Electronic Supplementary Information (ESI)

Recognition Triggered Assembly of Split Aptamers to Initiate Hybridization Chain Reaction for Wash-free and Amplified Detection of Exosomes

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1. Experimental Section

Chemicals and materials

DNA oligonucleotides, RPMI-1640 medium, phosphate buffer saline (PBS), and Dulbecco's phosphate buffered saline (DPBS) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The detailed sequences of the DNA oligonucleotide were listed in Table S1. According to the previously reported method,[1] the sequence design of split probe has shown in Fig.S1. A syringe-driven filter unit (0.22 μm) and 100 KD centrifugal ultrafiltration tube were provided by Millipore (USA). Yeast tRNA was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Exosome-depleted FBS was purchased from System Biosciences (USA). PNGase F, O-Glycosidase and tunicamycin were purchased from New England Biolabs (Beijing, China). The magnetic beads were purchased from Invitrogen (Thermo Scientific, USA). Ultrapure water was generated from a Millipore Milli-Q water purification system (Billerica, MA, USA) with an electrical resistance ≥18.2 MΩ•cm.

Instruments

The morphology of exosomes was characterized by transmission electron microscopy (FEI-Tecnai G2, USA) and scanning electron microscopy (Hitachi SU8000, Japan). The concentration of the exosomes was quantified via nanoparticle tracking analysis (NanoSight 300, UK) after 1000-fold dilutions in PBS. The size distribution and zeta potential of the exosomes were measured on a Malvern Zetasizer Nano ZS90 (Instruments UK). Agarose and native gel electrophoresis were imaged on a ChemiDoc XRS system (Bio-Rad, USA). Fluorescence measurements were performed on an F-7100 spectrophotometer (Hitachi, Japan). Emission spectra were recorded from 500 nm to 700 nm at 4 °C with an excitation wavelength of 488 nm. Cells and exosomes were imaged on a DMi8A laser scanning confocal microscope (Leica, Germany). Flow cytometry was performed on a Gallios, Beckman Coulter and data were analyzed using FlowJo software.

Cell culture

SMMC-7721 (human hepatocellular cancer), CCRF-CEM (human acute

lymphoblastic leukemia, T cell line), HepG2 (human hepatocellular cancer), HL-7702 (human normal hepatocytes), HeLa (human cervical cancer) and B16F1 cells (Mouse melanoma) were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% exosome-depleted FBS and 100 IU/mL penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2.

In the drug treatment experiments, the SMMC-7721 cells were cultured in DMEM culture medium at 37 °C for 24 h. Then 10 μg/mL of tunicamycin, which is a kind of drug that can inhibit the N-glycosylation of exosomes surface protein, was added in the above culture medium for another 48 h in a humidified incubator containing 5% CO_2 for two days at 37 °C.

Exosomes isolation and quantification

Exosomes were isolated from the cell culture medium by ultrafiltration at 4 °C as previously described method.[2] Briefly, cell supernatant was collected and centrifuged at 2,000 g for 20 min. Supernatant was filtered with a syringe-driven filter unit (0.22 μm), and pre-filtered fluid was collected and concentrated through a 100 KDa centrifugal ultrafiltration tube at 5,000 g for 30 min. To obtain exosomes, the filtered media was ultra-centrifuged at 160,000 g for another 2 h. The obtained exosomes were then resuspended in PBS (pH 7.4) and their concentration was measured by NTA. Samples were stored at -80 °C for further use.

Gel electrophoresis

Different samples were prepared in 200 μL eppendorf tube (enzyme-free) as follows: 1: 10 μL Marker, 2: 1 μM H1 and 1 μM H2 were mixed together at 4 ^{o}C for 6 h, 3: 1 μM trigger, 1 μM H1 and 1 μM H2 were mixed together at 4 ^{o}C for 6 h, 4: 0.5 μM split apt-a, 0.5 μM split apt-a, 1 μM H1 and 1 μM H2 were mixed together at 4 ^{o}C for 6 h, 5: 0.5 μM split C, 0.5 μM split apt-b, 1 μM H1 and 1 μM H2 were mixed together at 4 ^{o}C for 6 h. Different samples (10 μL) were assessed on 3% agarose or 12.5% polyacrylamide gels with 1 × TBE buffer (4.5 mM Tris, 4.5 mM boric acid and 0.1 mM EDTA, pH 7.9) at 4 °C. Electrophoresis was performed at 200 V for 10 min and 80 V for 45 min in dark place. Gels were SYBR Gold-stained and imaged on a ChemiDoc XRS system.

Detection of exosomes in the buffer

H1 and H2 were annealed by heating at 95 °C for 5 min, and cooling to room temperature in PBS to form stable hairpin structures. Under optimized conditions, different concentrations (0, 2.32×10^5, 5.80×10^5, 1.16×10^6, 2.32×10^6, 5.80×10^6, 1.16×10^7, 2.32×10^7, 5.80×10^7, 1.16×10^8, 2.32×10^8, 5.80×10^8, 1.16×10^9, 2.32×10^9 and 5.80×10^9 particle/mL) of exosomes were mixed with 50 nM of split apt-a, 50 nM of split apt-b, 200 nM of H1 and 200 nM of H2 in 200 μL eppendorf tube (enzyme-free). After incubation at 4 °C for 6 h in dark place, the fluorescence spectra were recorded on a FL-7100 spectrometer at an excitation wavelength of 488 nm. All measurements were performed on a minimum of three occasions.

To verify the target N-glycoprotein on surface of exosomes, PNGase F (2500 U/mL) were added to exosomes solution (2×10^8 particles/mL) with 1×glycoprotein buffer, 10% NP-40 at 37 °C for 1 h. Subsequently, the mixture of 50 nM of split apt-a, 50 nM of split apt-b, 200 nM of H1 and 200 nM of H2 probes were added in the above solution with a total volume of 100 μL of PBS (tRNA) buffer in 200 μL eppendorf tube (enzyme-free) out of light. Then, after incubation at 4 °C for 6 h, the fluorescence spectra of samples were measured on a FL-7100 spectrometer (the excitation wavelength was 488 nm, and emission wavelength were collected from 500 to 700 nm).

Flow cytometry assays of exosomes

Due to the small size of the exosome, Anti-CD63 antibody-magnetic bead conjugates (MB-CD63) with the diameter of 2.7 μm, were applied to capture the exosomes to perform the flow cytometry assays. Firstly, MB-CD63 were obtained by attaching the biotinylated CD63 antibody to streptavidin-modified magnetic beads. Exosomes were incubated with 100 μL 250 nM of split apt-a, 100 μL 250 nM of split apt-b, 100 μL 1000 nM of H1 and 100 μL 1000 nM of H2 to a final volume of 500 μL at 4 °C in 600 μL Eppendorf tube (enzyme-free) out of light. After 6 h, the 10^5 particles/mL MB-CD63 were added to above mixture for 30 min with gentle rotation. An external magnetic field was applied to remove the unbound exosomes by washing several times. The exosomes were analyzed with a flow cytometer by counting 10 000

events.

To verify the target protein, the exosomes were incubated with PNGase F (10000 U/mL), which can specifically hydrolyze N-glycoproteins at 37 °C for 1 h with a final volume of 500 μL containing 1×glycoprotein buffer, 10% NP-40. Then 10^5 particles/mL MB-CD63 were added to above mixture for 30 min with gentle rotation. An external magnetic field was applied to remove the unbound exosomes by washing several times. Next, 50 nM of split apt-a, 50 nM of split apt-b, 200 nM of H1 and 200 nM of H2 probes were added into the above-mentioned solution to bind exosomes and incubated at 4 °C for 6 h in 600 μL eppendorf tube (enzyme-free) out of light. After washing twice with buffer, the MB-CD63-exosomes were analyzed with a flow cytometer (Gallios, Beckman Coulter, USA).

To further verify the hypothesis that the target protein on the surface of the SMMC-7721 exosomes is the N-glycoproteins, O-glycosidase, which can catalyze the hydrolysis of O-glycoproteins, was used to treat exosome before flow cytometry assay. Briefly, the SMMC-7721 derived exosomes were incubated with 2000 unit/mL O-glycosidase at 37 °C for 1 h with a final volume of 500 μL containing 1×glycoprotein buffer, 10% NP-40, and then the procedure was the same as those described in the aforementioned experiment for PNGase F-treated exosomes.

Exosome imaging

Exosomes were incubated with 100 μL 250 nM of split apt-a, 100 μL 250 nM of split apt-b, 100 μL 1000 nM of H1 and 100 μL 1000 nM of H2 to a final volume of 500 μL at 4 °C in 600 μL Eppendorf tube (enzyme-free) out of light. After 6 h, fluorescent images were obtained on a Leica laser scanning confocal microscope under a 100×oil immersion lens.

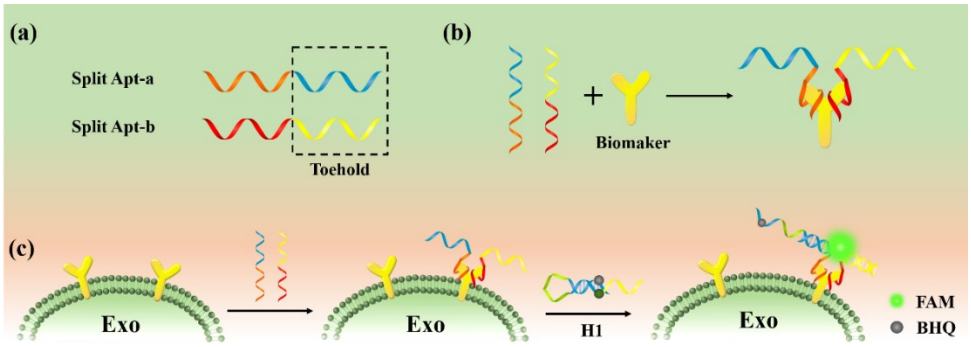
Analytical performance of the system in real samples

FBS solutions (10% and 20%) were prepared by diluting exosome-depleted FBS with PBS. Different concentrations (1.16×10^6, 2.32×10^6, 5.80×10^6, 1.16×10^7, 2.32×10^7, and 5.80×10^7 exosomes/mL) of exosomes were added into the FBS solutions at 4 °C in dark place. After 6 h, the fluorescent intensities were recorded.

The practicability of the platform was further investigated in clinical biological

samples. Human whole blood samples of healthy individuals and liver cancer patients were obtained from the First Affiliated Hospital, Zhengzhou University (Zhengzhou, China). All patients in the liver cancer group showed clinical symptoms and were examined using a blood screening test with positive results. Range indicators through the physical examination of healthy donors were within normal values. Written consent was obtained from all participants prior to enrollment, and the study was approved by the Ethics Committee of the First Affiliated Hospital at Zhengzhou University. Clinical serum was collected by centrifugation at 2,000 g for 20 min. After incubation with the probe at 4 °C in dark place for 6 h. The fluorescence data were statistically analyzed using OriginPro 2018.

2. Results and Discussion



Scheme S1. Design and sensing mechanisms of the non-amplifying system for exosomes detection.

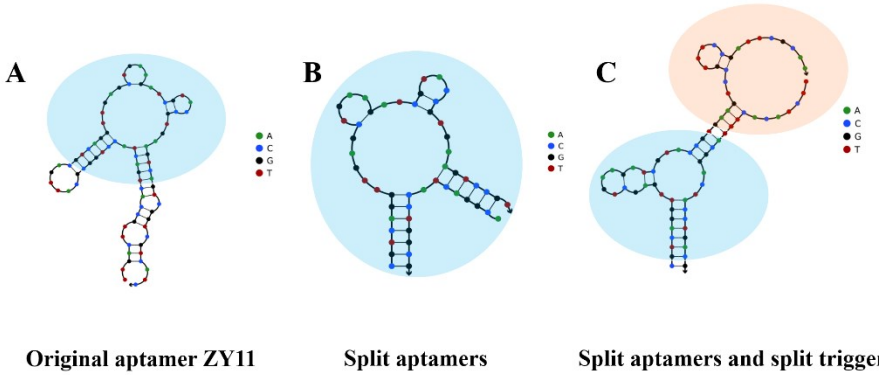


Fig. S1 Simulation the secondary structure of (A) original aptamer ZY11, (B) the two split aptamers and (C) the split triggers grafted to each end of the two split aptamers.

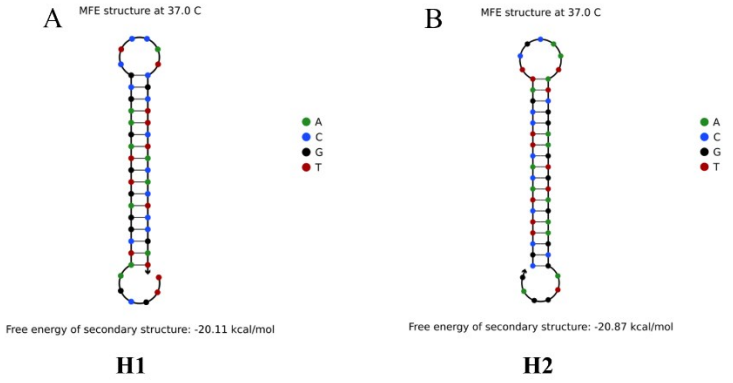


Fig. S2 Simulation the secondary structure of H1 and H2.

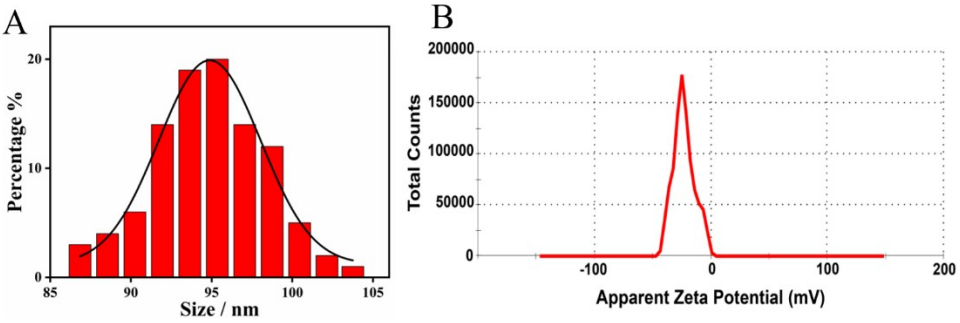


Fig. S3 (A) The size distribution and (B) zeta potential of SMMC-7721 exosomes.

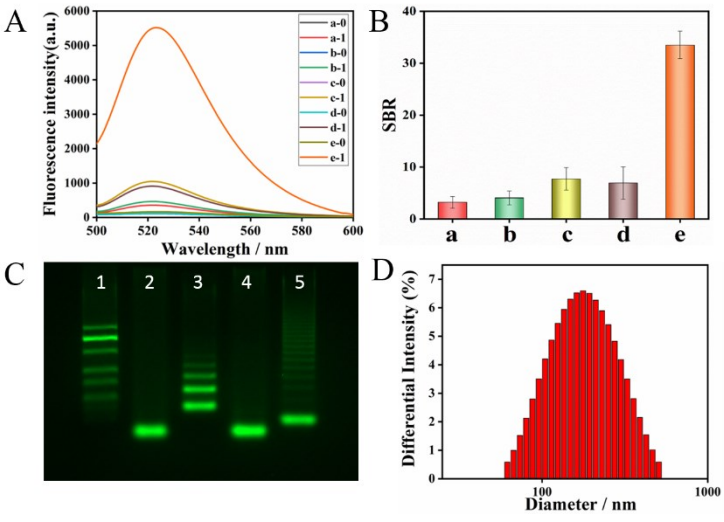


Fig. S4 (A) Fluorescence emission spectra under the indicated conditions: (a-0) H1, (a-1) exosomes+H1, (b-0) H1+H2, (b-1) exosomes+H1+H2, (c-0) split apt-a+H1+H2, (c-1) exosomes+split apt-a+H1+H2, (d-0) split apt-b+H1+H2, (d-1) exosomes+split apt-

b+H1+H2, (e-0) split apt-a+split apt-b+H1+H2, (e-1) exosomes+split apt-a+split apt-b+H1+H2. (B) Histogram of the ratios of fluorescent signal to background under different conditions. (C) Electrophoretic images under the indicated conditions: lane 1: Marker, lane 2: H1+H2, lane 3: Trigger+H1+H2, lane 4: split apt-a+split apt-b +H1+H2, lane 5: split C+ split apt-b+H1+H2. (D) The size distribution histogram of SMMC-7721 exosomes with HCR products analyzed by DLS.

The feasibility of the design was investigated by fluorescence spectra analysis. As shown in Fig. S4A (ESI†), when standard exosomes were mixed with the system, an obvious fluorescence signal is observed (e-1). This result suggested that the binding affinity of the split aptamers to the exosomes promoted the formation of an intact trigger that induced HCR. However, in the absence of the target, only weak background fluorescence is observed (e-0). Other control experiments all generated weak fluorescence signals in the presence of the target exosomes. The corresponding signal-to-background ratios of these groups are shown in Fig. S4B (ESI†). The feasibility of the design was further confirmed by agarose gel electrophoresis. Due to the large size of the exosomes, the target cannot be applied directly on gel electrophoresis, so strand split-C was chosen to simulate the target. The strand split-C contained two domains: one domain can hybridize with the aptamer region in the split-apt-b strand, and the other domain can form an intact HCR trigger strand with the split-apt-b strand. In Fig. S4C (ESI†), the brightness and distribution of bands for the intact-trigger/H1/H2 (line 3) are similar to the split-C/split apt-b/H1/H2 (line 5), with no obvious bands for H1 and H2 observed. This result suggested that a large molecular weight product of HCR was formed. DLS analysis was also used to investigate the formation of the HCR product on the surface of the exosomes. As shown in Fig. S4D, (ESI†), the diameter of the exosomes incubated with the system (196 nm) is larger than that of bare exosomes (122 nm). All these data suggested that the spilt-aptamer based HCR sensing system was applicable for the detection of exosomes.

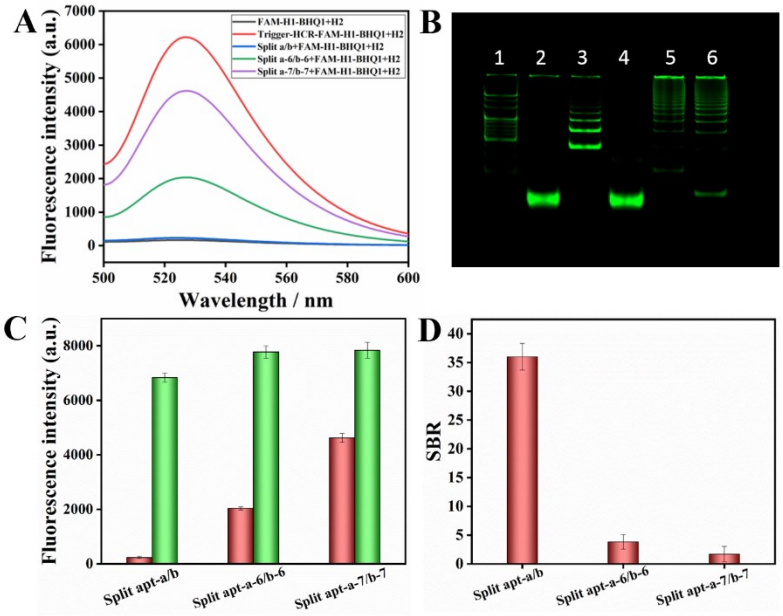


Fig. S5 Investigation the amplified ability of the nanoprobe. (A) Fluorescence emission spectra for the non-specific HCR response of the designed probe. (B) Native electrophoretic characterization of non-specific HCR reactions. Lane 1: Marker, Lane 2: H1+H2, Lane 3: Trigger+H1+H2, Lane 4: split apt-a+split apt-b+H1+H2, Lane 5: split apt-a-6+split apt-b-6+H1+H2, Lane6: split apt-a-7+split apt-b-7+H1+H2. (C) Histogram of fluorescence intensity of background (red bar) and signal (green bar) of the designed probe. (D) Histogram of signal-to-background ratios of (C).

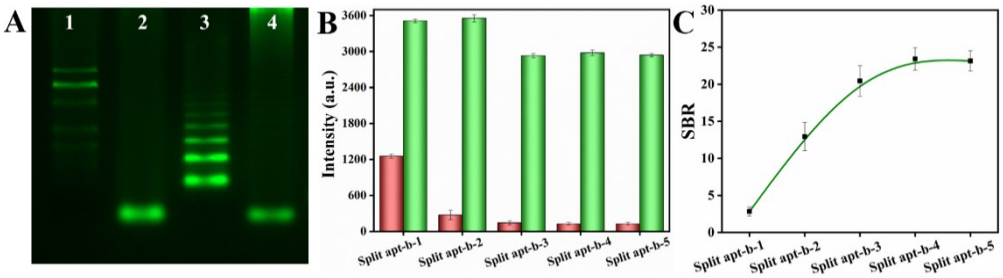


Fig. S6 The optimization of the splitting location of the trigger strand. (A) Electrophoretic image, Lane 1: Marker, Lane 2: H1+H2, Lane 3: Trigger+H1+H2, Lane 4: split apt-a+split apt-b-1+H1+H2. (B) The histogram of fluorescence intensity of background (red bar) and signal (green bar) with different split apt-b; (C) The signal-to-background ratio of (B).

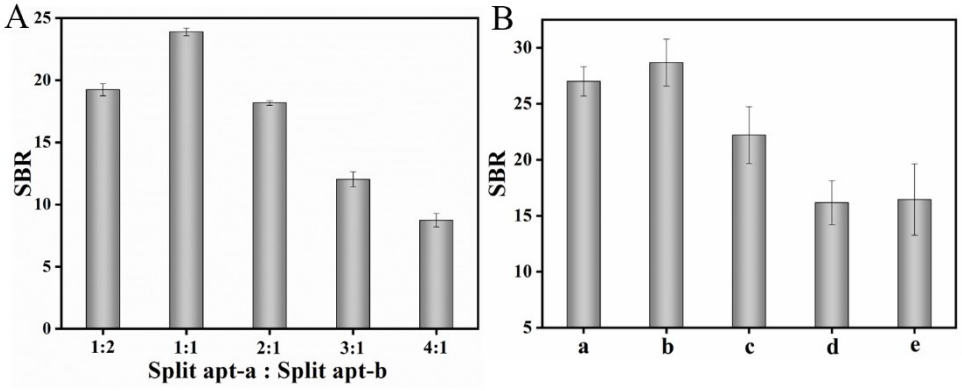


Fig. S7 The optimization of (A) the molar ratio and (B) concentration of split apt-a to split apt-b at (a) 25 nM, (b) 50 nM, (c) 100 nM, (d) 150 nM, and (e) 200 nM.

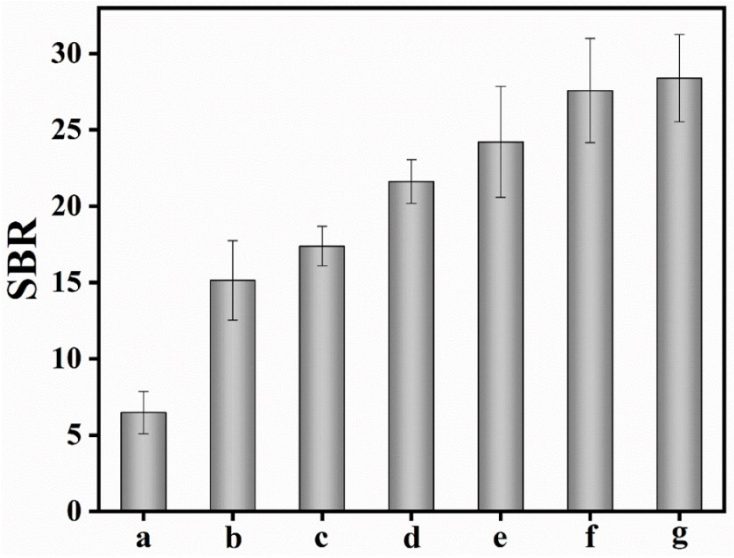


Fig. S8 The optimization of HCR reaction time. (a) 0.5h, (b) 1h, (c) 2h, (d) 3h, (e) 4h, (f) 6h and (g) 8h.

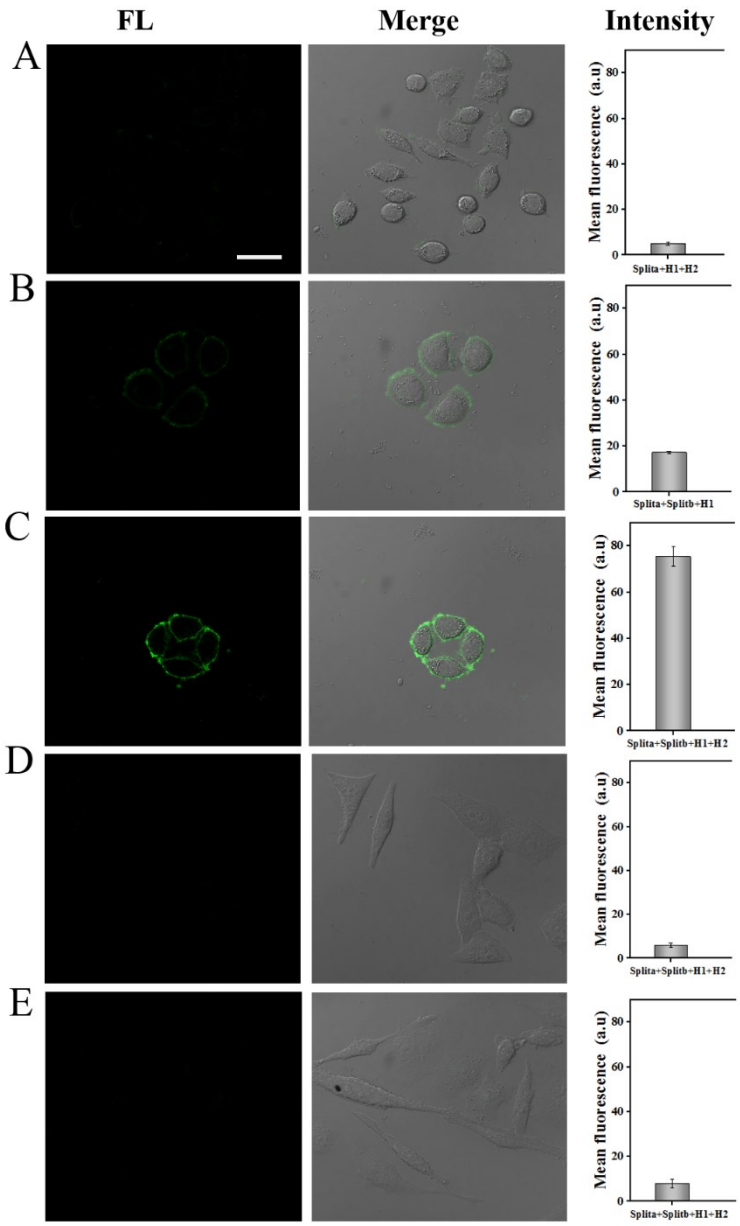


Fig. S9 Confocal microscopy images. (A) SMMC-7721 cells incubated with split apt-a, FAM-H1-BHQ1 and H2, (B) SMMC-7721 cells incubated with split apt-a, split apt-b and FAM-H1-BHQ1, (C) SMMC-7721 cells, (D) HepG2 cells, and (E) HL-7702 cells incubated with split apt-a, split apt-b, FAM-H1-BHQ1 and H2. Intensity represents the mean fluorescence intensity of FAM on cell membrane. Scale bar: 35 μm.

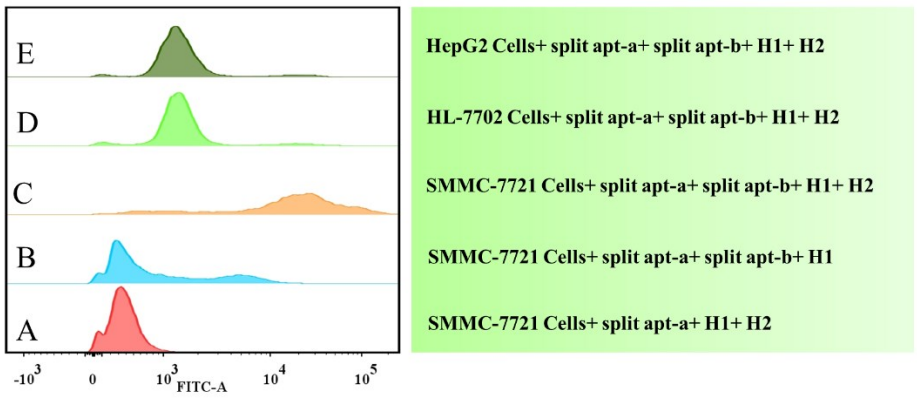


Fig. S10 Flow cytometry analysis under different conditions: (A) SMMC-7721 cells incubated with split apt-a, H1 and H2, (B) SMMC-7721 cells incubated with split apt-a, split apt-b and H1, (C) SMMC-7721, (D) HL-7702, and (E) HepG2 cells incubated with split apt-a, split apt-b, H1 and H2.

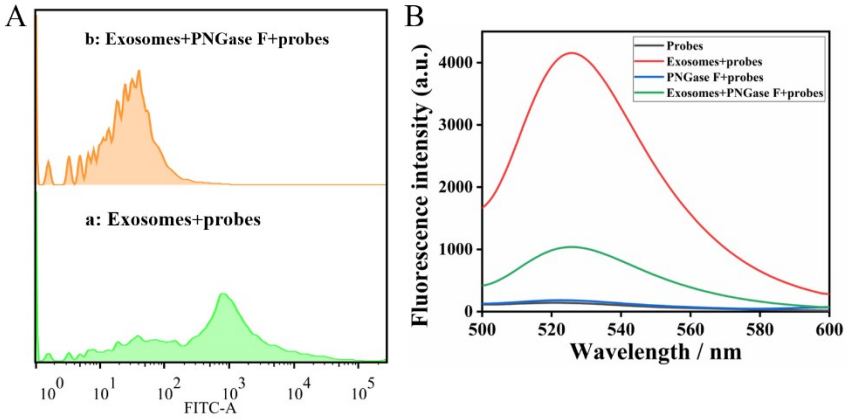


Fig. S11 (A) Flow cytometry assays of (a) untreated SMMC-7721 exosomes incubated with probes, (b) PNGase F treated SMMC-7721 exosomes incubated with probes. (B) Fluorescence emission spectra under the indicated conditions: the probe of split apt-a, split apt-b, H1 and H2 (black line), the mixture of exosomes with split apt-a, split apt-b, H1 and H2 (red line), the mixture of PNGase F with split apt-a, split apt-b, H1 and H2 (blue line), the mixture of exosomes, split apt-a, split apt-b, H1 and H2 and PNGase F (green line).

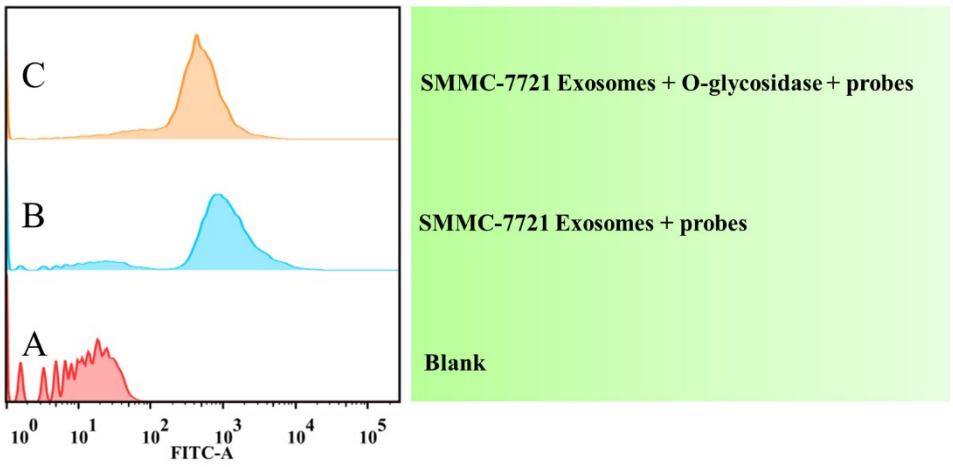


Fig. S12. Flow cytometry assays of (A) SMMC-7721 exosomes only, (B) untreated SMMC-7721 exosomes incubated with probes and (C) O-glycosidase treated SMMC-7721 exosomes incubated with probes.

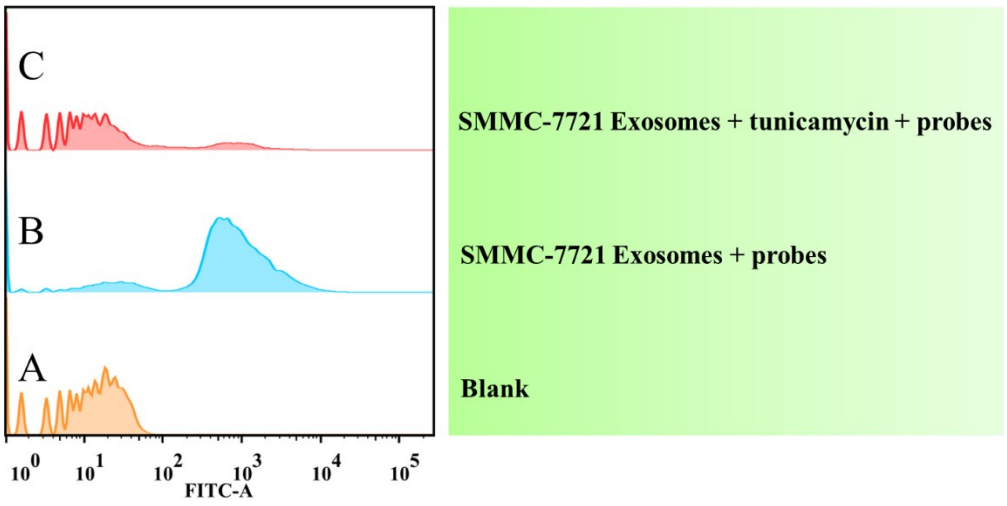


Fig. S13. Flow cytometry assays of (A) SMMC-7721 exosomes only, (B) untreated SMMC-7721 exosomes incubated with probes, (C) exosomes secreted from tunicamycin treated SMCC-7721 cells incubated with probes.

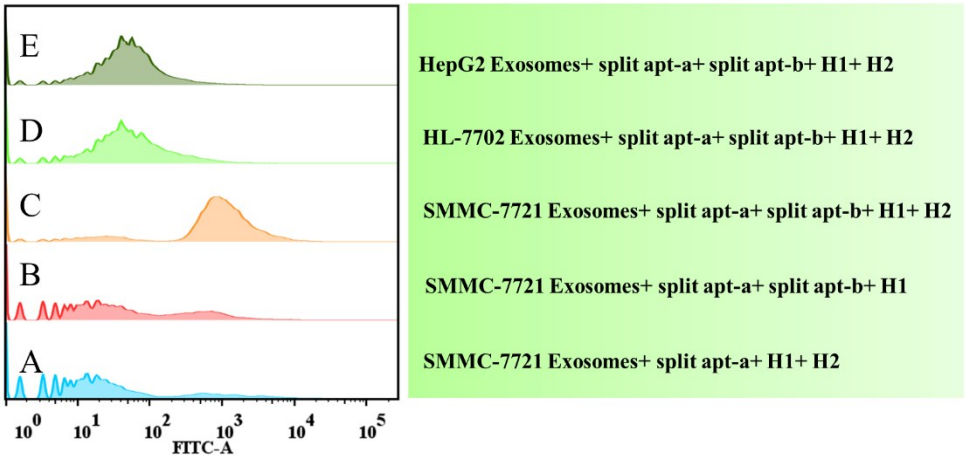


Fig. S14. Flow cytometry analysis under different conditions: (A) SMMC-7721 cells derived exosomes incubated with split apt-a, H1 and H2, (B) SMMC-7721 cells derived exosomes incubated with split apt-a, split apt-b and H1, (C) SMMC-7721 cells derived exosomes, (D) HL-7702 cells derived exosomes and (E) HepG2 cells derived exosomes incubated with split apt-a, split apt-b, H1 and H2.

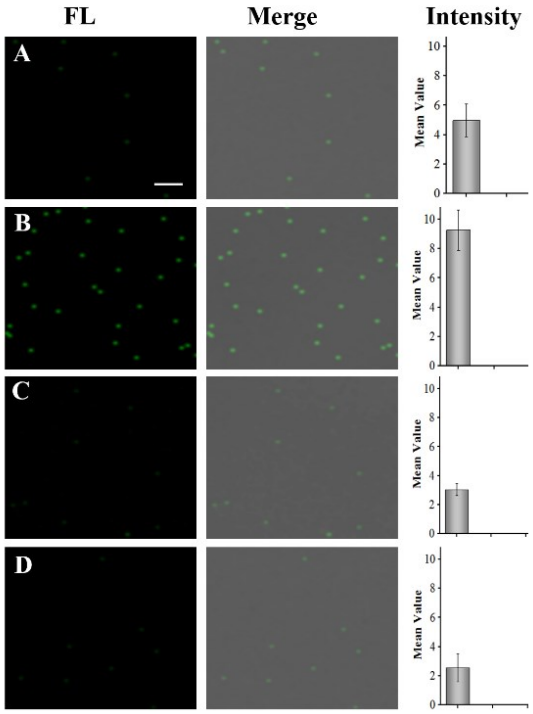


Fig. S15. Confocal microscopy. (A) SMMC-7721-exosomes incubated with split apt-a, split apt-b, and H1. (B) SMMC-7721-exosomes, (C) HepG2-exosome and (D) HL-7702-exosomes incubated with split apt-a, split apt-b, H1and H2. Intensity represents

the mean fluorescence intensity of FAM on exosomes. Images were taken through a 100×oil immersion lens (scale bar: 5 μm). Error bars indicate means ± SD (n = 3).

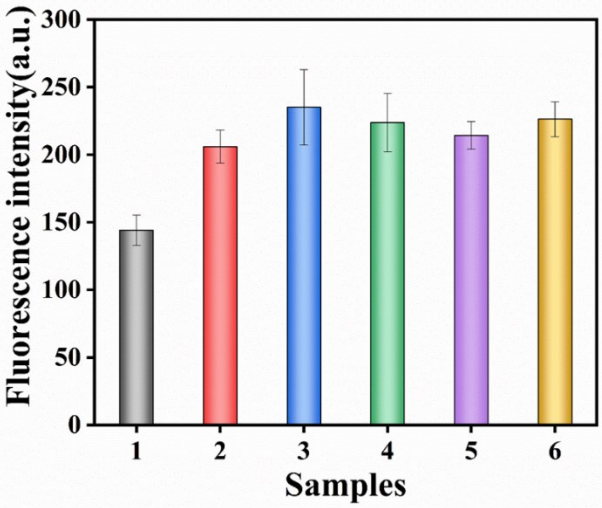


Fig. S16 The fluorescence intensity of probe incubated with different exosome-depleted clinical serum samples: 1, patient ID 3473450, 2, patient ID 3381601, 3 patient ID3568203, 4, patient ID 3473850, 5, patient ID 3453615.

Table S1. Sequences of oligonucleotides used in this work[a].

Name	Sequence(5' - 3')
Intact-Trigger	CGC TTC TAC ACT TCC GAT TCG CAA T
ZY11	TTGACTTGCCACTGACTACCTGGCGCATTGACGTCAGGTTGAGC TGAAGATCGTACCGTGAAGTCAGTCGGTCGTCATC
Split apt-a	CGT CAG GTT GAG CTG AAG ATC GTA CCG TGA AGT CCG TTT CCG ATT CGC AA
Split apt-b	TTC TAC ACT TTA CGG ACT ACC TGA CG
Split ctrl apt-a	CGT CAT TTT TCT TTT TAT TTT TCT TTT TAT TTT TAT TTT CCG ATT CGC AA
Split ctrl apt-b	TTC TAC ACT TTA TTT CTT ATT TTT CG
Split apt-b-1	CGC TTC TAC ACT TTA CGG ACT ACC TGA CG
Split apt-b-2	GCT TCT ACA CTT TAC GGA CTA CCT GAC G
Split apt-b-3	CTT CTA CAC TTT ACG GAC TAC CTG ACG
Split apt-a-6	CGT CAG GTT GAG CTG AAG ATC GTA CCG TGA AGT CCGT
Split apt-b-6	TT CTA CAC TTC CGA TTC GCA A AC GGA CTA CCT GAC G

Split apt-a-7	CGT CAG GTT GAG CTG AAG ATC GTA CCG TGA AGT CCG TTT CTA CAC TTC CGA TTC GCA A
Split apt-b-7	AC GGA CTA CCT GAC G
Split C	CGT CAG GTA GTC CGT TTC CGA TTC GCA A
Split apt-b-5	TCT ACA CTT TA CGG ACT ACC TGA CG
H1	TTG CGA ATC GGT AGT GTA GAA GCG CTC CAT CGC TTC TAC ACT TCC GAT
FAM-H1-BHQ1	TTG CGA ATC GGT(BHQ1)AGT GTA GAA GCG CTC CAT CGC TTC TAC ACT T(FAM)CC GAT
H2	CGC TTC TAC ACT TCC GAT TCG CAA TAT CGG AAG TGT AGA AGC GAT GGA G

[a]Two stem regions of the ZY11 aptamer are shown in pink and green, respectively, the recognition region of the aptamer is shown in brownness, the split trigger regions are shown in blue, the domain of split-C that hybridize with split-apt-b strand is shown in red.

Table S2. Comparison of the analytical performances with different methods.

Method	**Linear** (particles/mL)	**LOD** (particles/mL)	**Reference**
Aptamer-capped Fe_3O_4 NPs	0.4 ~ 6.0 × 10^8	3.58 × 10^6	3
Gold nanoparticles based on lateral flow immunoassay test	0.9 ~ 14.4×10^{10}	8.54×10^8	4
B-Chol anchor assay based HCR	0.23 ~ 23 × 10^7	2.2 ×10^6	5
A copper-mediated fluorescent signal amplification strategy	7.5 × 10^7 ~ 1.5 ×10^{10}	4.8 × 10^7	6

Aptasensor for electrochemical detection	$1 \times 10^6 \sim 1 \times 10^9$	1×10^6	7
DNA nanodevices for TIRF imaging	$10^6 \sim 10^{11}$	10^6	8
Split aptamer-based DNA nanostructure	**$2.32\times 10^5 \sim 5.80\times 10^8$**	**2.08×10^5**	**This work**

Table S3. Recovery of exosomes in serum samples using the proposed system.

Samples		**Add (particles/mL)**	**Found (particles/mL)**	**Recovery (%)**	**RSD (n=3, %)**
10% ED-FBS	1	1.16×10^6	1.11×10^6	95.7	1.63
	2	2.32×10^6	2.35×10^6	101.3	1.79
	3	5.80×10^6	6.02×10^6	103.8	2.35
20% ED-FBS	1	1.16×10^7	1.18×10^7	101.7	2.01
	2	2.32×10^7	2.42×10^7	104.3	1.97
	3	5.80×10^7	5.39×10^7	92.9	2.61

Table S4. Information of clinical blood samples.

Patient ID	Age(Years)	Gender(F/M)	Pathology
3473450	33	M	Malignant
3381601	42	M	Malignant
3568203	111	F	Malignant
3473850	56	M	Malignant
3453615	71	F	Malignant
4125326	67	F	Malignant
4324155	54	M	Malignant
4213514	49	M	Malignant
4324567	52	M	Malignant
4324790	65	F	Malignant
F15487257	NA	NA	No history of cancer
F15488023	NA	NA	No history of cancer
F15492462	NA	M	No history of cancer
F15487403	NA	NA	No history of cancer
F15493826	NA	M	No history of cancer
F15474219	NA	F	No history of cancer
F15464356	NA	NA	No history of cancer
F15467459	NA	M	No history of cancer
F15495718	NA	F	No history of cancer

F15484863	NA	NA	No history of cancer

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