Supporting information

Cascade signal amplification for sensitive detection of exosomes by integrating tyramide and surface-initiated enzymatic polymerization

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1.Experimental Section

Reagents and apparatus. The oligonucleotides were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China) and their sequences are shown in Table S1. The carboxylated magnetic beads (1.15 μm, 10 mg/mL) were gained from So-Fe Biomedicine Co., Ltd (Shanghai, China). TdT was purchased from Beyotime Co., Ltd (Haimen, China). CRC cell line (SW480) and human normal colorectal mucosal cell line (FHC) were from Beijing Beina Science & Technology Co., Ltd. (Beijing, China). Biotin-14-dATP was from Thermo Fisher Scientific (Waltham, MA, USA). HRP-conjugated streptavidin (SA-HRP) was from Sangon Biotech Co., Ltd (Shanghai, China). Cy5 tyramide was purchased from AAT Bioquest (Sunnyvale, CA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA). 1×phosphate-buffered saline (PBS), 1×PBS-Tween20 (PBST), and 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH=7.4) were from Solarbio Science & Technology Co., Ltd (Beijing, China). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), 2-(N-morpholino) ethanesulfonic acid buffer (MES) and other chemical reagents were from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared with ultrapure water (18 MΩ•cm).

The fluorescence intensities were carried out on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, USA). The size distribution and concentration of exosomes were tested by ZetaView (Particle Metrix, Diessen, Germany). Transmission electron microscopy (TEM) was performed on a HT7700 electron microscope (Hitachi, Japan) operating at an accelerating voltage of 120 kV. High-resolution transmission electron microscopy (HRTEM) was performed on a Tecnai G2 F20 electron microscope (FEI, USA) operating at a working voltage of 200 kV. The UV-vis absorption spectra of AuNPs and polyA-AuNPs were recorded on a UV-759 spectrophotometer (APL, China). The particle size of MBs and MBs-conjugation were measured using dynamic light scattering (DLS) technique on a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK)

Exosome isolation and characterization. Two different exosomes derived from SW480 and FHC cells were prepared as the previous reports.[1, 2] Briefly, the serum-free culture medium was centrifugated at 4℃ (500 g for 5 min and 2000 g for 15 min) to remove the cells and cellular debris, followed by filtering through 0.22 μm pore filter. The filtrate was then centrifugated at 120000 g at 4℃ for 2 h to discard large vesicles and protein aggregates. The precipitate was resuspended in PBS and centrifugated at 120000 g at 4 ℃ for 2 h. The final exosome pellet was dissolved in PBS and stored at -80℃ for further use. The exosomes isolated by the standard ultracentrifugation method were characterized by HRTEM, NTA and western blot.

HRTEM. 2.5 μL SW480 and FHC exosomes were loaded on a carbon-coated copper grid, negatively stained with 2.5 μL of 2% phosphotungstic acid for 10 min. The excess dye was removed using filter paper and left to dry at room temperature. The prepared samples were observed by HRTEM.

NTA. 30 μL SW480 and FHC exosomes were diluted 110 folds and 100 folds with PBS, respectively. Followed by filtrating through 0.22 μm filter. The size distribution and concentration of exosomes were measured by the ZetaView Particle Metrix.

Western blot analysis. The exosomes were lysed in RIPA buffer which contained protease inhibitors. The protein was denatured and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) with 10% gel concentration. Subsequently, the gels were transferred onto polyvinylidene fluoride (PVDF) membrane and then were blocked with 5% nonfat milk in TBST buffer.

Modification of MBs. The CD63 aptamer modified MBs were prepared by the standard protocols suggested by the manufacturer. Briefly, 200 μL of 10 mg/mL MBs were washed twice with 500 μL MEST (10 mM MES, pH=6, 0.05% Tween 20), 200 μL of 5 mg/mL EDC and 200 μL of 5 mg/mL NHS were added to the solution to activate -COOH groups on the MBs. After that, 10 μL of 100 μM amino-modified CD63 aptamer was added to the activated MBs solution and allowed to react for 3 h at 37 ℃. Finally, the MBs were blocked by 1% BSA for 30 min at 37 ℃ and then washed with PBST for 5 times. The MBs were dispersed in PBST and stored at 4 ℃.

PolyA-DNA attachment to AuNPs. AuNPs of 20 nm were synthesized by the previous reports,[3] and DNA-modified AuNPs were synthesized using low pH DNA loading assay.[4] The mixture of 20 μL of 10 μM polyA-tailed aptamer and 60 μL of 10 μM polyA-tailed primer was added to 200 μL prepared AuNPs. Then, 500 mM citrate-HCl buffer (pH=3) was added for a final concentration of 10 mM. After incubating at room temperature for 3 min, 10 μL of 1 M HEPES buffer was added to the solution to adjust the pH of AuNPs solution to neutral. The samples were allowed to react for 5 min and were subsequently washed three times by centrifuging at 8000 rpm, 4℃ for 10 min. The precipitate was stored in 200 μL PBS at 4℃ for use.

Immunogold experiment. 2 μL exosomes were mixed with 10 μL $polyA_{20}$-AuNPs and 10 μL ultrapure water, followed by incubating at 37℃ for 1 h. A 2.5 μL droplet of the mixture was placed on a carbon-coated copper grid for 15 min, and then 2.5 μL of 2% glutaraldehyde was placed on the cooper grids for 10 min. The excess dye was removed by filter paper and left to dry at room temperature. The prepared samples were observed by TEM.

Procedures for exosomes detection. 100 μL of target exosomes were added into 10 μL of 10 mg/mL CD63 aptamer modified MBs, followed by incubating for 1 h at 37℃ under stirring. After the MBs were washed three times with PBS, 50 μL of primer-Au-aptamer were added into MBs/exosomes conjugates and incubated for 1 h. The MBs/exosomes/primer-Au-aptamer sandwich complexes were then rinsed three times with PBS. The complexes were subsequently suspended in 10 μL of TdT reaction solution containing 15 U TdT, 6.5 μL reaction buffer and 0.5 μL of 0.4 mM biotin-dATP to trigger the primer extension. The products were magnetically isolated and then washed three times with PBS. After the solution was incubated with 100 μL

of streptavidin-HRP (2 μg/mL) at 37℃ for 30 min. 100 μL of 5 μM Cy5-tyramide containing H_2O_2 solution was added into the extended products and incubated at 37℃ for 20 min. After being washed with PBS, each sample was diluted with PBS. The fluorescence spectra was recorded from 655 nm to 800 nm under the excitation of a 600 nm laser.

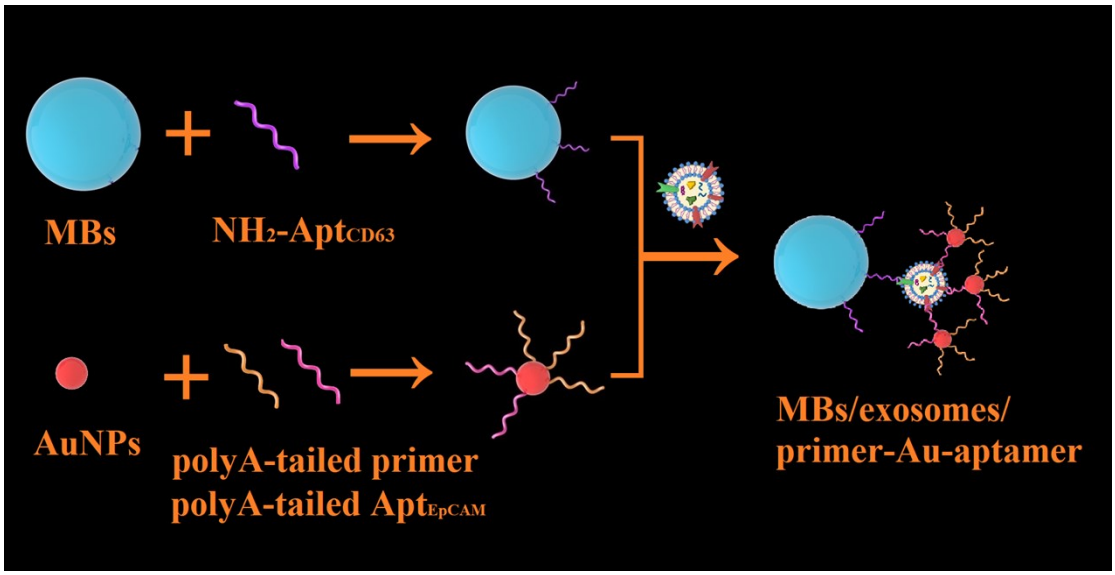
Gel electrophoresis. The products of TdT catalyzed primer-Au-aptamer were characterized by denaturing urea polyacrylamide (PAGE) and agarose gel electrophoresis. Urea PAGE was conducted in a vertical polyacrylamide gel system. After 6 μL samples were loaded in the denaturing urea polyacrylamide gel (2.52 g urea, 3 mL of 30% acrylamide stock solution, 120 μL of 50×TAE, 3 μL TEMED, 60 μL of 10% APS and 2.5 mL deionized water). The electrophoresis analysis was carried out in 1×TAE at 100 V for 1.5 h and stained with 3×YeaRed nucleic acid gel stain. The gels were finally photographed by the UV transilluminator system (Tanon-4100). The products of TdT catalyzed primer-Au-aptamer were run in 1% agarose gel using 1×TAE buffer at 100 V for 15 min, and the gels were visualized under white light.

Detection of exosomes from spiked serum. Clinical serum samples were collected from the central laboratory of the Yangpu Hospital, Shanghai, China. Written informed consent was obtained at the beginning of the study. The collected serum samples were centrifuged at 3000 rpm for 5 min and filtered through a 0.22-μm filter membrane. The concentration of the added exosomes was calculated using the linear regression equation in Fig.2b. All experiments on clinical samples were approved by the Medical Research Ethics Committee, Yangpu Central Hospital, Shanghai, China.

Table.S1 Oligonucleotides sequences used in the experiment

Name	Sequences (5' to 3')
PolyA$_{10}$-primer	AAAAAAAAAATTTTTATACGAACCTAGC
PolyA$_{20}$-primer	AAAAAAAAAAAAAAAAAAAATTTTTATACGAACCTAGC
PolyA$_{30}$-primer	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAATTTTTATACGAACCTAGC
PolyA$_{40}$-primer	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATTTTTATACGAACCTAGC
PolyA$_{10}$-EpCAM	CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTGTTTTTAAAAAAAAAA-P
PolyA$_{20}$-EpCAM	CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTGTTTTTAAAAAAAAAAAAAAAAAAAA-P
PolyA$_{30}$-EpCAM	CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTGTTTTTAAAAAAAAAAAAAAAAAAAAAAAAAAA-P
PolyA$_{40}$-EpCAM	CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTGTTTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-P
CD63-NH$_2$	CACCCCACCTCGCTCCCGTGACACTAATGCTATTTTT-NH$_2$
Random DNA	CCGTGTCTGGGGCCGACCGGCGCATTGGGTACGTTGTTGC-NH$_2$

The polyA-tailed aptamer used as a recognition unit for exosomes was comprised of three fragments: EpCAM aptamer for exosomes binding; a polyT strand (T_5), which has the lowest absorption at the surface of AuNPs to facilitate the EpCAM aptamer to adopt upright conformation;[5, 6] a polyA$_{20}$ tail modified with the phosphate group at the 3' end, which was served as an anchor block to bind with the AuNPs surface, and TdT-catalyzed extension was hindered by the 3′-P.[7]



Scheme.S1 Schematic illustration of the preparation process of MBs/exosomes/primer-Au-aptamer composite particles.

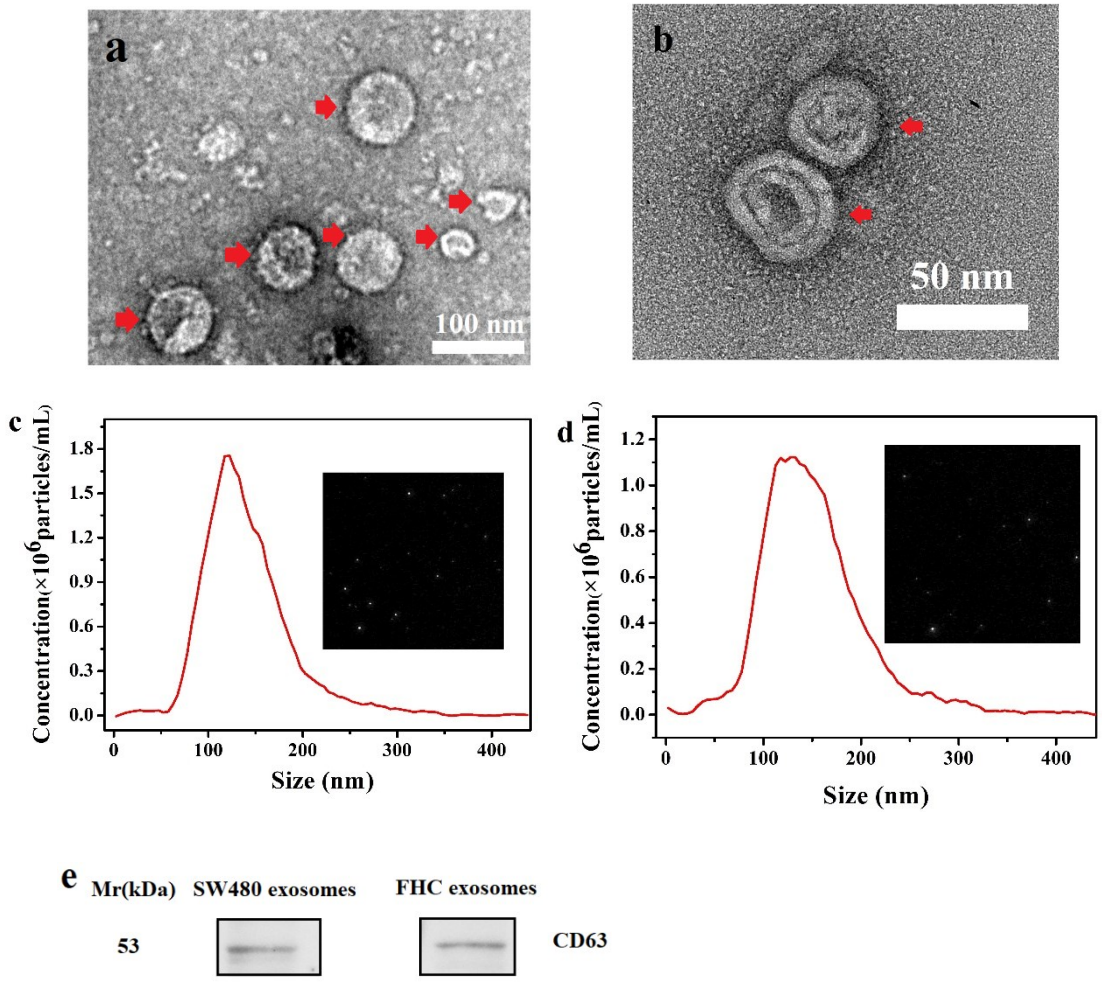


Fig.S1 Characterization of exosomes. a) HRTEM image of SW480 cells-derived exosomes. b) HRTEM image of FHC cells-derived exosomes. c) Size distribution of SW480 cells-derived exosomes by NTA. Inset: the SW480 exosomes with scattered light. d) Size distribution of FHC cells-derived exosomes by NTA. Inset: the FHC exosomes with scattered light. e) Western blot analysis of CD63 protein expression of SW480 and FHC cells-derived exosomes.

Table.S2 The concentration and average size of the purified exosomes.

The original cells of exosomes	Concentration (particles/μL)	Average size (nm)
FHC	2.2×10^6	133
SW480	3.3×10^6	130

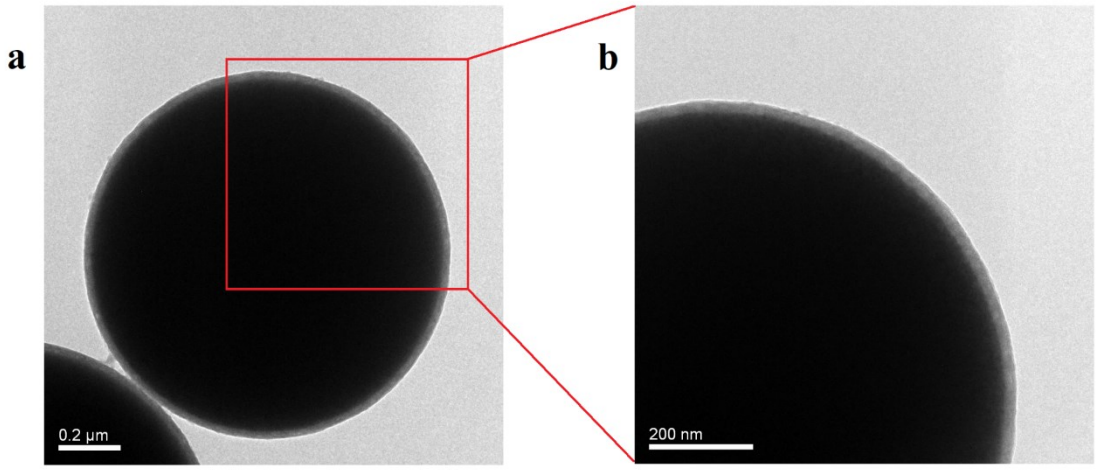


Fig.S2 a) The TEM image of the CD63 aptamer modified MBs. b) Enlarged view of the functionalized MBs.

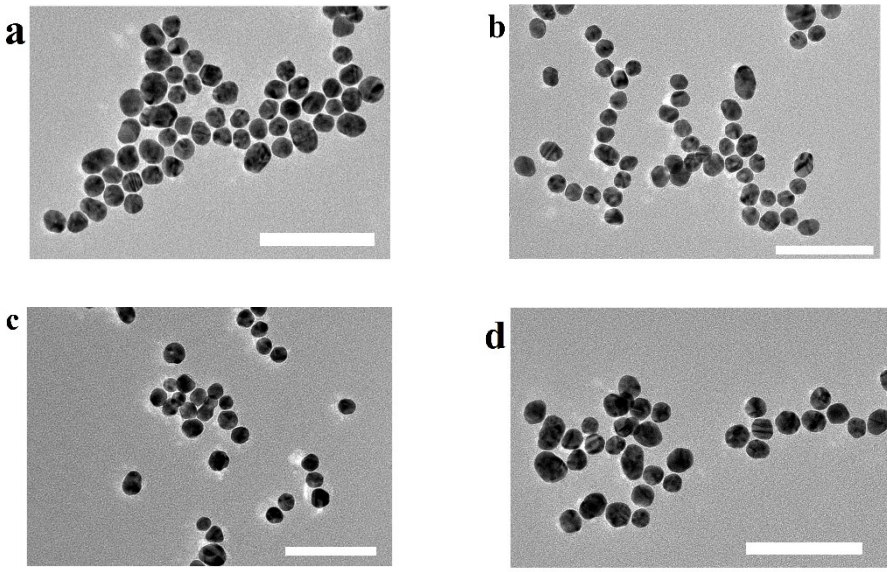


Fig.S3 HRTEM images of a) polyA$_{10}$-AuNPs, b) polyA$_{20}$-AuNPs, c) polyA$_{30}$-AuNPs, and d) polyA$_{40}$-AuNPs. Scale bar, 100 nm

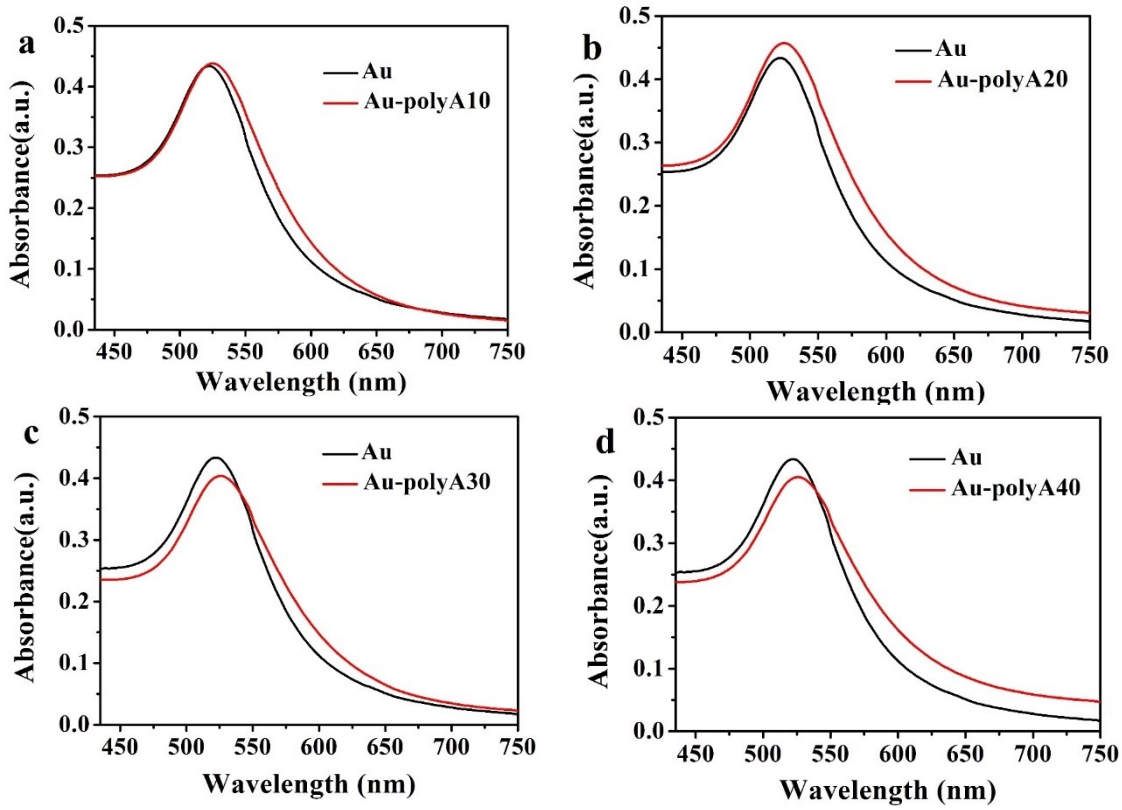


Fig.S4 UV-Vis absorption of AuNPs (black line) and polyA-AuNPs with different polyA block length (red line).

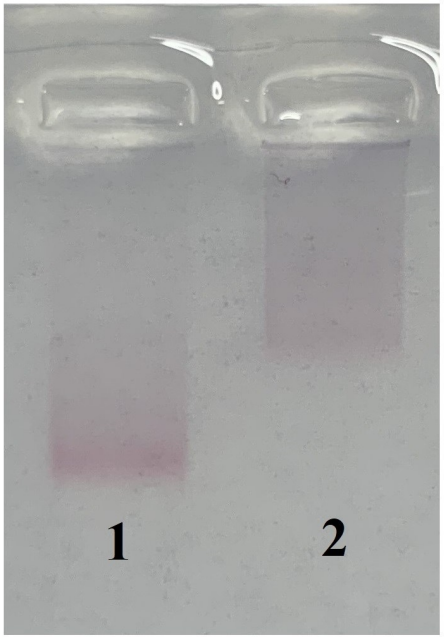


Fig.S5 The gel electrophoresis analysis of polyA$_{20}$-tailed AuNPs (lane 1) and TdT catalyzed AuNPs complex (lane 2).

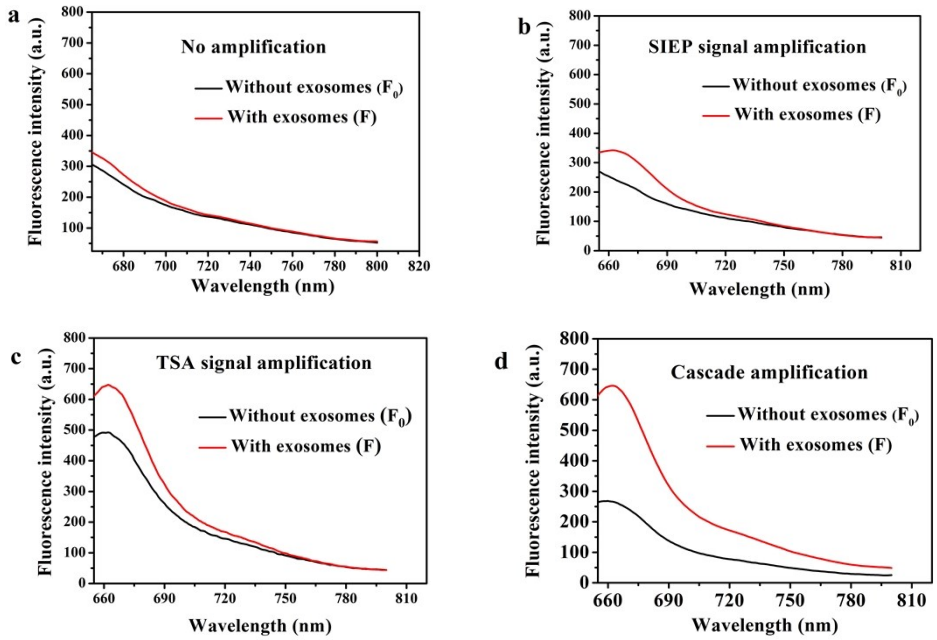


Fig.S6 The fluorescent spectrums of different amplifications (no amplification, SIEP amplification, TSA amplification and cascade amplification), where F is the fluorescence intensity with 2×10^4 particles/μl exosomes ($\lambda_{ex/em}$=600/666 nm) and F_0 is the fluorescence intensity without exosomes.

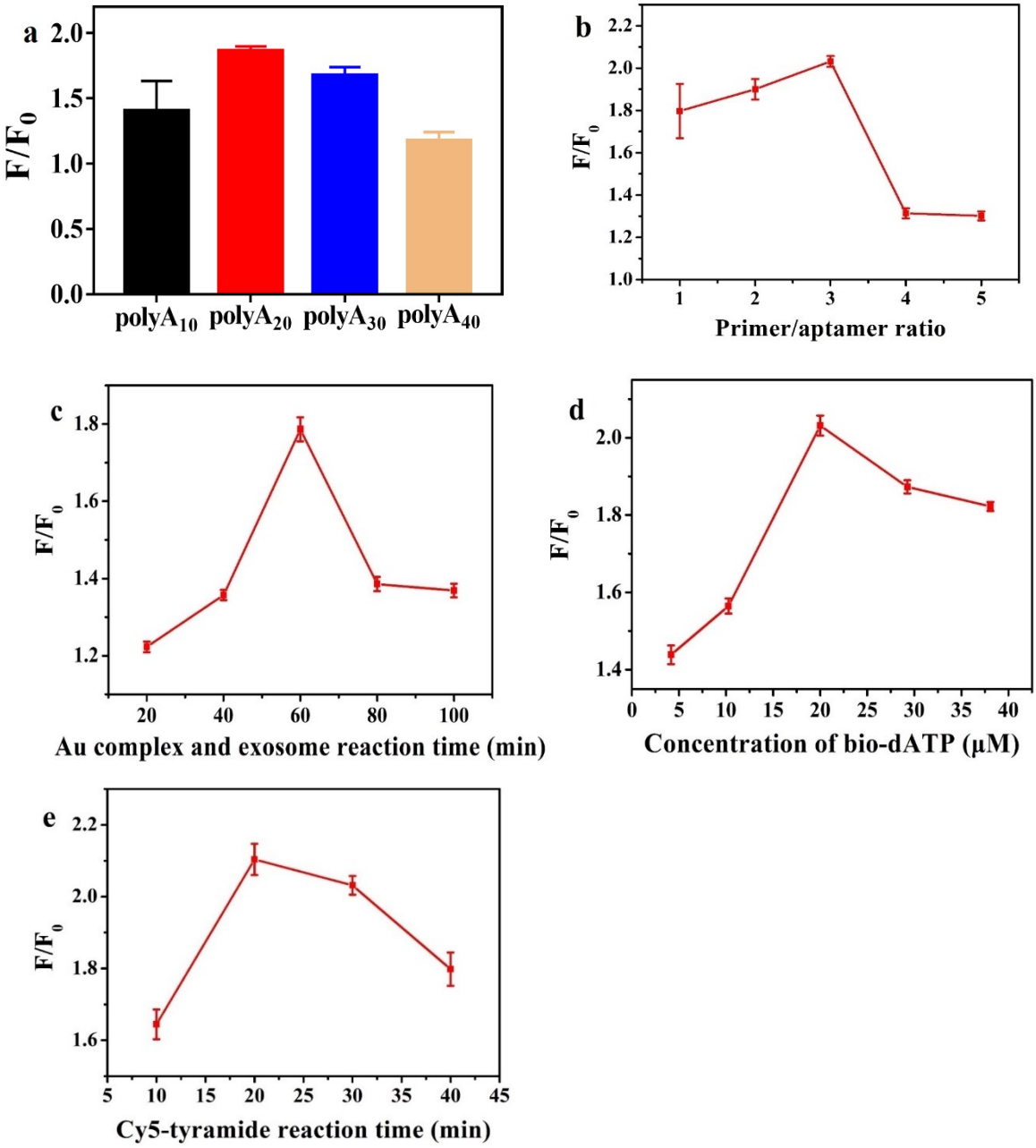


Fig.S7 Optimization of different detection conditions on the fluorescence ratio (F/F_0), where F is the fluorescence intensity with 10^4 particles/μL exosomes, F_0 is the fluorescence intensity without exosomes, at $\lambda_{ex/em}$=600/666 nm. (a) aptamer and primer with different polyA lengths (10, 20, 30, and 40). (b) primer and aptamer ratio in primer-Au-aptamer nanoconjugates (1:1, 2:1, 3:1, 4:1, and 5:1). (c) the reaction time between primer-Au-aptamer and exosomes (20, 40, 60, 80, and 100 min). (d) the concentration of bio-dATP (4.2, 10.3, 20.0, 29.3, and 38.1 μM). (e) Cy5-tyramide reaction time (10, 20, 30, and 40 min)

Since the key factor of our experiment is based on primer-Au-aptamer for signal amplification, the effect of the polyA length on the fluorescence signal was firstly

investigated by using the Au complex with different lengths of polyA (10,20, 30, and 40) at the same primer/aptamer ratio (1:1). As shown in Fig.S6a, AuNPs with $polyA_{20}$ exhibited the best performance. The longer length of polyA lowered the aptamer and primer density on the surface of AuNPs.[8] In our cascade amplification method, the increase of polyA length resulted in a reduction of the number of CD63 aptamers to capture exosomes and primers for signal amplification. Therefore, the fluorescence ratio decreased when the length of polyA was higher than 20. Interestingly, $polyA_{20}$ has a better fluorescence ratio than $polyA_{10}$, the reason for this phenomenon is probably that a more efficient and conducive hybridization of exosomes and aptamer-Au-primer could be achieved when the aptamer density in AuNPs was lower.

The effects of primer/aptamer ratio and the reaction time between primer-Au-aptamer complex and exosomes on signal ratio were also studied. The fluorescence ratio gradually increased with an increased primer/aptamer ratio and reached the highest signal ratio when the primer/aptamer ratio was 3:1 (Fig.S6b). The decrease of fluorescence ratio could be explained that excess primers occupied the binding sites of aptamer and AuNPs,[9] resulting in the decreasing number of AuNPs bound to exosomes. Therefore, the optimal ratio of primer/aptamer was 3:1. 30 min was chosen as the best incubation time of primer-Au-aptamer (Fig.S6c). The optimal concentration of biotin-dATP was found to be 20 μM (Fig.S6d). The incubation period of Cy5-tyramide was optimized by using exosomes samples and blank at different incubation time, the deposition time for Cy5-tyramide was fixed at 20 min (Fig.S6e).

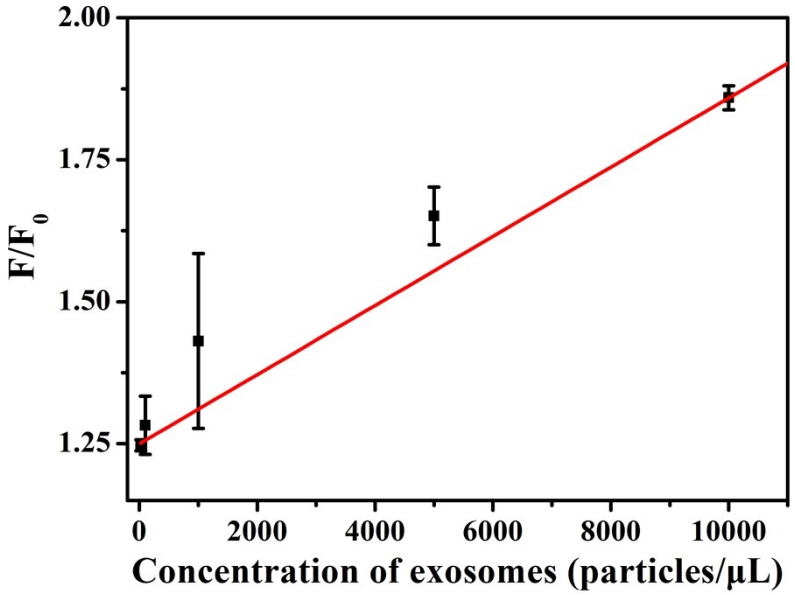


Fig.S8 The linear relationship between F/F_0 ($\lambda_{ex/em}$=600/666 nm) and the concentration of exosomes in the range of 25-1×10^4 particles/μL. The error bars represent the standard deviation of three independent assays.

Table.S3 Comparison of different exosomes detection methods.

No.	Technique	Strategy	LOD (particles/ μL)	Linear range of detection (particles/μL)	Time (h)	Ref
1	Colorimetry	Hemin/G-quadruplex toward H_2O_2 reduction	394	8.3×10^2-5.3×10^4	~1	[10]
2	Colorimetry	SIEP signal amplification	6700	9.75×10^3-1.95×10^6	~4.5	[11]
3	Electrochemical	CTSDR-based catalytic molecule machine	17.2	1×10^5-5×10^7	>1.8	[12]
4	Electrochemiluminescence	DNA walking machine	60	2×10^2-7.5×10^4	>11.5	[13]
5	Electrochemiluminescence	Multivalency Interface and g-C_3N_4 @Galinstan-PDA	31	50-10^5	>4	[14]
6	Fluorescence	Branched rolling circle amplification	42.7	10^2-10^6	>8	[15]
7	Fluorescence	DNA dendrimer self-assembly	1160	1.75×10^3-7×10^6	~4.5	[16]
8	Fluorescence	Fluorescence polarization assay	500	5×10^2-5×10^5	~0.5	[17]
9	Fluorescence	Hybridization displacement reaction	340000	5×10^5-5×10^7	~3.6	[18]
10	Fluorescence	Zirconium–phosphate coordination chemistry	7600	1.68×10^4-4.2×10^7	~2.5	[19]
11	Fluorescence	TSA-SIEP cascade signal amplification	12.8	25-2.5×10^4	~3.5	Our work

Table.S4 Recovery experiments of exosomes in clinical serum samples.

Samples	Value added (particles/μL)	Value founded (particles/μL)	Recovery (%)	RSD (%, n=3)
Serum 1	100	114.69	114.69	1.68
Serum 2	1000	1014.50	101.50	4.84
Serum 3	10000	10272.77	102.73	1.48

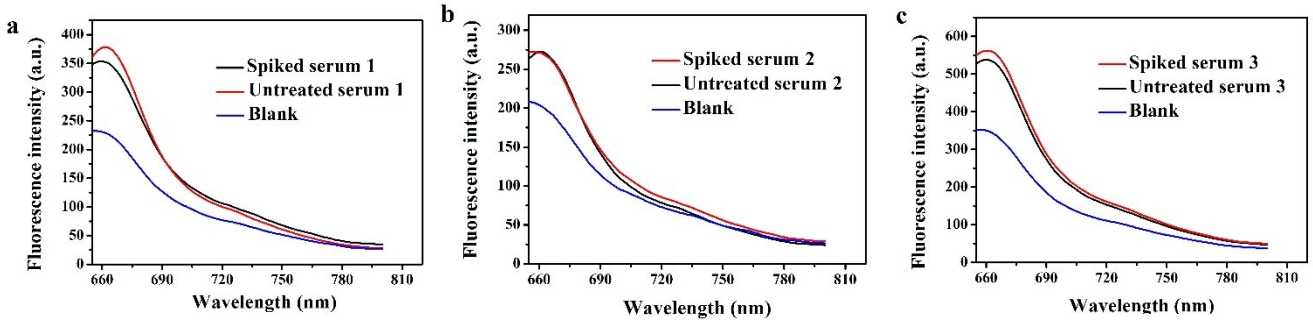


Fig.S9 The fluorescent spectrums of the spiked serum samples and equivalent untreated serum samples.

3.References:

1. H. Valadi, K. Ekström, A. Bossios, M. Sjöstrand, J. J. Lee and J. O. Lötvall, *Nature cell biology*, 2007, **9**, 654.
2. H. Shao, H. Im, C. M. Castro, X. Breakefield, R. Weissleder and H. Lee, *Chem. Rev.*, 2018, **118**, 1917-1950.
3. I. E. Paul, A. Rajeshwari, T. Prathna, A. M. Raichur, N. Chandrasekaran and A. Mukherjee, *Analytical Methods*, 2015, **7**, 1453-1462.
4. X. Zhang, M. R. Servos and J. Liu, *J. Am. Chem. Soc.*, 2012, **134**, 7266-7269.
5. J. J. Storhoff, R. Elghanian, C. A. Mirkin and R. L. Letsinger, *Langmuir*, 2002, **18**, 6666-6670.
6. W. Li, Y. Dong, X. Wang, H. Li and D. Xu, *Biosens. Bioelectron.*, 2015, **66**, 43-49.
7. X.-Y. Li, Y.-C. Du, Y.-N. Pan, L.-L. Su, S. Shi, S.-Y. Wang, A.-N. Tang, K. Kim and D.-M. Kong, *Chem. Commun.*, 2018, **54**, 13841-13844.
8. D. Zhu, P. Song, J. Shen, S. Su, J. Chao, A. Aldalbahi, Z. Zhou, S. Song, C. Fan and X. Zuo, *Anal. Chem.*, 2016, **88**, 4949-4954.
9. Y. Zhu, H. Wang, L. Wang, J. Zhu and W. Jiang, *ACS applied materials & interfaces*, 2016, **8**, 2573-2581.
10. Y. Zhou, H. Xu, H. Wang and B.-C. Ye, *Analyst*, 2020, **145**, 107-114.
11. Z. Huang, Q. Lin, X. Ye, B. Yang, R. Zhang, H. Chen, W. Weng and J. Kong, *Talanta*, 2020, **218**, 121089.
12. Y. Cao, L. Li, B. Han, Y. Wang, Y. Dai and J. Zhao, *Biosens. Bioelectron.*, 2019, **141**, 111397.
13. Q.-M. Feng, P. Ma, Q.-H. Cao, Y.-H. Guo and J.-J. Xu, *Chem. Commun.*, 2020, **56**, 269-272.
14. Y. Zhang, F. Wang, H. Zhang, H. Wang and Y. Liu, *Anal. Chem.*, 2019, **91**, 12100-12107.
15. R. Huang, L. He, S. Li, H. Liu, L. Jin, Z. Chen, Y. Zhao, Z. Li, Y. Deng and N. He, *Nanoscale*, 2020, **12**, 2445-2451.
16. M.-L. Gao, F. He, B.-C. Yin and B.-C. Ye, *Analyst*, 2019, **144**, 1995-2002.
17. Z. Zhang, C. Tang, L. Zhao, L. Xu, W. Zhou, Z. Dong, Y. Yang, Q. Xie and X. Fang, *Nanoscale*, 2019, **11**, 10106-10113.
18. J. Chen, H.-M. Meng, Y. An, X. Geng, K. Zhao, L. Qu and Z. Li, *Talanta*, 2020, **209**, 120510.
19. L. Wang, Y. Yang, Y. Liu, L. Ning, Y. Xiang and G. Li, *Chem. Commun.*, 2019, **55**, 2708-2711.