Supplementary material

Supplemented experimental section

Assembly of DNA tweezer:

The DNA tweezer structure was firstly formed by mixing of sequence (i–iv) with a molar ratio of 1:1. Then, the mixture was heated up to 95 °C and slowly cooled down to room temperature.

Assembly of HP:

The mixture containing 20 μL HP (10 μM) was heated up to 95 °C and slowly cooled down to room temperature.

Determination of the extracted sEVs concentrations:

Nanoparticle tracking analysis (NTA) were performed to determine the concentration of sEVs by NTA, which was performed using the Nanosight NS300 followed by protocol according to fomer references [1, 2].

Experimental parameters optimization:

The sEVs were incubated for 40 min in 200 μL of PBS buffer with 0.5 μM C2 probe and 1 μM C1 probe, respectively. Following that, a hairpin (150 nM) and DNA tweezers (200 nM) were added to the mixture in 10 mM PBS (pH 7.4) containing 0.5 M NaCl. Following a three-hour incubation under different experimental temperatures, the sample was analyzed using a fluorescence spectrometer. Other experimental parameters optimization follows the former procedures.

Table S1. The sequences of used oligonucleotides in this work

Title	**Sequences (5' to 3')**
C1	AGC TCG GTA GAC TCA GGA TTT TTT TTT
C1	Cholesterol-TTT TTT TTT TCC TGA GTC TAC GTA GC
HP	CGG TAG CCT GTG CTA CCG AGC T
ii	GTT GGA GCG ACA TTA GAG AGC TAC AA-FAM
iii	DABCYL-GTA GCC TCC TGT CCT ATC TAT GAT GG
i	CTA ATG TCG CTC CAA CAA CCA TCA TAG ATA GGA C
iv	TTG TAG CAC AGG CTA CCG

Table S2. A brief comparison of the proposed method with former ones

Title	Mechanism	Target molecule(s)	Low limit of detection (particles/μL)	Enzymes	Advantages and disadvantages	Ref
The method	Proximity ligation+ DNA tweezer	CD63 and lipid bilayer	57	No	Advantages: high stability and anti-interference capability to free CD63 protein or cell lysis; high sensitivity; no enzymes were used. Disadvantages: could only be applied for sEVs detection.	
AcmPLA	Proximity ligation assisted RCA	CD63 and lipid bilayer	10^3	T4 DNA ligase, phi29 polymerase, antibody,	Advantages: high stability and anti-interference capability to free CD63 protein or cell lysis; Disadvantages: five probes and two enzymes were used in this method; low sensitivity	[1]
AID-Cas	CRISPR-Cas12a assisted dual cycles	CD63 protein	10^2	Cas12a, polymerase, Nt.BbvCI, T7 RNA polymerase	Advantages: high sensitivity; Disadvantages: low anti-interference capability to free CD63 protein	[2]
Allosteric probe based	SDA	CD63 protein	10^2	DNA polymerase, endonuclease	Advantages: high sensitivity; Disadvantages: low anti-interference capability to free CD63 protein	[3]

RCA, rolling circle amplification; SDA, strand displacement amplification; AcmPLA, aptamer–cholesterol-mediated proximity ligation assay; AID-Cas, allosteric probe-initiated dual cycle amplification-assisted CRISPR-Cas12a.

References:

1. X. Zhao, C. Luo, Q. Mei, H. Zhang, W. Zhang, D. Su, W. Fu and Y. Luo, *Anal. Chem.*, 2020, **92**, 5411-5418.
2. X. Zhao, L. Zeng, Q. Mei and Y. Luo, *ACS Sens*, 2020, **5**, 2239-2246.
3. Y. Zhou, F. Yu, H. Cheng and L. Ning, *ACS Omega*, 2021, **6**, 17776-17781.