Supporting Information

A three-dimensional multipedal DNA walker for ultrasensitive detection of tumor exosomes

Huizhen Wang,[a] Kejing Wan,[a] Yue Zhou,[b] Xiaoxiao He*,[a] Dinggeng He*,[a] Hong Cheng,[a] Jin Huang,[a] Ruichen Jia,[a] Kemin Wang*[a]

[a] State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Biology, College of Chemistry and Chemical Engineering, Hunan University, Key Laboratory for Bio-Nanotechnology and Molecule Engineering of Hunan Province, Changsha 410082, China.

[b] Hunan Branch Center, National Tissue Engineering Center of China, Translational Medical Center, Central Laboratory, Hunan Cancer Hospital/The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha, China

Experimental Section

Chemicals and Materials. The streptavidin-modified micrometer-scale sepharose beads (MBs, 34 μm; biotin binding capacity, >300 nmol/mL medium) were purchased from GE Healthcare Bio-Sciences (Sweden). Dulbecco's phosphate-buffered saline (D-PBS) was purchased from Sigma−Aldrich. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, cholera toxin and 0.22 μm syringe-filters were purchased from Thermo Scientific HyClone (MA, U.S.A.). DNA Marker (25~500 bp) and Triton X-100 was purchased Sangon Biotechnology Inc. (Shanghai, China). Filter (300 mesh) was purchased from Tmall (China). Anti-human-CD63 and Anti-human-Alix was obtained from Liaoning Rengen Biosciences Co. Ltd (China). CEM (human acute lymphoblastic leukemia) cells, Ramos (human Burkitt's lymphoma) cells and MCF-7 (human breast cancer) cells used in this study were obtained from the Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All oligonucleotides were synthesized by Sangon Biotechnology Inc. (Shanghai, China). DNA sequences used in this article were listed in **Table S1**. All the chemicals were at least of analytical reagent grade unless otherwise specified. All aqueous solutions were prepared with ultra-pure MilliQ water (18.2 MΩ cm, Millpore Co).

The reaction buffers used in this work were listed as below:

Tris-HCl (50 mM, 8 mM $MgSO_4$, pH 7.5);

TNaKT buffer (20 mM Tris, 140 mM NaCl, 5 mM KCl, pH 7.5);

Binding buffer (20 mM Tris, 1 M NaCl, 1 mM EDTA, 0.0005% Triton X-100, pH 7.5).

1×TBE (pH = 8.0, containing 89.0 mM Tris-HCl, 89.0 mM H_3BO_3, and 2.0 mM EDTA·2Na).

Preparation of Three-dimensional (3D) Multipedal DNA Walker. Prior to fabrication of 3D multipedal DNA walker, Apt_{PTK7}-catalyst (20 μM), biotin-coated anchored DNA (biotin-aDNA, 10 μM), biotin labeled hairpin DNA1 (biotin-H1, 10 μM) and 5-carboxy fluorescein (FAM) modified hairpin DNA2 (FAM-H2, 10 μM) were annealed by heating to 95 °C for 5 min, followed by slowly cooling down to room temperature (25 °C) for at least 2 h before use. Micrometer-scale beads (MBs) were washed three times with binding buffer to remove the ethanol solution and then filtered by the filter (300 mesh) to remove large sizes of MBs for subsequent flow cytometry analysis.

DNA modified MBs, including Apt_{PTK7}-catalyst/aDNA modified MBs and H1 coated MBs conjugations, were prepared as follows. Briefly, for Apt_{PTK7}-catalyst/aDNA modified MBs (Apt_{PTK7}-catalyst/aDNA-MBs), 10 μL Apt_{PTK7}-catalyst (20 μM) and 10 μL aDNA (10 μM) were incubated together in Tris-HCl buffer to produce duplex DNA (e.g., Apt_{PTK7}-catalyst/aDNA), which were annealed by heating to 95 °C for 5 min, followed by slowly cooling down to room temperature (25 °C) for at least 2 h before use. Then, 100 μL Apt_{PTK7}-catalyst/aDNA (5 μM) was incubated with 50 μL MBs resuspended in 350 μL TNaKT buffer. After shaking for 0.5 h at room temperature, the resulted Apt_{PTK7}-catalyst/aDNA-MBs was washed three times with binding buffer, followed by resuspension in 500 μL TNaKT buffer, and stored at 4 °C for further use. For H1 coated MBs conjugations (H1-MBs), 50 μL biotin-H1 (10 μM) was incubated with 50 μL MBs resuspended in 400 μL TNaKT buffer. After shaking for 0.5 h at room temperature, the following operations for H1-MBs were the same as the above-mentioned procedures.

EXs Isolation and Characterization. All cells were cultured in DMEM media containing 10% EXs-depleted FBS, 1% penicillin and streptomycin, and then incubated in a humidified atmosphere of 5 wt %/vol CO_2 at 37 °C. After 48 h of incubation, EXs were collected from cell culture supernatant and isolated based on a standard ultracentrifugation protocol with some modification.[50] In brief, the intact cells, cell debris, and large microvesicles were removed sequentially by centrifugation

at 300 g for 10 min, 2,000 g for 20 min, and 10,000 g for 45 min, respectively. The supernatant containing EXs was filtered through a 0.22 μm filter. Then, the filtered supernatant was centrifuged at 100,000 g for 1.5 h at 4 °C to obtain EXs sediment. Next, the EXs sediment was washed and resuspended in 1× PBS and centrifuged again for 1.5 h at 4 °C. Finally, the obtained EXs were resuspended in 1× PBS and stored at −80 °C for further use.

The morphology of isolated EXs was characterized by transmission electron microscopy (TEM). In brief, isolated EXs were fixed with 2% paraformaldehyde for 1 min. After that, the mixture was loaded on formvar carbon-coated copper grids for 5 min, negatively stained with 2% phosphotungstic acid for 110 min and then washed twice with 1× PBS. Finally, the prepared sample was dried completely at room temperature and imaged by a Tecnai G2 20 STwin (FEI, Czech Republic). The total number and size distribution of EXs was measured by nanoparticle tracking analysis (NTA) using Nanosight NS300 instrument (Malvern).

Gel Electrophoresis. Experimental feasibilities, including CHA reaction and binding force of Apt_{PTK7}-catalyst, were determined respectively. Briefly, for investigation of CHA reaction, 1 μM H1, 1 μM H2 and 100 nM catalyst (free catalyst or Apt_{PTK7}-catalyst) were incubated in 10 μL TNaKT buffer for 1 h at 37 °C. For studying the binding force between Apt_{PTK7}-catalyst and different lengths of anchored DNA, 1 μM Apt_{PTK7}-catalyst were incubated with 1 μM anchored DNA over different hybridization lengths ranging from 8 to 17 bp, which were annealed by heating to 95 °C for 5 min, followed by slowly cooling down to room temperature (25 °C) for at least 2 h before use. Subsequently, 10 μL reaction samples mentioned-above were mixed with 2 μL 6 × loading buffer and 2 μL SYBR Gold. The resulted mixtures (10 μL) were then separated by gel electrophoresis and run at 80 V for 1 h in 1 × TBE buffer. These separated products were photographed by Azure C600 multifunctional imaging system (Azure Biosystems, U.S.A.).

Preparation of Multipedal EXs. First, CEM EXs were added into the TNAKT buffer containing Apt_{PTK7}-catalyst/aDNA-MBs and incubated at 25 °C for 15 min. Many PTK7 aptamers conjugated on MBs could specifically bind the PTK7 proteins on the surface of CEM EXs to construct spider-like multipedal DNA walkers. After a low speed-separation by vortex shaker, the sediment was discarded and the supernatant containing multipedal DNA walkers was obtained. Finally, the resulted

multipedal DNA walkers were subjected to the following flow cytometry measurement.

Flow Cytometry Measurement. Generally, the multipedal DNA walkers were added into the TNaKT buffer (200 μL) including H1-MBs and FAM-H2 (1 nM), and incubated at 37 °C for 4 h. These MBs were then washed three times with TNaKT to stop the reaction and immediately determined by a FACSCalibur device (BD Biosciences, U.S.A.). During flow cytometry analysis, a certain number of FAM-H2 immobilized H1-MBs conjugations (FAM-H2/H1-MBs) were collected and their fluorescence intensities were measured by the FL1 channel using 488 nm laser excitation.

Practical Sample Investigation. The preparation of the fetal serum sample was operated according to the previous work.[1] First, the fetal bovine serum was centrifuged at 100,000 g for 2.5 h at 4 °C to remove EXs. Next, different quantities of isolated CEM EXs were spiked into the 60% EXs-depleted serum sample. The detection procedure in serum was the same as the procedure in the buffer described above.

To evaluate the performance of this biosensor in clinical application, serum samples from four lymphoma patients were collected from Hunan Cancer Hospital/The Affiliated Cancer Hospital of Xiangya School of Medicine (Changsha 410013, Hunan, China) and two acute leukemia patients were collected from the Xiangya Hospital of Central South University (Changsha 410013, Hunan, China). Before experiments, the EXs extracted from human serum were isolated by differential ultracentrifugation according to previously described protocols.[2] First, the collected serum was centrifuged twice at 3000g for 10 min. Then, the supernatant was filtered with a 0.22 μm filter to obtain clean serum. Finally, the EXs pellets were obtained by ultracentrifugation at 120000g for 70 min and stored at -80 °C for further use. After that, the subsequent recognition and detection procedures for serum EXs were the same as the procedure in buffer.

Ethics Statement

Clinical samples from four lymphoma patients were collected from Hunan Cancer Hospital/The Affiliated Cancer Hospital of Xiangya School of Medicine and two acute leukemia patients were collected from the Xiangya Hospital of Central South University. All experiments were performed in accordance with the Guidelines of Clinical Sample Management Rules of Hunan Cancer Hospital and Xiangya Hospital

of Central South University, which were reviewed and approved by the Ethics Committee at Hunan Cancer Hospital and Xiangya Hospital of Central South University. Informed consents were received from the blood donors of this project.

Statistical Analysis

The data were expressed as the mean ± standard error. All data were processed by SPSS 22.0 statistical software. The results were analyzed by t-test and one-way analysis of variance (one-way ANOVA) to determine differences between groups. The significance level was set to $P<0.05$.

Table S1. Oligonucleotides used in this work.[a]

Name	Sequence (from 5 to 3)
H1	GTCAGTGAGCTAGGTTAGATGTCGCCAT GTGTAGACGACATCTAACCTAGC
Biotin-H1	Biotin-GTCAGTGAGCTAGGTTAGATGTCGCCAT GTGTAGACGACATCTAACCTAGC
H2	AGATGTCGTCTACACATGGCGACATCTA ACCTAGCCCATGTGTAG
FAM-H2	FAM-AGATGTCGTCTACACATGGCGACATCTA ACCTAGCCCATGTGTAG
FAM-BHQ1-H2	AGATGTCGT(FAM)CTACACATGGCGACA TCTAACCTAGCCCATGTGTAG-BHQ1
Catalyst	CGACATCTAACCTAGCTCACTGAC
cDNA1	SH-TTTTTTTTTGTCAGTGAGCTAGGTTAGAT GTCG
Control DNA	SH-TTTTTTTTTNNNNNNNNNNNNNNNNNN NNNN
FAM-cDNA2	FAM-CGGTTAGATCG
Apt_{PTK7}-catalyst	ATCTAACTGCTGCGCCGCCGGGAAAATA CTGTACGGTTAGATCG$(T)_{18}$CGACATCTA ACCTAGCTCACTGAC
FAM-Apt_{PTK7}-catalyst	FAM-ATCTAACTGCTGCGCCGCCGGGAAAATA CTGTACGGTTAGATCG$(T)_{18}$CGACATCTA ACCTAGCTCACTGAC
Apt_{PTK7}-$catalyst_{12T}$	ATCTAACTGCTGCGCCGCCGGGAAAATA

	CTGTACGGTTAGATCG(T)$_{12}$CGACATCTA ACCTAGCTCACTGAC
Apt$_{PTK7}$-catalyst$_{21T}$	ATCTAACTGCTGCGCCGCCGGGAAAATA CTGTACGGTTAGATCG(T)$_{21}$CGACATCTA ACCTAGCTCACTGAC
Apt$_{PTK7}$-catalyst$_{35T}$	ATCTAACTGCTGCGCCGCCGGGAAAATA CTGTACGGTTAGATCG(T)$_{35}$CGACATCTA ACCTAGCTCACTGAC
Control aptamer	ATCTAACTGTCTTTTTTTTTTCCCCCCTC TCACGGTTAGATCG(T)$_{18}$CGACATCTAAC CTAGCTCACTGAC
Control catalyst	ATCTAACTGCTGCGCCGCCGGGAAAA TACTGTACGGTTAGATCG(T)$_{18}$TTCTCCTC CCTTCCTTCTCTCTCT
HL_8	Bio-TTTTTTTTTTTTTTTTTTTCGATCTAA
HL_9	Bio-TTTTTTTTTTTTTTTTTTTCGATCTAAC
HL_{10}	Bio-TTTTTTTTTTTTTTTTTTTCGATCTAACC
HL_{12}	Bio-TTTTTTTTTTTTTTTTTTTCGATCTAACC GT
HL_{14}	Bio-TTTTTTTTTTTTTTTTTTTCGATCTAACC GTAC
HL_{17}	Bio-TTTTTTTTTTTTTTTTTTTCGATCTAACC GTACAGT
HL_{20}	Bio-TTTTTTTTTTTTTTTTTTTCGATCTAACC GTACAGTATT
Apt$_{MUC1}$-catalyst$_{21T}$	TTTTTGCAGTTGATCCTTTGGATACCCTG G(T)$_{21}$CGACATCTAACCTAGCTCACTGAC
HL-MUC1$_{10}$	Bio-

	TTTTTTTTTTTTTTTTTTTTCCAGGGTATC
HL-MUC1$_{12}$	Bio-TTTTTTTTTTTTTTTTTTTTCCAGGGTATCCA
HL-MUC1$_{15}$	Bio-TTTTTTTTTTTTTTTTTTTTCCAGGGTATCCAAAG

[a] HL, Hybridization length;

HLn, anchored DNA with n pairs of hybridization length.

Table S2. Comparison of properties of various DNA walkers.

Walking mechanism	"Body" of walker	Track	Numbers of "legs"	Ref.
Strand displacement	Cleat	Microparticle	1	3
Strand displacement	Single strand DNA	Microparticle	2	4
Light control/strand displacement	DNA double-strand helix	1D DNA	2	5
Cleavage by DNAzyme/strand displacement	Streptavidin	2D DNA origami	3	6
Strand displacement	Trigonal arrangement of DNA double helices	2D DNA origami	3	7
Strand displacement	Exosomes	Microparticle	302	**This work**

The number of "legs" anchored on per EX was measured by fluorescence method. The schematic of detection procedures was depicted in **Fig. S2A**. Firstly, FAM-labeled Apt$_{PTK7}$-catalyst was anchored on MBs by anchored DNA. In the presence of CEM EXs, an excess amount of Apt$_{PTK7}$-catalyst can recognize the PTK7 protein on the surface of CEM EXs to generated multipedal DNA walker with many fluorescence molecules. After a low speed separation by vortex shaker, the fluorescence of the supernatant containing multipedal DNA walker was measured. The resulting fluorescence value can be used to evaluate the concentrations (C) of FAM-labeled Apt$_{PTK7}$-catalyst on CEM EXs in the supernatant after comparing the standard curve in **Fig. S2B**. By using the concentrations of FAM-labeled Apt$_{PTK7}$-

catalyst in the supernatant, we could calculate the number of "legs" according to the equation ($N=C \times V \times NA/N_{EXs}$). C is the Apt_{PTK7}-catalyst concentrations in the supernatant after separation; V is the reaction volume; N_A is the Avogadro constant (~ 6.02×10^{23}/mol).

Table S3. Comparison of currently available biosensing assays for the detection of EXs.

Methods	LOD (particles μL^{-1})	Ref.
A catalytic molecule machine-driven electrochemical method	17.20	8
DNAzyme walker amplified electrochemical assay	13	9
An aptamer-binding DNA walker electrochemiluminescence biosensor	60	10
Electrochemical aptasensor based on click chemistry	96	11
Ti3C2 MXenes nanosheets catalyzed ECL biosensor	1.25×10^2	12
Bridging exosome and liposome through zirconium-phosphate coordination chemistry	7.60×10^3	13
Molecular recognition-based DNA nanodevices	10^3	14
Copper-Mediated Signal Amplification	4.8×10^4	15
Superparamagnetic conjunction-molecular beacon (SMC-MB) platform	1×10^2	16
SERS assay by assembling gold nanoparticles in triangular pyramid DNA (TP-Au NPs).	1.1×10^2	17
Aptamer-based fluorescence method	1.0×10^5	18

ECL biosensor based on multivalency interface and g-C_3N_4 coated liquid metal nanoprobe	31	19
Zr-based metal-organic frameworks electrochemical detection	7.83×10^3	2
Exosomes-based multipedal DNA walker	1	**This work**

Table S4. Recovery results of CEM EXs in 60% fetal serum by multipedal DNA walker. All measurements were performed in triplicate.

Spiked amount (particles μL^{-1})	Detected amount (particles μL^{-1})	Recovery (%)	SD (%)	RSD (%)
723.08	573.47	79.31	2.45	3.09
72.31	71.37	98.71	5.45	5.51
7.23	6.59	91.13	7.98	8.75

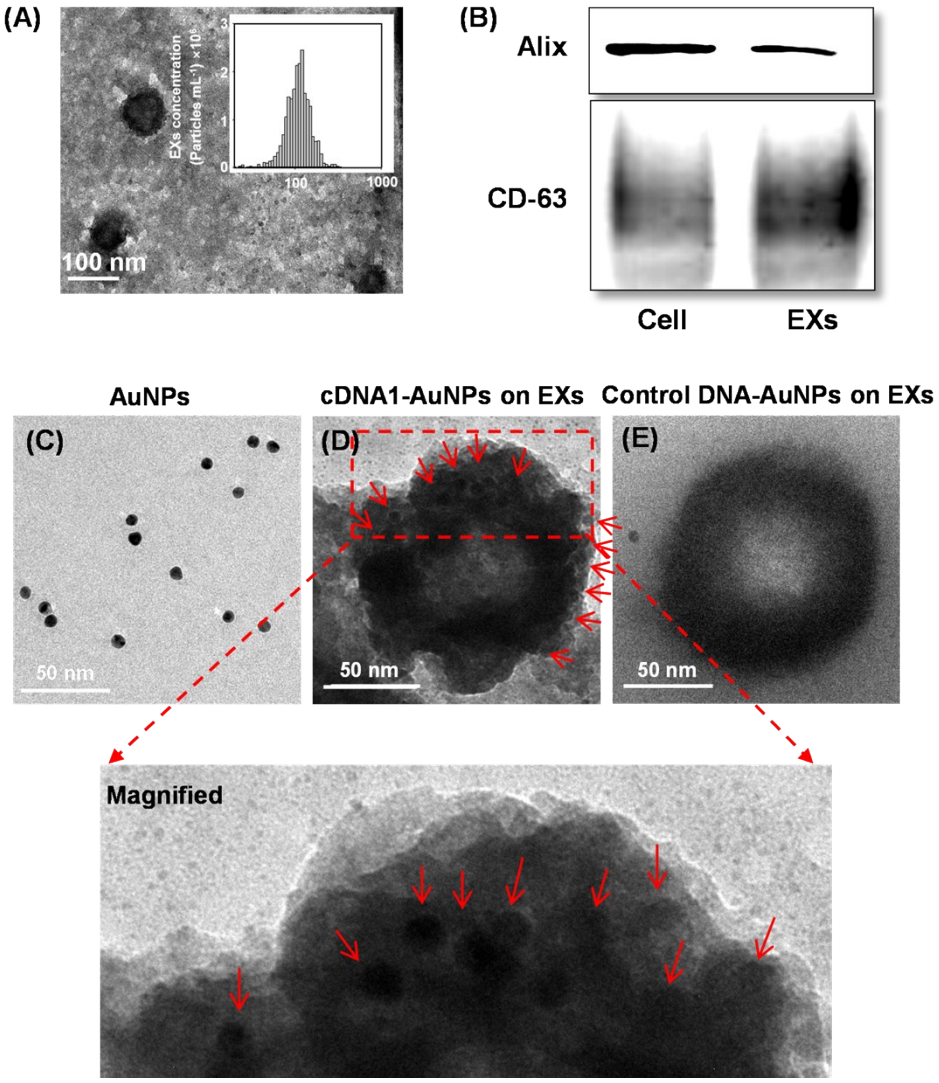

Fig. S1 Characterization of CEM EXs. (A) TEM images and NTA analysis (Inset) of the isolated EXs. (B) Western blot image of CD-63 and Alix from cell lysates and their corresponding Exs (the measurement was performed in triplicate). TEM image the assembly of cDNA1-AuNPs (D) and control DNA-AuNPs (E) on CEM EXs surface after incubation with Apt_{PTK7}-catalyst. (C) TEM image of AuNPs. Red arrows represented AuNPs.

To investigate whether the multipedal DNA walker was formed, AuNPs modified with cDNA1 (cDNA1-AuNPs) was used, which could be intuitively observed by TEM. This designed cDNA1 was completely complementary to the catalytic sequence in AptPTK7-catalyst. Besides, control DNA was used as a negative control. If a multipedal DNA walker was formed, there would be many cDNA1-AuNPs around the EXs because of the strong binding between cDNA and catalytic sequence. As expected, when using cDNA1-AuNPs, a large number of AuNPs were bound on EXs surface (Fig. S1D, ESI†). By contrast, negligible assembly was observed with control DNA-modified AuNPs (Fig. S1E, ESI†). These results

indicated that the multipedal DNA walker could be successfully prepared due to the stable binding between aptamer in AptPTK7-catalyst and proteins on EXs surface.

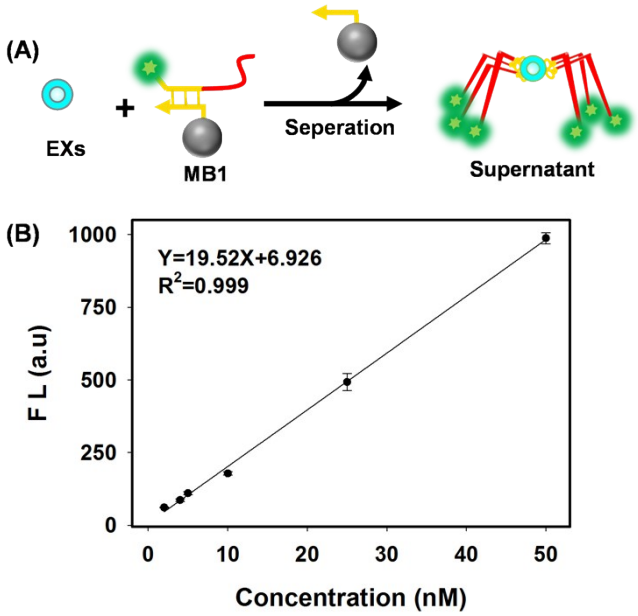

Fig. S2 (A) The schematic of detection procedures for evaluating the number of DNA legs. (B) The standard fluorescence curve of FAM-labeled Apt_{PTK7}-catalyst regarded as DNA legs. Error bars: SD (n = 3).

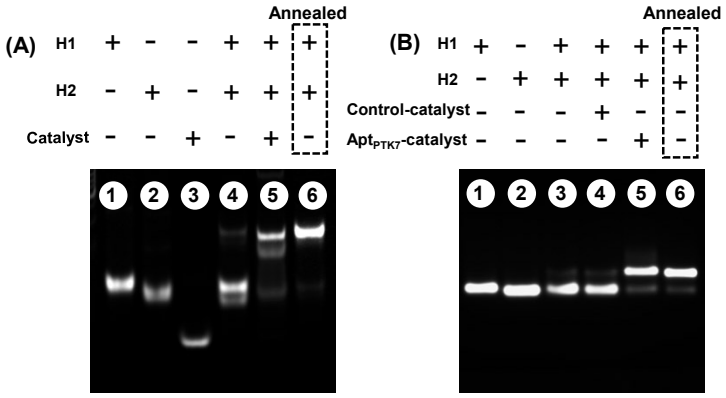

Fig. S3 Gel electrophoresis analysis of catalytic reaction after incubation with free catalyst (A) and Apt_{PTK7}-catalyst (B). Annealed assembly (dotted rectangle, lane 6 in A and B).

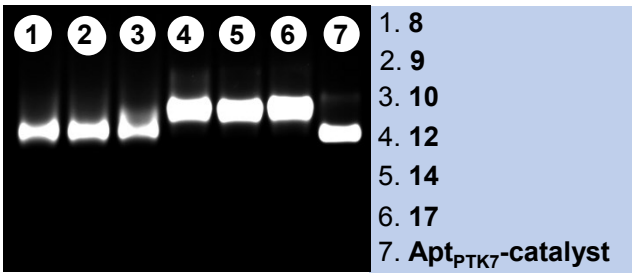

Fig. S4 Investigation of hybridization length of the anchored Apt$_{PTK7}$-catalyst by 2% agarose gel electrophoresis. All measurements were performed in triplicate.

Prior to the application of Apt$_{PTK7}$-catalyst in detecting tumor EXs, the binding force between PTK7 aptamer in Apt$_{PTK7}$-catalyst and anchored DNA over different hybridization lengths have be verified. The results from gel electrophoresis revealed that binding force tended to be stable when hybridization length was over 10 bp. In order to ensure that the target EXs had a better recognition effect, we first chose 12 bp of hybridization length to roughly test the detection capability of this method for EXs.

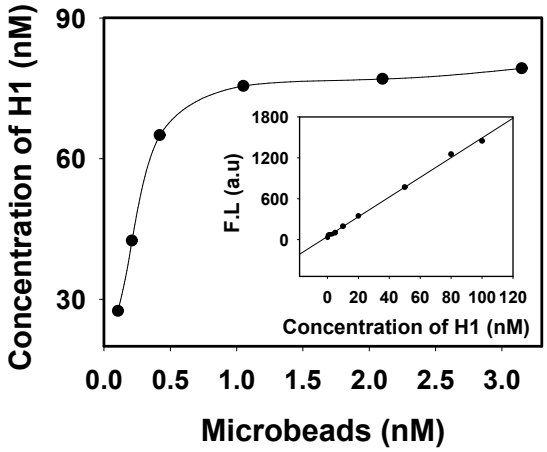

Fig. S5 Determination of the amounts of hairpin on MBs.

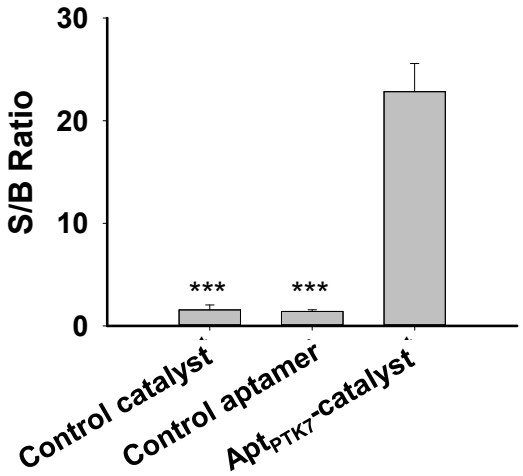

Fig. S6 Specificity studies of the DNA walker toward Apt$_{PTK7}$-catalyst and control subjects (control catalyst and control aptamer) after incubation with CEM EXs. The concentration of EXs was 9.4×10^3 particles μL^{-1}. Compared with the Apt$_{PTK7}$-catalyst group, ***P<0.001. Error bars: SD (n = 3),

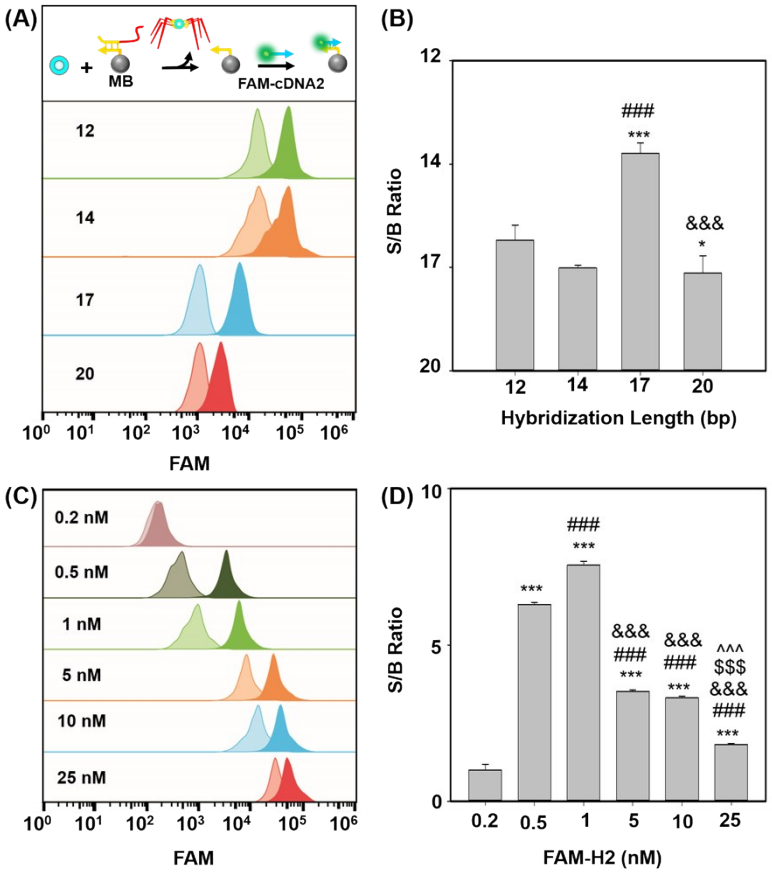

Fig. S7 Optimization of assay parameters. (A) Optimization of hybridization lengths of Apt_{PTK7}-catalyst anchored on MBs by flow cytometry after incubation with CEM EXs. Upper Inset: the schematic of detection mechanism. (B) The corresponding S/B ratio of panel (A). Compared with the 12 bp group, *P<0.05, ***P<0.001. Compared with the 14 bp group, $^{###}$P<0.001. Compared with the 17 bp, $^{\&\&\&}$P<0.001. (C) Optimization of the concentrations of FAM-H2 by flow cytometry after incubation with CEM EXs. (D) The corresponding S/B ratio of panel (C). Compared with the 0.2 nM group, ***P<0.001. Compared with the 0.5 nM group, $^{###}$P<0.001. Compared with the 1 nM group, $^{\&\&\&}$P<0.001. Compared with the 5 nM group, $^{\$\$\$}$P <0.001. Compared with the 10 nM group, ^^^P <0.001. The concentration of EXs was 9.4×10^3 particles μL^{-1}. Error bars: SD (n = 3).

The binding force of various hybridization lengths on the recognition performance of EXs were first investigated by using cDNA2 labeled with FAM at its terminal (FAM-cDNA2), which was complementary to anchored DNA. Upon encountering the target CEM EXs, PTK7 aptamer identified PTK7 proteins on a whole EXs to produce a multipedal DNA walker, which was accompanied by the disassembly of Apt_{PTK7}-catalyst from MBs. After that, FAM-cDNA2 was hybridized to anchored DNA to produce fluorescence signals (Upper in **Fig. S6A**). As shown in **Fig. S6A** and **B**, with increasing hybridization length, DNA duplex with gradual stability were generated and the background signals were expected to decrease stepwise. To get a high signal-to-background (S/B) ratio, 17 bp of hybridization length was selected for EXs recognition. As demonstrated in the principle, the fluorescence signal came from

FAM-H2. Therefore, the binding amount of FAM-H2 should be optimized to get a better S/B ratio (**Fig. S6C** and **D**). The fluorescence intensities increased with the addition of increasing dose of FAM-H2 and reached a maximum signal at 1 nM.

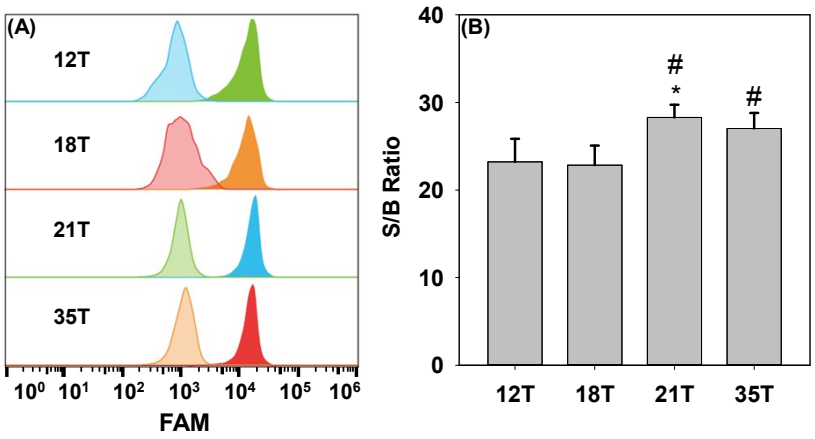

Fig. S8 Optimization of the poly-T linker between PTK7 aptamer and catalyst in Apt_{PTK7}-catalyst. (A) Fluorescence histograms of the DNA walker after incubation with CEM EXs with the additional of Apt_{PTK7}-catalyst over different T spaces (12, 18, 21, 35 T). (B) The corresponding S/B ratio of panel (A). Compared with the 12 T group, *P<0.05. Compared with the 18 T group, $^{\#}$P<0.05. The concentration of EXs was 9.4×10^3 particles μL^{-1}. Error bars: SD (n = 3).

The length of poly-T linker in Apt_{PTK7}-catalyst was the key factor on walking. As shown in **Fig. S7**, 21 T bases were long enough to ensure the multipedal DNA walker traveled well.

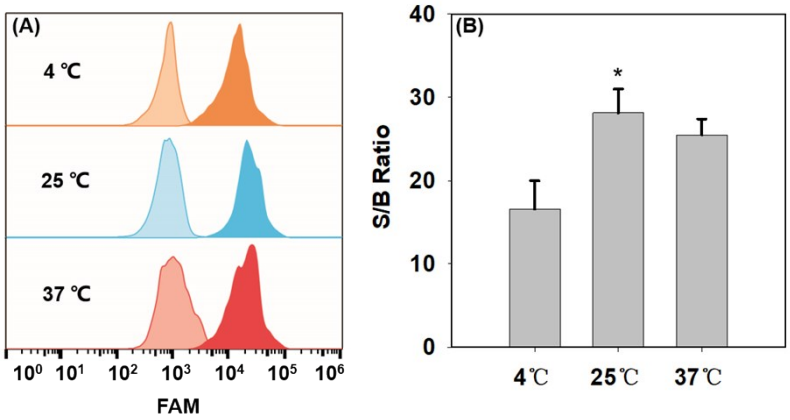

Fig. S9 Optimization of the recognition temperature of CEM EXs. (A) Fluorescence histograms of CEM EXs recognition after incubation with the Apt_{PTK7}-catalyst anchored at different temperature (4 ℃, 25 ℃, 37 ℃). (B) The corresponding S/B ratio of panel (A). Compared with the 4 ℃ group, *P<0.05. The concentration of EXs was 9.4×10^3 particles μL^{-1}. Error bars: SD (n = 3).

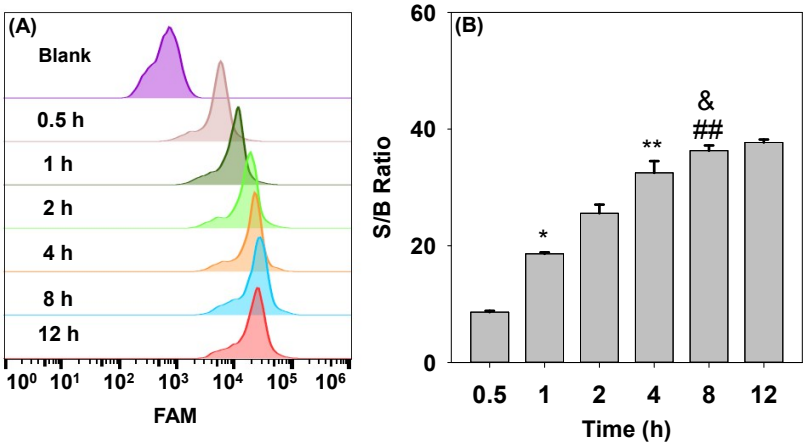

Fig. S10 Optimization of the walking time. (A) Fluorescence histograms of the DNA walker after incubation with CEM EXs at different reaction time ranging from 0.5 to 12 h. (B) The corresponding S/B ratio of panel (A). Compared with the 0.5 h group, *P<0.05, **P<0.01 Compared with the 1 h group, $^{\#}$P<0.05, $^{\#\#}$P<0.01. Compared with the 2 h group, $^{\&}$P<0.05. The concentration of EXs was 9.4×10^3 particles μL^{-1}. Error bars: SD (n = 3).

The walking time was also investigated (**Fig. S9**). It was suggested that the S/B ratio raised rapidly within 4 h, and then it went up slowly and was gradually stable. Therefore, 4 h was utilized as an optimal walking time.

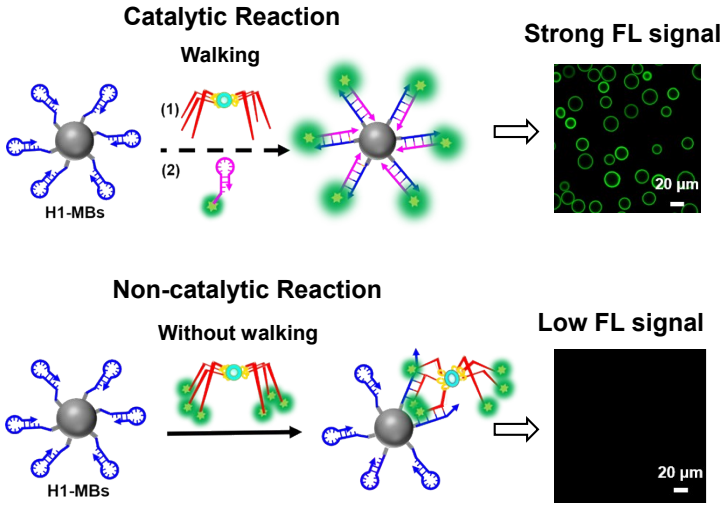

Fig. S11 Schematic and fluorescence confocal image of catalytic reaction and non-catalytic reaction after incubation with CEM EXs.

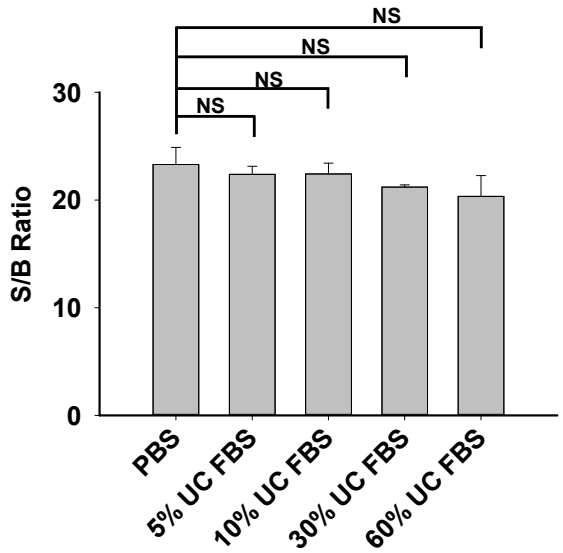

Fig. S12 Detection of CEM EXs in PBS and different complex biosamples. UC FBS represents the ultracentrifuged fetal bovine serum. Compared with the PBS group, NS: $P>0.05$. The concentration of EXs was 3.7×10^3 particles μL^{-1}. Error bars: SD (n = 3).

Given the complex biological environment that could interfere or even decrease the detection performance of the multipedal DNA walker, we thus chose ultracentrifuged EXs-free FBS (UC FBS) as a mimetic complex biological environment to investigate its practical application. As shown in **Fig. S10**, the results generated from EXs spiked in 5%, 10%, 30% and 60% UC FBS were almost the same as those in PBS buffer, suggesting that the proposed strategy was suitable for target EXs detection in a complex biological environment.

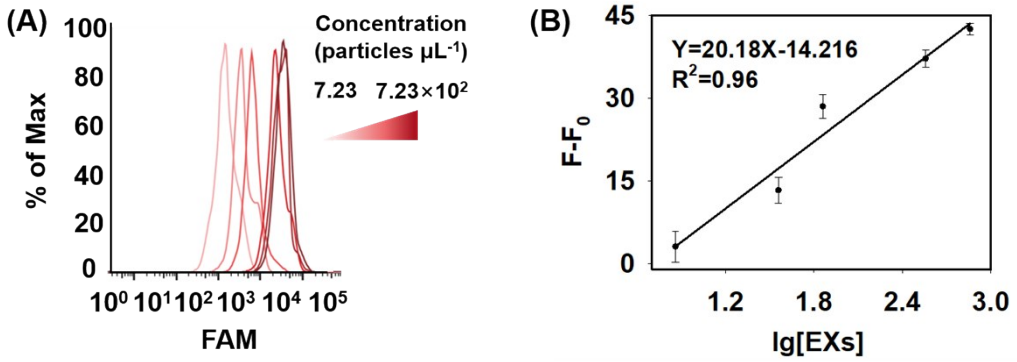

Fig. S13 Fluorescence histograms of catalytic reaction in 5% UC FBS over various concentrations of CEM EXs and their corresponding calibration curves. Error bars: SD (n = 3).

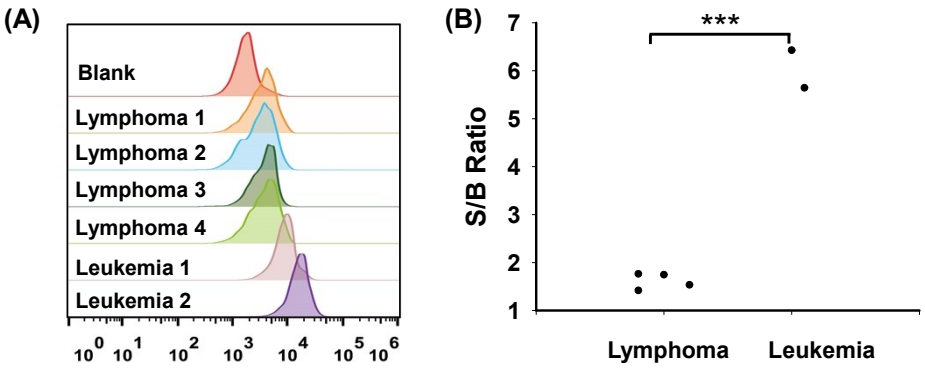

Fig. S14 Response of the DNA walker to EXs obtained from clinical sample. (A) Fluorescence histograms and (B) scattered dot plots show the significant expression of PTK7-positive EXs from acute leukemia patients (n = 2) compared to lymphoma patients (n = 4). The concentration of EXs was 4.6×10^4 particles μL^{-1}. Error bars: SD (n = 3), ***P<0.001.

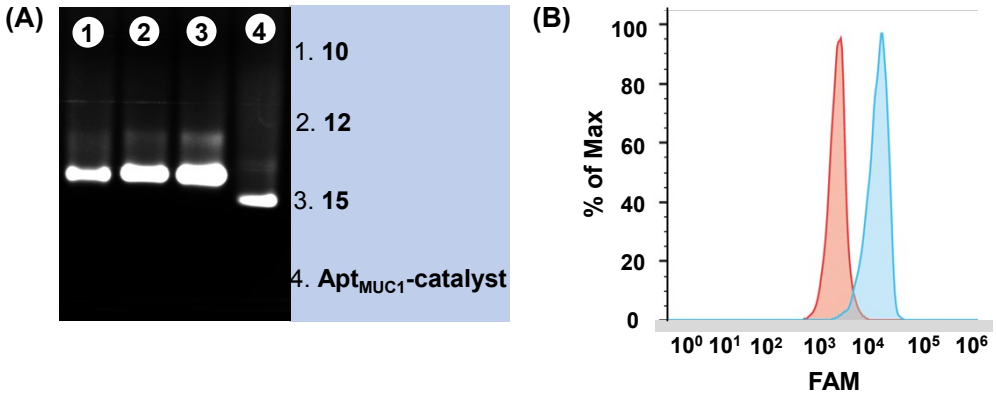

Fig. S15 Versatility investigation of the DNA walker by detecting MCF-7 EXs using Apt_{MUC1}-catalyst. (A) Investigation of hybridization length of the anchored Apt_{MUC1}-catalyst by 3% agarose gel electrophoresis. (B) Flow cytometry assay of Apt_{MUC1}-catalyst without (red) and with (blue) MCF-7 EXs (9.4×10^3 particles μL^{-1}). All measurements were performed in triplicate.

To further demonstrated the versatility of this DNA walker, we changed the PTK7-target aptamer into MUCI-target aptamer to detect MCF-7 (human breast cancer) EXs, which express a abundance of MUC1 protein.[20] Prior to the application of Apt_{MUC1}-catalyst in detecting EXs, the binding force between MUC1 aptamer in Apt_{MUC1}-catalyst and anchored DNA over different hybridization lengths have be verified (**Fig. S13A**). In order to ensure that the target EXs had a better recognition effect, we first chose 10 bp of hybridization length to roughly test the detection capability of this method. The fluorescence intensity of Apt_{MUC1}-catalyst after adding MCF-7 EXs was much higher than that of the blank, indicating that the DNA walker can be a versatile platform for sensitive detection of other targets EXs (**Fig. S13B**).

Reference

1. Doldán, X.; Fagúndez, P.; Cayota, A.; Laíz, J.; Tosar, J. P. Electrochemical sandwich immunosensor for determination of exosomes based on surface marker-mediated signal amplification. Anal. Chem. **2016**, 88, 10466-10473.

2. Sun, Z.; Wang, L.; Wu, S.; Pan, Y.; Dong, Y.; Zhu, S.; Yang, J.; Yin, Y.; Li, G. An electrochemical biosensor designed by using Zr-based metal–organic frameworks for the detection of glioblastoma-derived exosomes with practical application. Anal. Chem. **2020**, 92, 3819-3826.

3. Jung, C.; Allen, P. B.; Ellington, A. D. A simple, cleated DNA walker that hangs on to surfaces. ACS Nano **2017**, 11, 8047-8054.

4. Jung, C.; Allen, P. B.; Ellington, A. D. A stochastic DNA walker that traverses a microparticle surface. Nat. Nanotechnol. **2016**, 11, 157-163.

5. Liu, M.; Hou, R.; Chen, J.; Loh, L. Y.; Sreelatha, S.; Tey, J. N.; Wen, J.; Wang, Z. Autonomous synergic control of nanomotors. ACS Nano **2014**, 8, 1792–1803.

6. Lund, K.; Manzo, A. J.; Dabby, N.; Michelotti, N.; Johnson-Buck, A.; Nangreave, J.; Taylor, S.; Pei, R.; Stojanovic, M. N.; Walter, N. G.; Winfree, E.; Yan, H. Molecular robots guided by prescriptive landscapes. Nature **2010**, 465, 206-210.

7. Gu, H.; Chao, J.; Xiao, S. J.; Seeman, N. C. A proximity-based programmable DNA nanoscale assembly line. Nature **2010**, 465, 202-205.

8. Cao, Y.; Li, L.; Han, B.; Wang, Y.; Dai, Y.; Zhao, J. A catalytic molecule machine-driven biosensing method for amplified electrochemical detection of exosomes. Biosens. Bioelectron. **2019**, 141, 111397.

9. Zhao, L.; Sun, R.; He, P.; Zhang, X. Ultrasensitive detection of exosomes by target-triggered three-dimensional DNA walking machine and exonuclease III-assisted electrochemical ratiometric biosensing. Anal. Chem. **2019**, 91, 14773-14779.

10. Feng, Q.; Ma, P.; Cao, Q.; Guo, Y. An Aptamer-binding DNA walking machine for sensitive electrochemiluminescence detection of tumor exosomes. Chem. Commun. **2020**, 56, 269-272.

11. An, Y.; Jin, T.; Zhu, Y.; Zhang, F.; He, P. An ultrasensitive electrochemical aptasensor for the determination of tumor exosomes based on click chemistry. Biosens. Bioelectron. **2019**, 142, 111503.

12. Zhang, H.; Wang, Z.; Zhang, Q.; Wang, F.; Liu, Y. Ti3C2 MXenes nanosheets catalyzed highly efficient electrogenerated chemiluminescence biosensor for the detection of exosomes. Biosens. Bioelectron. **2019**, 124-125, 184-190.

13. Wang, L.; Yang, Y.; Liu, Y.; Ning, L.; Xiang, Y.; Li, G. Bridging exosome and liposome through zirconium-phosphate coordination chemistry: a new method for exosome detection. Chem. Commun. **2019**, 55, 2708-2711.

14. He, D.; Ho, S. L.; Chan, H. N.; Wang, H.; Hai, L.; He, X.; Wang, K.; Li, H. W. Molecular-recognition-based DNA nanodevices for enhancing the direct visualization and quantification of single vesicles of tumor exosomes in plasma microsamples. Anal. Chem. **2019**, 91, 2768-2775.

15. He, F.; Wang, J.; Yin, B. C.; Ye, B. C. Quantification of exosome based on a copper-mediated signal amplification strategy. Anal. Chem. **2018**, 90, 8072-8079.

16. Li, P.; Han, W.; Kong, Y.; Bao, W.; Zhang, J.; Zhang, W.; Gu, Y. A dual functional platform composed of superparamagnetic conjunctions and molecular beacons. ACS Sens. **2019**, 4, 1433−1441.

17. Zhang, X.; Liu, C.; Pei, Y.; Song, W.; Zhang, S. Preparation of a novel raman probe and its application in the detection of circulating tumor cells and exosomes. ACS Appl. Mater. Interfaces. **2019**, 11, 28671-28680.

18. Yu, X.; He, L.; Pentok, M.; Yang, H.; Yang, Y.; Li, Z.; He, N.; Deng, Y.; Li, S.; Liu, T.; Chen, X.; Luo, H. An aptamer-based new method for competitive fluorescence detection of exosomes, Nanoscale. **2019**, 11, 15589-15595.

19. Zhang, Y.; Wang, F.; Zhang, H.; Wang, H.; Liu, Y. Multivalency interface and g-C3N4 coated liquid metal nanoprobe signal amplification for sensitive electrogenerated chemiluminescence detection of exosomes and their surface proteins. Anal. Chem. **2019**, 91, 12100-12107.

20. Wang, L.; Pan, Y.; Liu, Y.; Sun, Z.; Huang, Y.; Li, J.; Yang, J.; Xiang, Y.; Li, G. Fabrication of an aptamer-coated liposome complex for the detection and profiling of exosomes based on terminal deoxynucleotidyl transferase-mediated signal amplification. ACS Appl. Mater. Interfaces. **2020**, 12, 322-329.