Electronic Supporting Information

Construction of streptavidin-based dual-localized DNAzyme walker for disease biomarker detection

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1. Reagents

Sodium chloride (NaCl), potassium chloride (KCl), sodium hydroxide (NaOH), sodium nitrate (NaNO$_3$), magnesium chloride hexahydrate (MgCl$_2$·6H$_2$O), lead dichloride (PbCl$_2$), lead nitrate (Pb(NO$_3$)$_2$), acetic acid (HAc) and hydrochloric acid (HCl) were purchased from Chron Chemicals Co., Ltd (Chengdu, China). D-biotin, 5×TBE buffer and 10×PBS buffer were commercially obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). Streptavidin was obtained from New England Biolabs Ltd. (Beijing, China). Thrombin was obtained from Roche Diagnostics GmbH Mannhein (Germany). Tris(hydroxymethyl)aminomethane (Tris), ethylene diamine tetra acetic acid disodium salt dihydrate (EDTA·2Na), Tris(2-carboxyethyl)phosphine (TCEP) and dithiothreitol (DTT) were purchased from Aladdin Industrial Co., Ltd. (Shanghai, China). Recombinant human serum was from Zhengzhou Cell to Antibody&Antigen Biotechnology. Co., Ltd (Zhengzhou, China). Gold nanoparticles (Au NPs) were from Sigma Aldrich (St. Louis, MO, USA).

The oligonucleotides used in this work were ordered from Shenggong Bioengineering Co., Ltd. (Shanghai, China) with the sequences list in Table S1:

Table S1. Sequences of all DNA oligonucleotides applied in this work.

Name	Sequence (5′- 3′)
8-17E substrate	TTTTTTTTTTTTTTCACTAT/rA/GGAAGAGAT
fragment-2 (thrombin)	AGTCCGTGGTAGGGCAGGTTGGGGTGACTTTTTTTTTT TTTTTTTTTTTTTTTTGAGACCGTCGAAATAGTG
fragment-1 (thrombin)	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTC TTCTCCGAGCCGGGTCTCTTTTTTTTTTTTTTTTTTTTTT TTGGTTGGTGTGGTTGG
fragment-1 (microRNA-21)	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT TCTCCGAGCCGGGTCTGTTTTTTTTTTTTTTTTTTTTTTTTT

	TATCAGTCTGCAC
fragment-2 (microRNA-21)	GGGTGGGTCAAC**CAGACC**GTCGAAATAGTG
microRNA-21	UAGCUUAUCAGACUGAUGUUGA
microRNA-21 SNP1	UAGC**A**UAUCAGACUGAUGUUGA
microRNA-21 SNP2	UAGC**A**UAUC**U**GACUGAUGUUGA
microRNA-21 SNP3	UAGC**A**UAUC**U**GACUG**U**UGUUGA
hairpin-21	CAGACTGATGTTGACCCTGATCAATTCAACATCAGTCTGATAAGCTA

2. Instruments

The gel was stained by GelRed and then imaged with ChampGel 7000 (China). All the spectral absorption and fluorescence measurements in this experiment were completed with a BioTek H1M (USA). Ultrapure water was prepared by a purification system from PINCHENG (Pure Technology CO., Ltd., China). The Zeta potential was measured by Zetasizer Nano ZS (Malvern Instruments Ltd., England). The surface morphology of the AuNPs was explored with a scanning electron microscope (SEM, JEOL, JSM-7500F).

3. Polyacrylamide gel electrophoresis (PAGE) analysis

Due to the large molecular weight of streptavidin, the original 16% polyacrylamide was replaced with a lower concentration of 9% polyacrylamide in our study. To prepare 9% polyacrylamide gel, 2 mL of 5×TBE buffer and 3 mL of 30% Acr-Bis (29:1) was added into 5 mL ultrapure water, and then 6 μL of N,N,N',N'-tetramethylethylenediamine (TEMED) and 100 μL of 10% ammonium persulfate (APS) were rapidly added into the solution to trigger the polymerization of acrylamide for 20 min at room temperature. Electrophoresis was conducted at 120 V for about 30 min, and then the gel was stained with GelRed and imaged.

4. Preparation of free unipedal, streptavidin-localized unipedal, streptavidin-localized bipedal, streptavidin-localized tripedal and streptavidin-localized quadrupedal of DNAzyme walkers

Diverging from the conventional multipedal DNA walker design, in which the pedal existed in a free state or tethered on nanoparticles. Streptavidin was employed as the carrier for pedals, consisted of DNAzyme fragment-1, DNAzyme fragment-2 and target protein. Specifically, free unipedal DNAzyme walker, streptavidin-localized unipedal, bipedal, tripedal and quadrupedal DNAzyme walkers were prepared. Streptavidin is known to possess four equivalent sites to rapidly and strongly recognize biotin, which ensures the stability of the characteristic structure of the proposed quadrupedal DNAzyme walker, maintaining the consistency among its quadrupedal. Meanwhile, in order to contribute to the formation of various pedals based on streptavidin, the concentrations of the DNAzyme fragment-1 are pivotal in this process. Following a series of optimization procedures, a series concentrations of DNAzyme fragment-1 including 1 μM, 0.75 μM, 0.5μM, and 0.25 μM were mixed with 250 nM streptavidin at room temperature to form quadrupedal, tripedal, bipedal, and unipedal DNAzyme walkers, respectively. Subsequently, in the preparation of the quadrupedal DNAzyme walker and its successful operation, further optimization is required for the concentrations of the formed streptavidin-localized four

fragments-1 and the auxiliary factor Pb^{2+}. The prepared pedals were stored

in a 4 °C for subsequent use.

5. Preparation of DNA-AuNPs track.

The preparation of the DNA-AuNPs track for the DNAzyme walker was conducted by conjugating thiol-labeled DNAzyme's substrate strands onto AuNPs with the assistance of gold-sulfur bonds. To prepare the DNA-AuNPs track for the DNAzyme walker responsive to thrombin, 20 µL, 100 uM substrate strands labeled with a FAM molecule at its 3′-end and a dimercaptoyl group at its 5′-end were mixed with 2 µL, 5 mM tris(2-carboxyethyl)phosphine (TCEP) and the obtained mixture was incubated for 1 h at 37 °C. Through such a process, the dimercaptoyl group was redacted to thiol group. Next, 500 µL AuNPs (1.16 nM, d = 20 nm) were mixed with 20 µL, 100 µM redacted substrate. This solution was incubated at room temperature overnight after sonicating for 1 min. To improve the oligonucleotide loading efficiency, 20 µL, 3 M NaCl was solely added into the solution for three more times. After each increment, the solution was sonicated for 10 s followed by 1 h incubation at room temperature, and this process is repeated for three times. Then, the obtained mixture was centrifuged for 3 times with 13500 rpm to separate the unloaded substrate strands. Lastly, the prepared DNA-AuNPs track solution was re-dispersed in 500 µL 0.1 M phosphate buffer solution (PBS) (pH = 7.4).

6. Measurement of loading amount for substrate strands on per AuNP

To ensure the seamless functioning of the DNAzyme walker, it is significant to determine the optimal number of substrate strands on the prepared DNA-AuNPs track (Figure S7). To assess this parameter, 10 µL of a 1 nM solution of the prepared DNA-AuNPs track was dispersed in 80 µL of distilled water. Subsequently, 10 µL of 200 mM DDT was added, and the mixture was stored overnight at room temperature in the dark to facilitate the complete displacement of the substrate DNA strand on the DNA-AuNPs track from AuNPs. Given that the 3'-end of the substrate DNA strand is modified with a fluorophore (FAM), the fluorescence originally quenched by AuNPs is restored upon detachment from AuNPs. The number of loaded substrate strands on the DNA-AuNPs track can be calculated from the detected fluorescence intensity.

7. Analytical procedure

To evaluate the operation of the constructed DNAzyme walker responding to thrombin, the fluorescence increase was measured, from sample and blank solutions in real time. Unless otherwise stated, sample solutions contained 0.1 nM DNA-AuNPs track, 10 nM aptamer-labeled DNAzyme fragment-2, 0.25 mM streptavidin labeled split DNAzyme fragment-1, 150 mM NaCl and specified concentrations of thrombin in 100 μL of 100 mM PBS (pH = 8.2). Blank solutions contained all other components except thrombin. Solutions were transferred to a 96-well assay plate, which was then loaded onto the fluorescence microplate reader for real-time fluorescence detection. The fluorescence was measured for a total of 2 h by using 485 nm for excitation and 521 nm for emission. The operation of the motor responding to thrombin was monitored. Similarly, to evaluate the operation of the constructed DNAzyme walker responding to microRNA-21, different concentrations of microRNA-21 were mixed with 0.1 nM DNA-AuNPs track, 10 nM hairpin 21, 10 nM DNAzyme attached with microRNA-21 complementary sequence, 150 mM NaCl in 100 μL of 500 mM Tris-HAc (pH = 8.2). The blank solution contains all other components except microRNA-21.
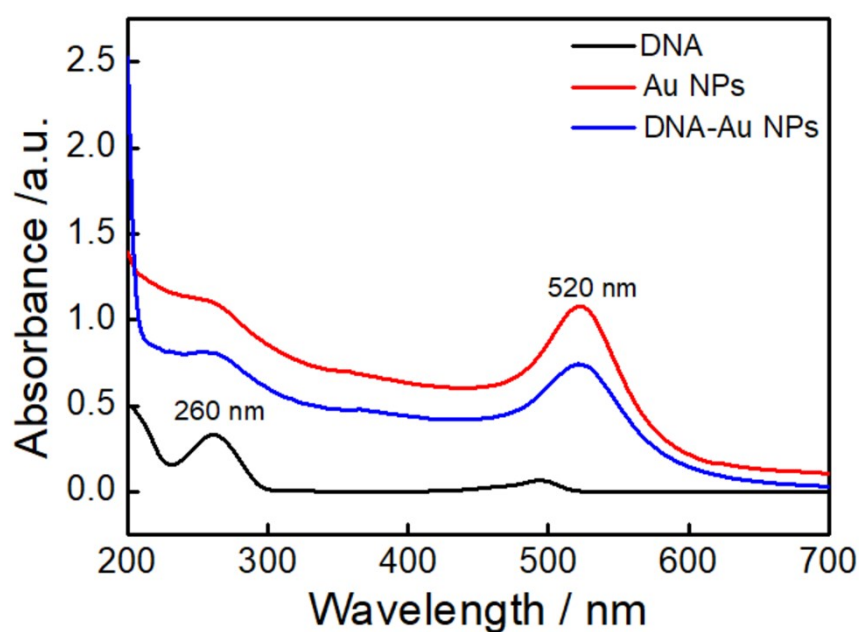
8. Characterization of the AuNPs

To produce high-performance DNAzyme walkers and simultaneously minimize errors arising from different batches of walkers, AuNPs were employed as carriers for substrate strands. The role of AuNPs is pivotal in the fabrication process of DNAzyme walkers. Consequently, scanning electron microscopy (SEM) characterization was performed for the AuNPs utilized in this study. The results, depicted in Figure S1, showcase the morphology of the AuNPs. Evidently, these AuNPs exhibit a well-defined 3D spherical structure, with a remarkably uniform particle size averaging around 20 nm in diameter. The uniformity of these AuNPs serves as a crucial foundation for fabricating DNAzyme walkers with superior performance.
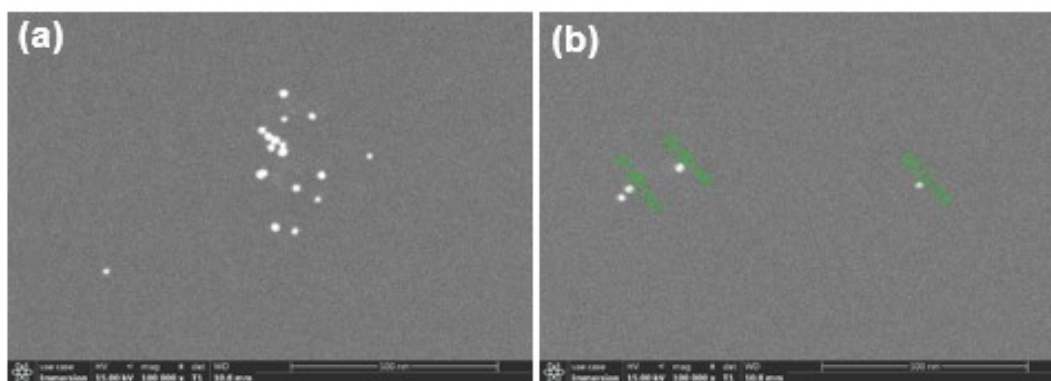
Figure S1 SEM characterization of AuNPs.

## 9. UV-visible absorption spectroscopy measurement

Figure S2 further characterizes the fabricated DNA-AuNPs track using ultraviolet-visible (UV-vis) absorption spectroscopy. Notably, the absorption peak of the DNA solution alone is situated at 260 nm, while the absorption peak of the AuNPs is at 520 nm. Upon testing the synthesized DNA-AuNPs track, a pronounced blue shift phenomenon was observed.

Figure S2 UV-visible absorption spectroscopy measurement of substrate DNA strands, AuNPs and DNA-AuNPs track.

# 10. Potential determination of AuNPs with and without DNA

A preliminary characterization of the fabricated DNA-AuNPs track was conducted using a Zeta potential analyzer, as depicted in Figure S3. The measured AuNPs displayed a robust negative potential, primarily attributed to the accumulation of a substantial amount of negative charges on their surface. In the case of the DNA-AuNPs structure of the 3D track based on AuNPs, a significant negative potential was observed due to the attachment of numerous negatively charged DNA strands onto the surface of the AuNPs. These findings provide evidence for the successful construction of DNA-AuNPs track.
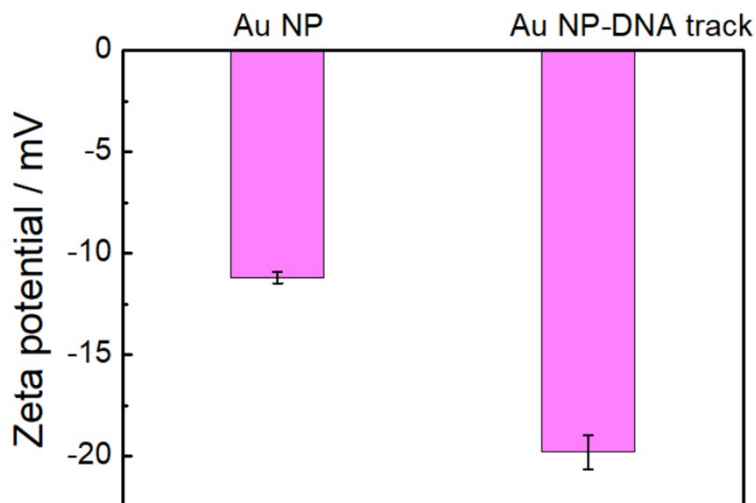
**Figure S3** Measurement of AuNP surface potential in the absence and presence of substrate DNA strand.

11. The loading amount of substrate strands on AuNPs for different batches
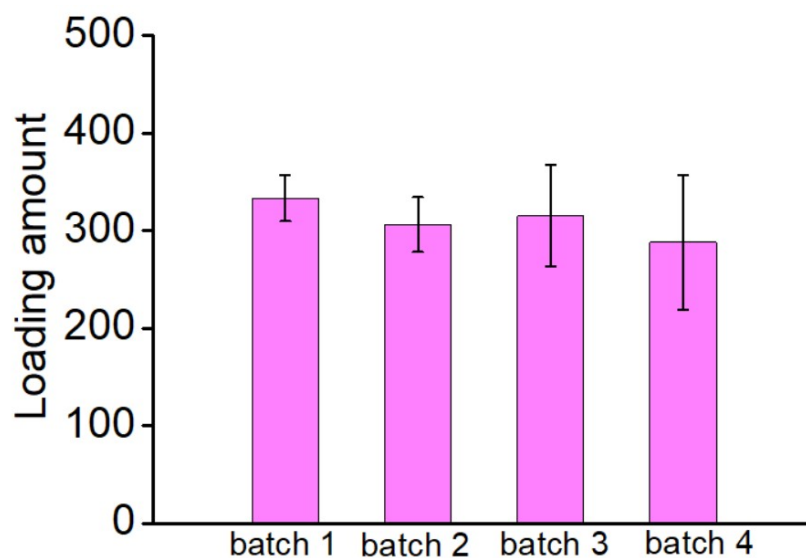
Figure S4. Four batches of functional DNA-AuNP tracks were synthesized on different days for determination of loading amount. 20 mM DTT was used for releasing the substrate from functional AuNPs. Error bars show the standard deviation of three experiments.

12.PAGE analysis

To validate the successful fabrication of our proposed streptavidin-localized quadrupedal DNAzyme walker, a 9% polyacrylamide gel electrophoresis was employed for structural characterization, as depicted in FigureS5.
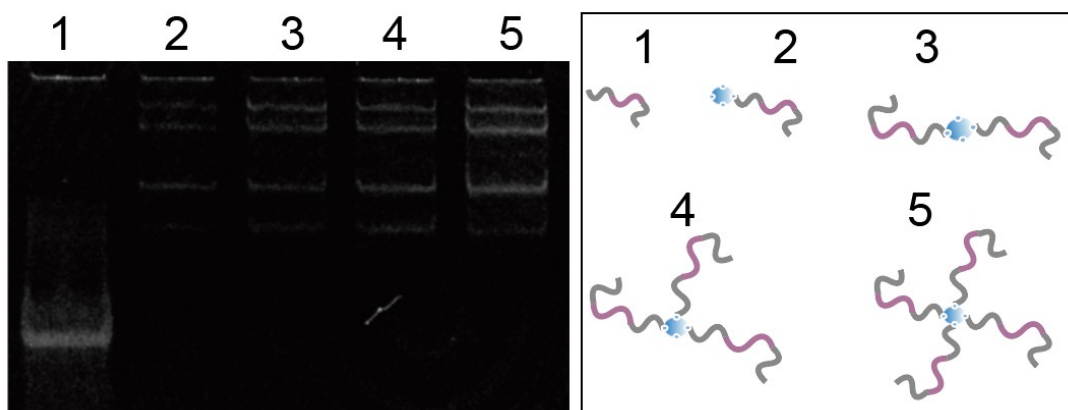

Figure S5. The PAGE result of the preparation of streptavidin-localized fragment-1. Lane 1 represented free fragment-1, displaying a single band. Lanes 2 to 5 represent the outcomes of different input ratios of streptavidin to fragment-1. Distinct and prominent four bands are evident in each lane, denoted as streptavidin-localized single fragment-1, streptavidin-localized two fragments-1, streptavidin-localized three fragments-1, and streptavidin-localized four fragments-1 from bottom to top.

13. **Design principle of the dual-localized DNAzyme walker and single-localized DNAzyme walker**
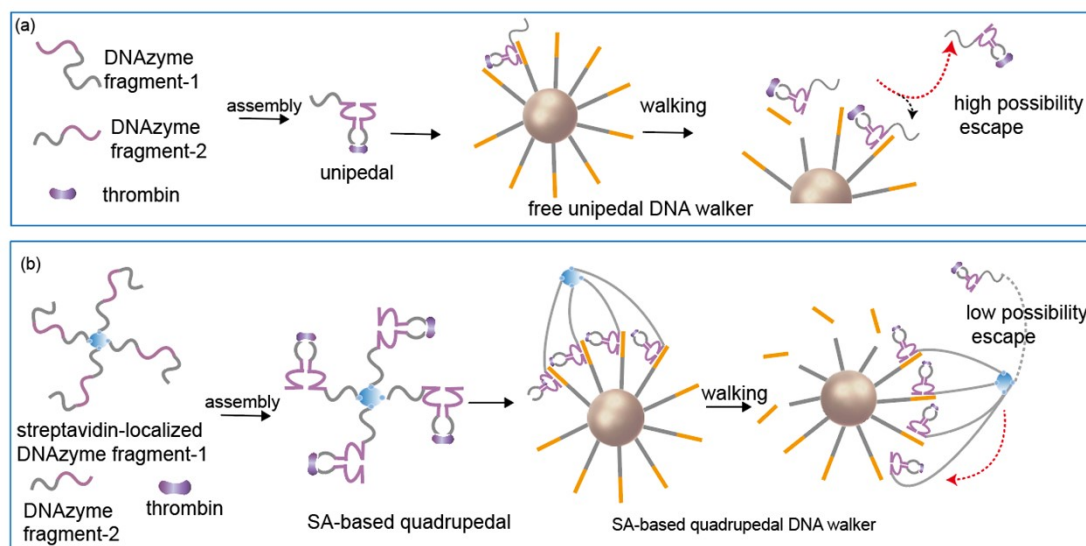
Figure S6. Design principle of the (a) single-localized DNAzyme walker and (b) dual-localized DNAzyme walker.

Unlike traditional DNAzyme walkers, the dual-localized DNAzyme walker (dlDW) has four identical walking pedals, and they exhibit a temporal offset in their initial binding to the substrate (Figure S6). This feature ensures that the quadrupedal structure maintains a consistently high local concentration around the DNA-AuNPs track, effectively minimizing the pedal detachment from the track.
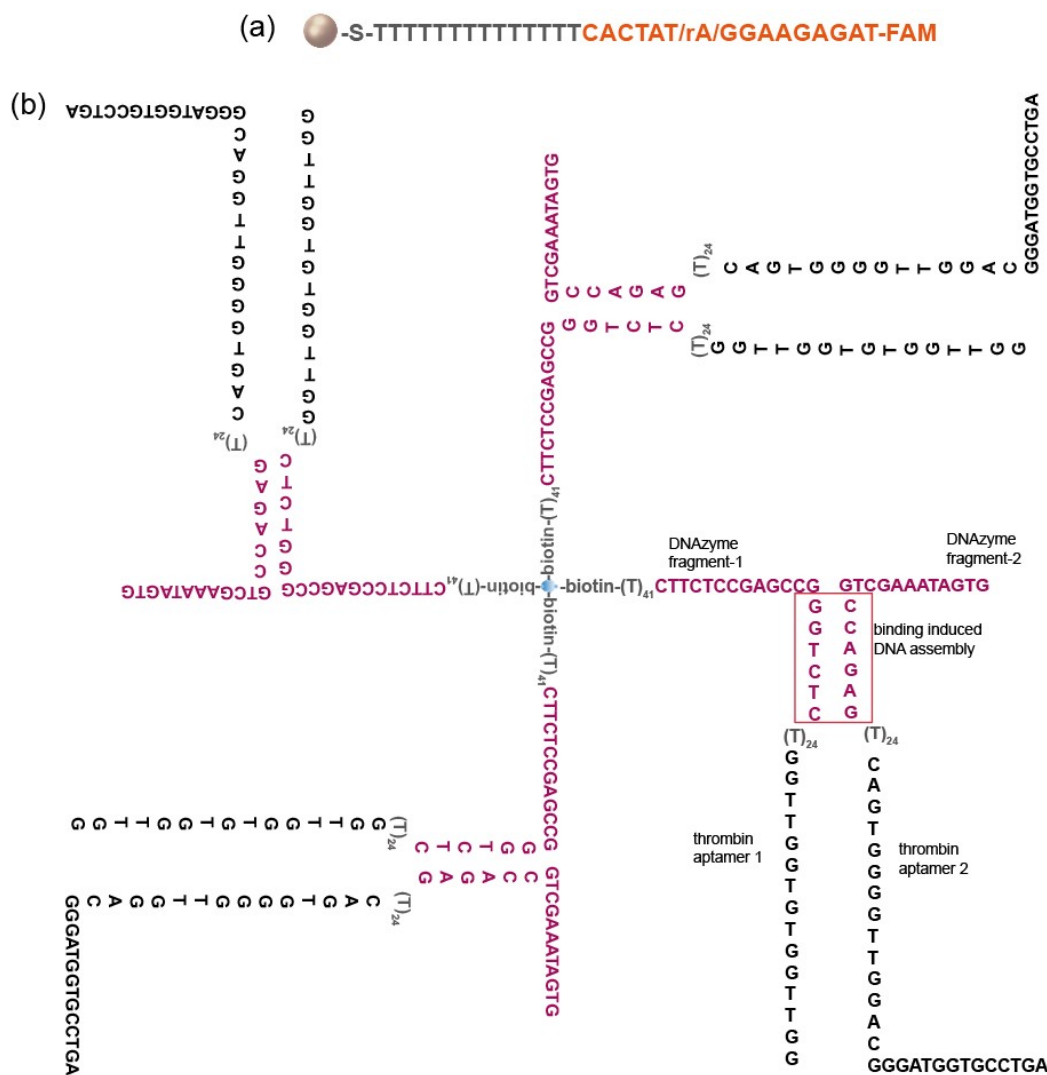
Figure S7. Sequence design of (a) substrate located on AuNPs and (b) streptavidin-localized DNAzyme four fragments-1 and assembled DNAzyme fragment-2 with the assistance of target protein.

The dlDW consists of three main components: (1) a quadrupedal structure with four fragments-1 based 8-17E DNAzyme positioned on one streptavidin as power components for walking, (2) multiple fragments-2 of split DNAzyme are free to be recruited for the assembly, and (3) A walking track contained substrate strands modified on the surface of AuNPs.

To enhance the accessibility of the DNAzyme to substrates, 14 thymines (poly-T) at the 5'-end of the substrate strand was introduced to

help maintain an upright orientation of the substrate chain on the AuNP surface (Figure S7a). Additionally, for monitoring changes in fluorescence signals to detect the operational status of the DNAzyme walker, a FAM molecule was attached to the 3' end of the substrate strand.

For precise respond to the external trigger (thrombin), affinity ligands (aptamer) were conjugated to the fragments-1 and fragments-2 for the external trigger recognition. To prevent independent assembly, only 6 complementary base pairs are designed on the fragments (Figure S7b). Simultaneous recognition of the thrombin by both fragments increases the local concentration of complementary fragments, allowing the two split DNA fragments in close proximity and stabilize the split DNAzyme for activation. Once anyone of pedals hybridize with substrates, the other pedals rapidly bind and cleave the substrates on the DNA-AuNPs track.

14. Walking rate and assembly time of free unipedal DNAzyme walker, streptavidin-localized unipedal DNAzyme walker, streptavidin-localized bipedal DNAzyme walker, streptavidin-localized tripedal DNAzyme walker and streptavidin-localized quadrupedal DNAzyme walker
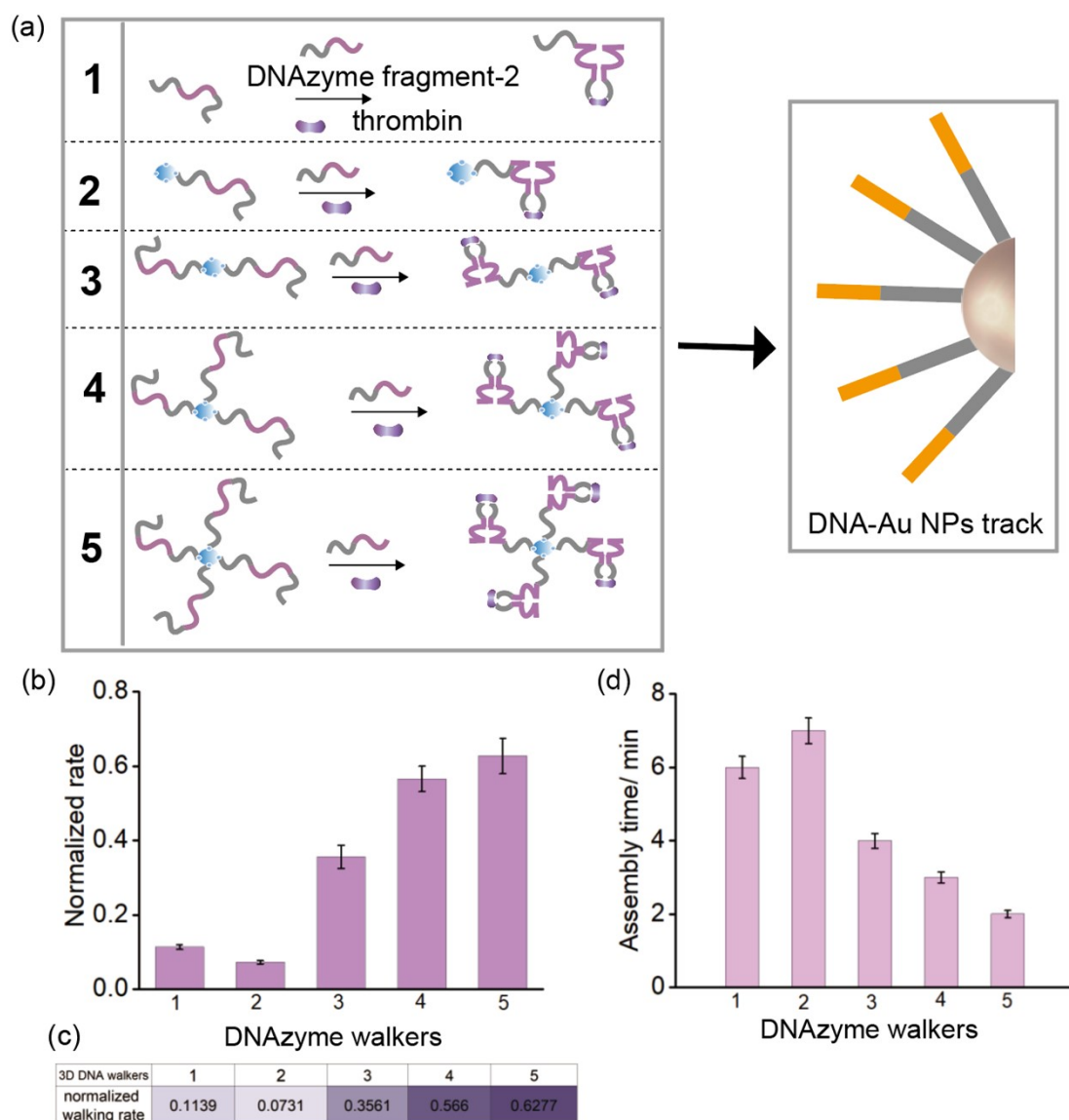
Figure S8. (a) Preparation of various pedals. (b) and (c) are the walking rate (V_0) of free unipedal DNAzyme walker (1), streptavidin-localized unipedal DNAzyme walker (2), streptavidin-localized bipedal DNAzyme walker (3), streptavidin-localized tripedal DNAzyme walker (4) and streptavidin-localized quadrupedal DNAzyme walker (5). (d) is the assembly time of DNAzyme walkers of different structures.
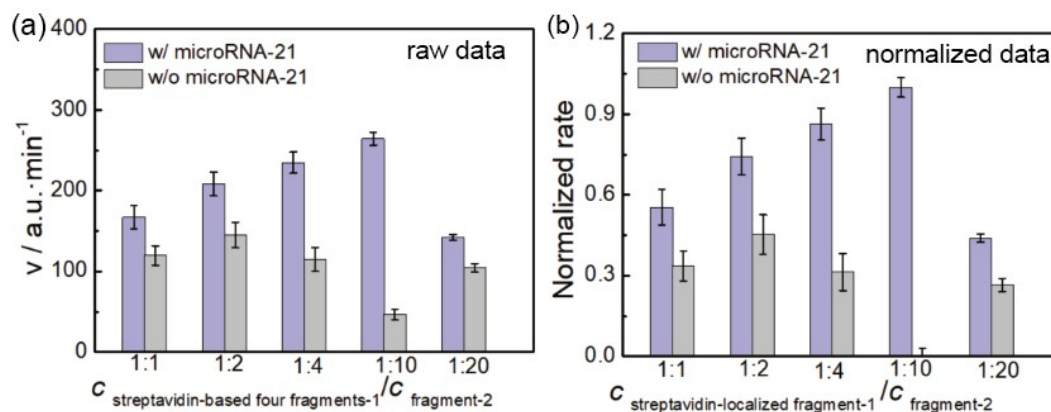
15. Optimization of the concentration ratio of streptavidin-localized four fragments-1 and fragment-2 for thrombin triggered
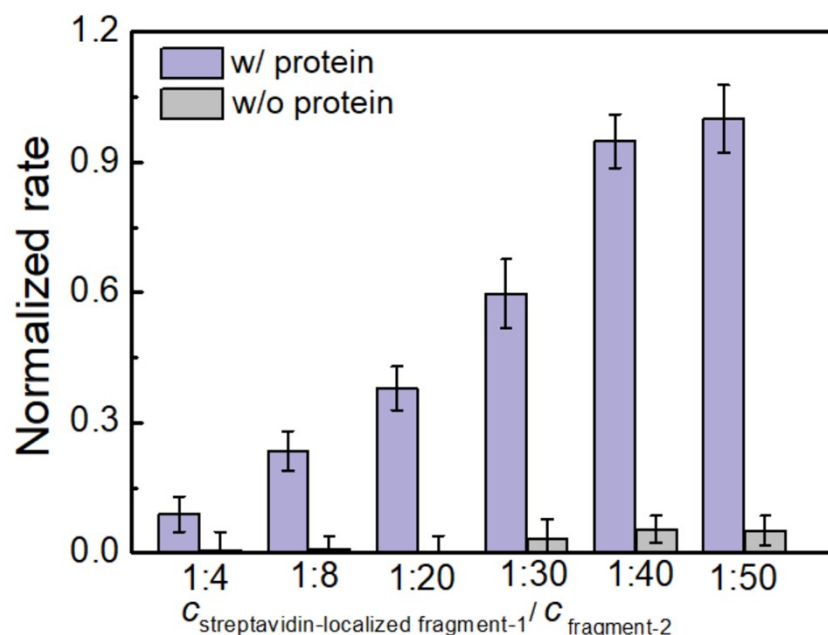
Figure S9. Optimization of the concentration ratio of streptavidin-localized four fragments-1 and DNAzyme fragment-2 toward thrombin as target.

In Figure S9, It can be observed that as the concentration ratio of streptavidin-localized four fragment-1 to fragment-2 gradually decreases, the signal in the control group increases. It should be noted that the acquisition of this experimental data was obtained by fixing the concentration of streptavidin-localized four fragment-1 and only altering the concentration of fragment-2 to adjust the ratio between them. Therefore, when the ratio of streptavidin-localized four fragment-1 to fragment-2 decreases, the concentration of fragment-2 in the entire reaction system is higher. As the assembly of the walking strand localized on the streptavidin surface relies on the localized increase in concentrations of fragment-1 and fragment-2 of the split DNAzyme, even in the absence of the target protein, the increased concentrations of fragment-1 and fragment-2 are slightly to induce non-specific assembly. This non-specific assembly is the main cause of the elevation in background signal.

16. Optimization of concentration ratio between streptavidin-localized DNAzyme four fragments-1 and free DNAzyme fragment-2 toward microRNA-21 as the trigger
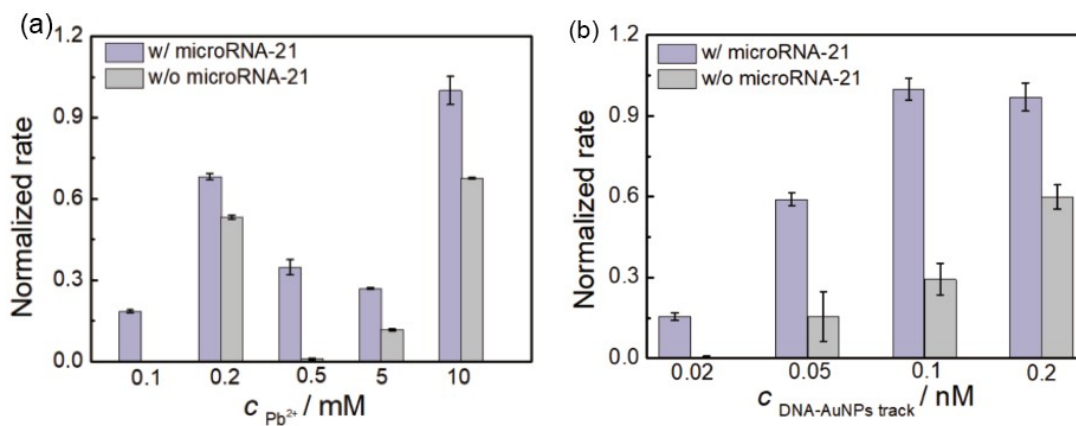

Figure S10. Optimization of the concentration ratio of streptavidin-localized four fragments-1 and fragment-2 for microRNA-21 detection. (a) Raw result, and (b) normalized result. Abbreviations: w/ = with, and w/o = without.

Figure S8 demonstrates the optimization of the ratio of streptavidin-localized four fragment-1 to fragment-2 when microRNA-21 is used as the target. As the ratio decreases, the fluorescence signal intensity gradually increases until the ratio is 1:10, after which the fluorescence signal intensity decreases. In addition, it can be found that the intensity of the background signal does not change significantly as the ratio decreases, but the lowest background signal is displayed at 1:10. Due to the normalization of walking rate, the background signals appear to be quite different, but in fact, the differences between these sets of signals are not very obvious. The reason for the elevated background signal may be the introduction of the assistant hairpin H21, if the H21 is annealed and there is still a part that has not fully formed the hairpin structure, it will be very easy to bring a non-specific response signal.
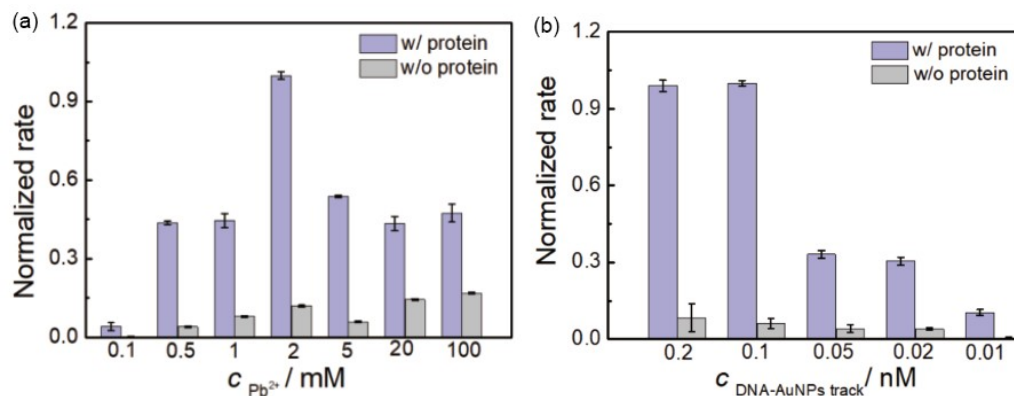
17. Optimization of concentrations of Pb^{2+} and DNA-AuNPs track toward microRNA-21 as target

Figure S11. Optimization of (a) Pb^{2+} concentration and (b) DNA-AuNPs track concentration of DNAzyme walker for microRNA-21 detection. Abbreviations: w/ = with, and w/o = without.

18. Optimization of the concentrations of Pb^{2+} and DNA-AuNPs track for the constructed DNAzyme walker toward thrombin as target
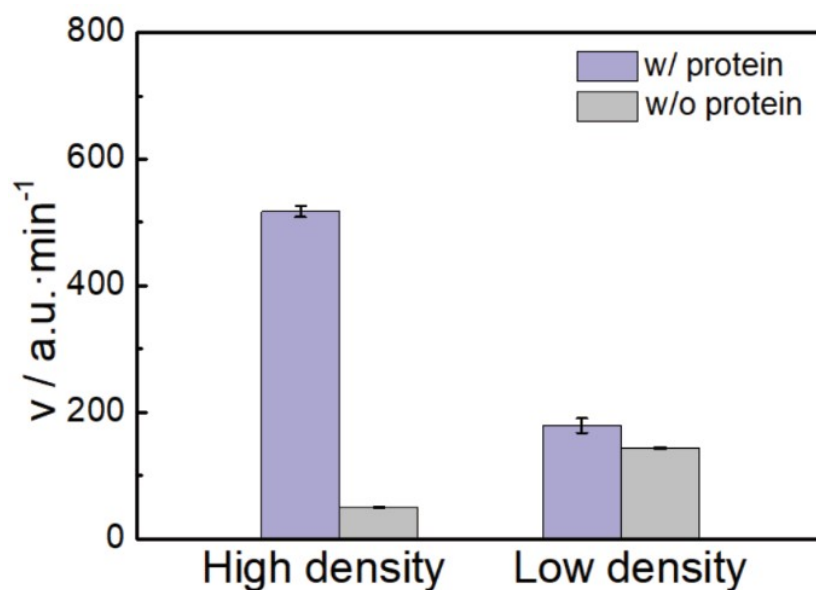

Figure S12. Optimization of (a) Pb^{2+} concentration and (b) DNA-AuNPs track concentration for the constructed DNAzyme walker toward thrombin as target. Abbreviations: w/ = with, and w/o = without.

In order to further achieve the optimal catalytic activity of the constructed DNAzyme walker toward thrombin as target, an optimization process was conducted regarding the concentration of Pb^{2+} (while keeping the system incubated with 20 nM streptavidin). As depicted in Figure S12(a), a gradual decrease in Pb^{2+} concentration was observed, resulting in a slight reduction in fluorescence signal intensity. Intriguingly, beyond a threshold concentration of 2 mM, the signal intensity exhibited a noticeable increase. Consequently, 2 mM as the optimal concentration of Pb^{2+} was selected for this study.

Similarly, the optimally used amount of prepared DNA-AuNPs tracks needed to be determined during delivery (Figure S12b). High-density DNA-AuNPs tracks were introduced at concentrations of 0.2 nM, 0.1 nM, 0.05 nM, 0.02 nM, and 0.01 nM. Fluorescence kinetic monitoring results revealed that the concentration of 0.1 nM DNA-AuNPs track was more suitable for our experimental results.

19. Optimization of loading density for substrate strand located on AuNPs

Figure S13. Optimization of density of substrate DNA mobilized on AuNP. Abbreviations: w/ = with, and w/o = without.

To achieve optimal operational efficiency of the walker, the loading concentration of substrate strands on AuNPs was carefully optimized. As illustrated in the figure S13, two types of DNA-AuNPs tracks with different loading amounts of substrate strands were successfully prepared, categorized as high density (approximately 330 substrate strands on per AuNP) and low density (approximately 180 substrate strands on per AuNP). The results indicated that the high-density DNA-AuNPs track exhibits superior operational efficiency. Therefore, the high-density DNA-AuNPs track was selected for subsequent study.
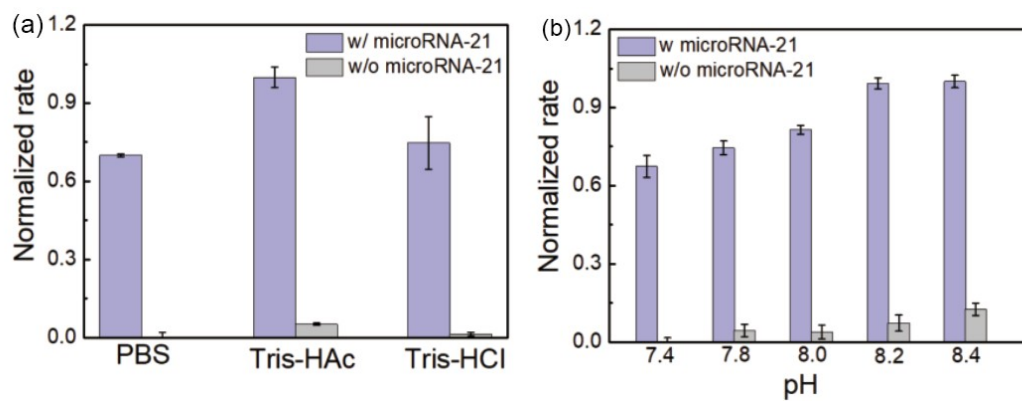
20. Selection of buffer and optimization of the pH of buffer toward protein as target
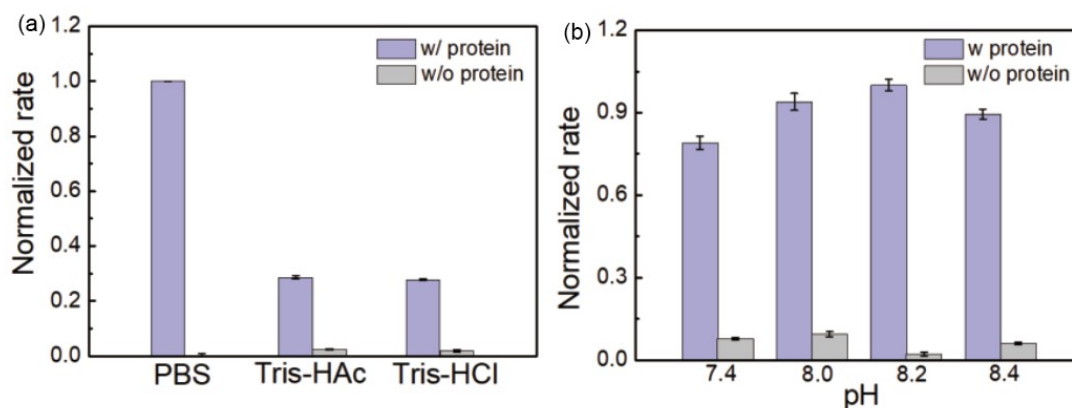
Figure S14. (a) Selection of buffer solution and (b) optimization of pH for the selected buffer solution (PBS). Abbreviations: w/ = with, and w/o = without.

To maintain the stability of the reaction system environment and ensure the consistency of the reaction, it is necessary to add an appropriate buffer solution to the system. Here, three buffer solutions were selected for screening, namely PBS, Tris-HCl, and Tris-HAc. As shown in the Figure S14a, our constructed DNAzyme walker can efficiently operate in PBS, while the operational efficiency in Tris-HCl and Tris-HAc is less ideal. Based on these results, the suitable pH range was further optimized for the reaction system, choosing a range from 7.4 to 8.4. The results, as shown in the figure S14b, indicate that pH = 8.2 has the optimal signal-to-background ratio. In conclusion, PBS with a pH of 8.2 was ultimately selected as the buffer solution for the reaction system.

21. Selection of buffer **and** optimization of the pH of buffer toward microRNA-21 as the trigger

Figure S15 (a) Selection of buffer solution and (b) optimization of pH for the selected buffer solution (Tris-HAc) for microRNA-21 detection. Abbreviations: w/ = with, and w/o = without.

22. Operation performance of the dual-localized DNAzyme walker using microRNA-21 as direct trigger
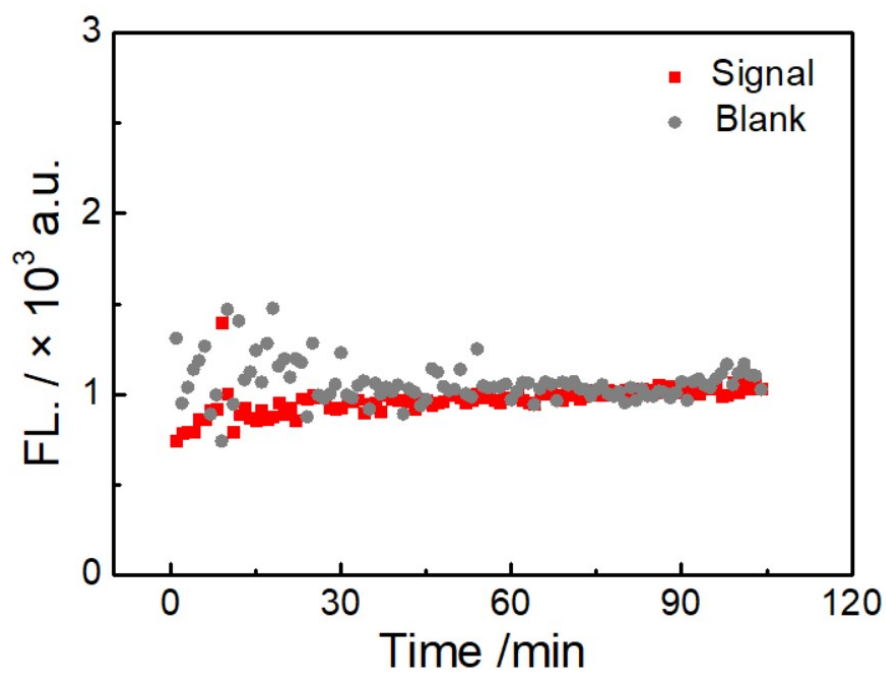
**Figure S16.** Operation performance of the dual-localized DNAzyme walker using microRNA-21 as direct trigger.

23. Definition of initial walking rate (V_0)

$$V_0 = F_t - F_0 / t$$

- V represents the catalytic rate.

- F_t is the fluorescence intensity at a specific time point 't' on the fluorescence kinetics curve before reaching equilibrium under optimal conditions.

- F_0 represents The initial fluorescence intensity recorded on the fluorescence kinetics curve

The calculation of V_0 is based on the value of 10th min of the fluorescence kinetic curves of each DNAzyme walker, mainly because all DNAzyme walkers have been assembled and started walking, but none of them have reached a fluorescence signal plateau at the 10th min.
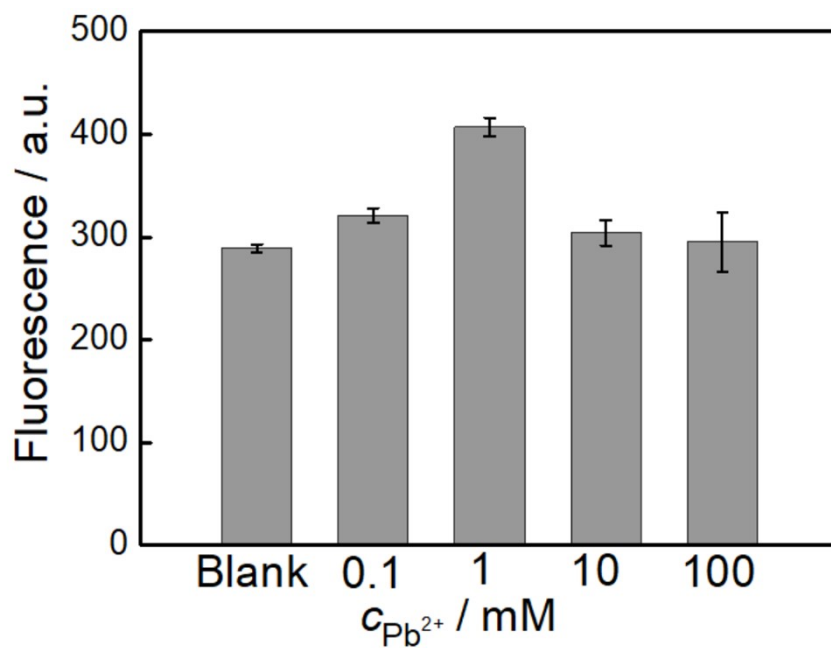
24 The stability of the DNA-AuNPs track in different concentrations of recombinant human serum
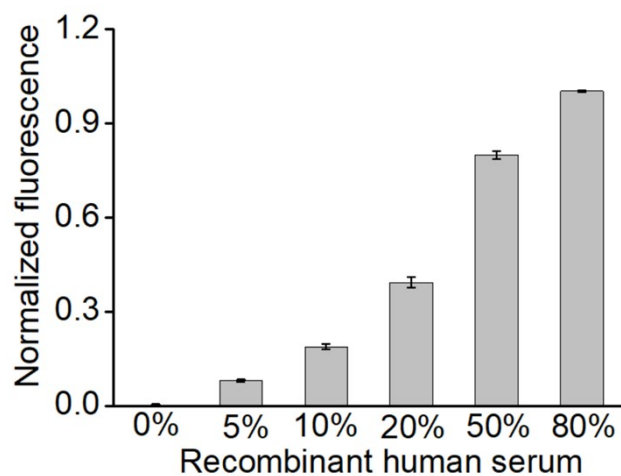
Figure S17. The stability of the dIDW in different concentrations of recombinant human serum, including 0%, 5%, 10%, 20% , 50% and 80%. After 1 hour incubation, each group of functional DNA-AuNPs tracks without DNAzyme fragments and external target was transferred into the spectrometer for end-point reading.

25. The stability of the DNA-AuNPs track in different concentrations of Pb^{2+}

Figure S18. The stability of the dlDW in different concentrations of Pb^{2+}, including 100 mM, 10 mM, 1 mM, 0.1 mM and blank.

26.Detection process of thrombin in 5% recombinant human serum.

To evaluate the clinical potential of the developed dlDW, its analytical performance in recombinant human serum was assessed (Figure S17). Thrombin was employed as the external target, and the fluorescence response of the dlDW demonstrated proportionality to the concentration of each biomarker target within 30 min. Additionally, a linear relationship spanning from 10 pM to 2 nM was observed for thrombin in 5% recombinant human serum. Subsequently, a recovery experiment was conducted using the standard addition method, indicating recovery rate of thrombin in 5% recombinant human serum ranged between 98% and 120% (Table S2). Considering that the substrates track in the dlDW contained one ribonucleic acid base, the stability of the dlDW was then evaluated at different serum concentrations. Although the DNA-AuNsP track becomes less stable with increasing concentration, dlDW remains relatively stable in 5% recombinant human serum (Figure S17). Thus, the performance in biological matrices verified the dlDW, with potential applications in clinical diagnostics.
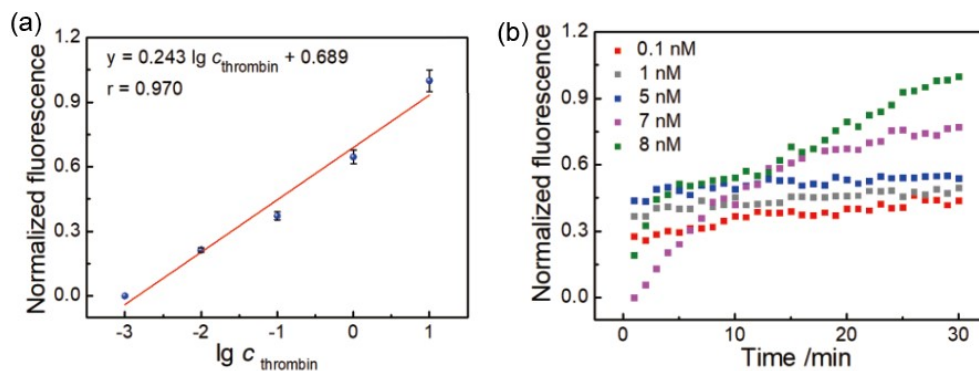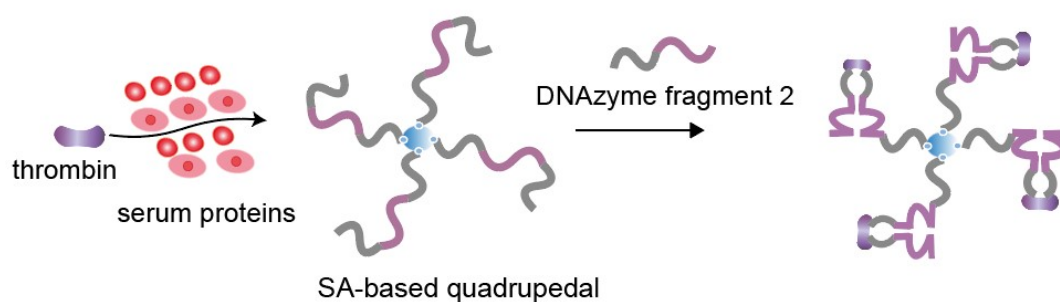
Figure S19. Scheme illustration of the detection process of thrombin in 5% recombinant human serum.

Table S2. Determination of thrombin in 5% recombinant human serum with the proposed method

Added concentration/ nM	Found concentration/ nM	Recovery/ %
0.1	0.12	120
1	0.98	98
5	5.2	104
7	8.1	115
8	8.8	110