multipedal DNA walking nanomachine driven	2	10 aM to 1 pM	**2.8 aM**	8
Twice-walking strategy	2	0.1 fM to 200 fM	**68 aM**	this work

12. Investigation of Reproducibility.

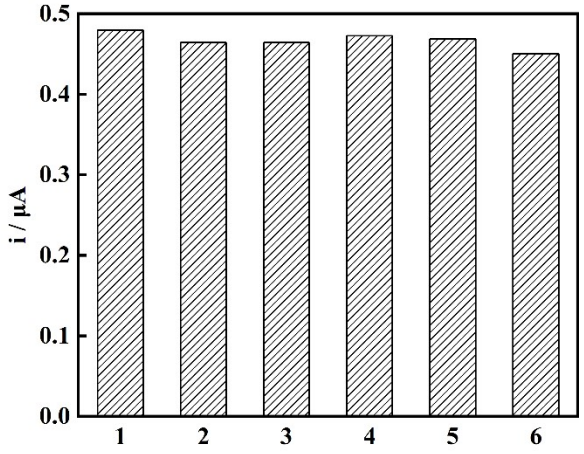


Fig. S6 Reproducibility of the proposed biosensor with six electrodes (50 fM miRNA-141).

Reference

1. L. Xu, S. Lei, Z. Liu, G. Ouyang, L. Zou and B. Ye, Anal. Chim. Acta., 2019, **1087**, 29-35.

2. S. Lei, L. Xu, Z. Liu, L. Zou, G. Li and B. Ye, Anal. Chim. Acta., 2019, **1081**, 59-64.

3. S. Lei, Y. Ji, Q. Zhang, G. Li, L. Zou, G. Chai, J. Mao, J. Zhang, and B. Ye, Tal., 2024, **272**, 125756.

4. K. Shi, B. Dou, C. Yang, Y. Chai, R. Yuan, and Y. Xiang, Anal. Chem., 2015, **87**, 8578-8583.

5. Hua Chai, and Peng Miao, Anal. Chem., 2019, **91**, 4953-4957.

6. Z. Xu, Y. Chang, Y. Chai, and H. Wang, and Ruo Yuan, Anal. Chem., 2019, **91**, 4843-4888.

7. D. Li, X. Zhang, Y. Chai, and R. Yuan, Anal. Chem., 2023, **95**, 1490-1497.

8. P. Miao, Anal. Chem., 2023, **95**, 6760-6764.