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A simple and sensitive method for exosomes detection based on steric hindrance-controlled signal amplification

Electronic Supplementary Information

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Experimental Section

Materials

Fetal Bovine Serum (FBS) was obtained from Invitrogen Trading Co., Ltd (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin solution, phosphate buffer saline (PBS, containing NaCl 136.89 mM; KCl 2.67 mM; Na_2HPO_4 8.1 mM; KH_2PO_4 1.76 mM, pH 7.4), carboxyl magnetic beads (MBs), streptavidin (SA, from Streptomyces avidinii) were purchased from Sangon Biotech Co., Ltd (Shanghai, China). All oligonucleotides used in this work were also synthesized and purified by Sangon (Table S1). Sigma-Aldrich provided us with N-hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), 4-Morpholineethanesulfonic acid (MES) and Thioflavin T (ThT) while dATP and dGTP were supplied by Takara. Terminal deoxynucleotidyl transferase (TdT) enzyme with reaction buffer and $CoCl_2$ solution was bought from New England Biolabs Co., Ltd (Beijing, China). The experimental water was filtered by Millipore water purification system.

Cell culture

Hela cells were cultured in DMEM medium containing 10 % (v/v) FBS and 1 % (v/v) penicillin-streptomycin under the environment of 37 ℃ and 5 % CO_2. When the cells grew to 80 % area of the culture dish in good condition, discarded the supernatant. After washing with PBS twice, the medium containing 1 % exosomes-free special serum was added and cultured for another 48 hours (Other conditions remained unchanged). Then the cell culture supernatant was collected for further isolation and extraction of Hela exosomes.

Exosomes extraction

According to our previous reports,[1-3] we used the gold standard gradient centrifugation method with minor adjustments for the collection of exosomes. Firstly, the supernatant was centrifuged at 5000 g for 10 min to ensure preliminary removal of cells, cell debris and other large particles. The newly obtained supernatant was centrifuged again at 10000 g for 30 min to remove other biological vesicles, the regained supernatant was filtered through a 0.22 μm filter and filtrate was next used for ultracentrifugation. The sediment was collected after centrifuging for 2 hours at 120000 g and the precipitated exosomes can be resuspended in 200 μL PBS by blowing the bottom of the centrifuge tube repeatedly. The extracted exosomes were stored at -80 ℃ for subsequent use. The concentration, size and morphology of the exosomes were characterized by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM).

Modification of carboxyl magnetic beads with CD63 aptamer

The magnetic beads were first cleaned three times with 25 mM MES solution (pH 6.0) to completely remove the remaining preservation buffer. Next, 200 μL of freshly prepared 0.01 M NHS and 0.1 M EDC solutions were added respectively and maintained on the oscillator at 1000 rpm for 2 h to activate carboxyl groups of MBs. Then 500 μL 3' aminated CD63 aptamer (5 μM) was put in after washing MBs with MES solution for three times and kept reacting overnight at 1000 rpm. Finally, CD63 aptemer modified MBs were washed with PBS three times to remove the free CD63 aptamer and resuspended in 1 mL PBS, stored at 4 ℃.

Detection different concentrations of exosomes

First of all, 10 μL CD63-MBs were incubated with 10 μL Hela exosomes of different concentrations on a 1000 rpm oscillator at 37 ℃ for 30 min to fully capture exosomes. Secondary, the magnetic beads were repeatedly cleaned with PBS three times to reduce the interference caused by nonspecific adsorption. Then 5 μL P1 (5 μM) were added and incubated for 30 min to make P1 insert into the phospholipid bilayer of exosomes. Next, 5 μL streptavidin modified P2 (SA-P2, 5 μM) was added directly and incubated for another 30 min. Afterwards, 10 μL mixture containing dATP/dGTP (dATP : dGTP = 2 : 3), TdT buffer, $CoCl_2$ solution and TdT enzyme was added and reacted at 37 ℃ for 2 h, then heated at 95 ℃ for 5 min to denature the TdT enzyme. After the reaction system was naturally cooled to room temperature, ThT solution was added and incubated at 37 ℃ for 35 min. Depending on the folding topology, G-quadruplex DNA can recognize the ThT in the groove, stack it on the top/bottom quartet or allow intercalation between the G-quartet planes since ThT is a cationic dye. In all these, optimum stabilization can be expected from electrostatic, van der Waal's, hydrogen-bonding, π-stacking, and other noncovalent interactions.[4] The final fluorescence intensity was measured by Infinite 200Pro.

Characterization of exosomes and CD63-MBs

The collected exosomes are diluted with PBS to a suitable concentration and NTA technique is used to determine the size and concentration of exosomes. As shown in Fig. S2A, the size uniformity of Hela cell exosomes is decent, with an average diameter of 145.9 nm, and the concentration of purified exosomes can reach 1.65×10^7 particles/μL. The morphology of exosomes can be observed directly by transmission electron microscopy (TEM) (Fig. S2B), which is consistent with previous reports. Whether the carboxyl magnetic beads are successfully modified with amino CD63 aptamer is characterized by zeta potential (Fig. S2C). The zeta potential of the magnetic bead is negative due to the negative charges of carboxyl group. After modified with CD63 aptamer, the zeta potential of magnetic beads becomes more negative due to the abundant negative charges carried by the phosphate skeleton of aptamer, so we think that CD63 aptamer has been adequately connected to the

magnetic beads. Moreover, the average diameter of CD63-MBs becomes larger after capturing the exosomes (exosomes-CD63-MBs) by the measurement results of dynamic light scattering (DLS), indicating favorable target recognition and capture capability of CD63-MBs (Fig. S2D).

Optimization of conditions

In order to improve the performance of the method to obtain the best detection capability, we optimize the following three main factors: TdT mediated polymerization reaction time (t_{TdT}), incubation time of ThT with G-quadruplexes (t_{ThT}) and ThT concentration (C_{ThT}). Taking the time cost into consideration, we first optimize the TdT reaction time and the incubation time of ThT with G-quadruplexes. As Fig. S3A shows, when the TdT reaction time reaches 2 hours, the fluorescence intensity reaches the highest value and remains stable. Besides, when ThT incubates with G-quadruplexes for 35 minutes, we find out the highest fluorescence signal (Fig. S3B). In order to ensure that the generated G-quadruplexes can incubate with sufficient ThT to achieve the strongest signal intensity, we adjust the concentration of ThT. With the increase of ThT concentration from 5 μM to 0.5 mM, the fluorescence intensity also rises until it enters a plateau (Fig. S3C). Based on the above, 2 hours of TdT reaction time, 35 minutes of ThT incubation time and 0.5 mM ThT are selected as the optimal reaction conditions and are applied to the following experiments.

Table S1. DNA sequences used in this work

Name	Sequence (5’ to 3’)
probe 1 (P1)	GTA ACT GTT TCC TTC - cholesterol
probe 2 (P2)	biotin - GAA GGA AAC AGT TAC
CD63 aptamer	CAC CCC ACC TCG CTC CCG TGA CAC TAA TGC TA - NH_2
random DNA	GTC CTT ATT CAG TTG ACG GTG CTG TAT ATA TAT A - NH_2

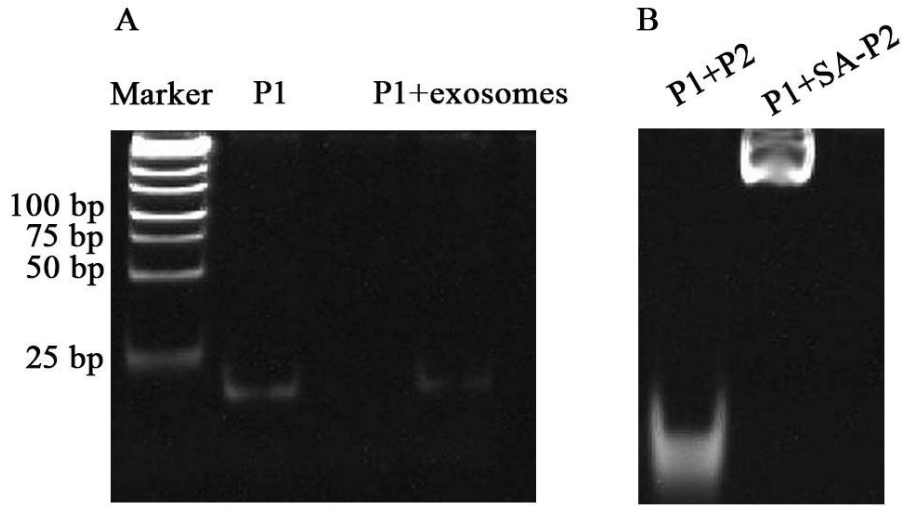

Fig. S1. Electrophoresis analysis of the different products to prove P1 can embed into exosome membrane (A) and the little effect of streptavidin modification for the binding of SA-P2 to P1 (B).

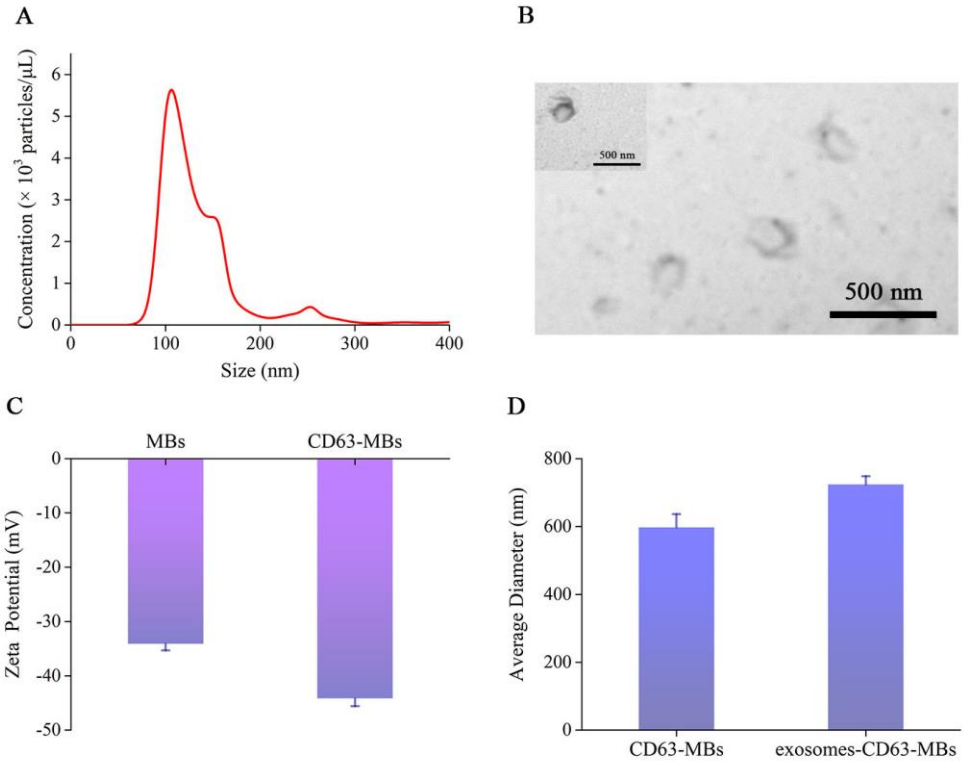

Fig. S2. (A) The concentration and size distribution of Hela exosomes analyzed by NTA. (B) Transmission electron microscopy image of exosomes. The scale bar is 500nm. (C) Zeta potential of MBs before and after the modification with CD63 aptamer. (D) Average diameter comparison of CD63-MBs and exosomes-CD63-MBs.

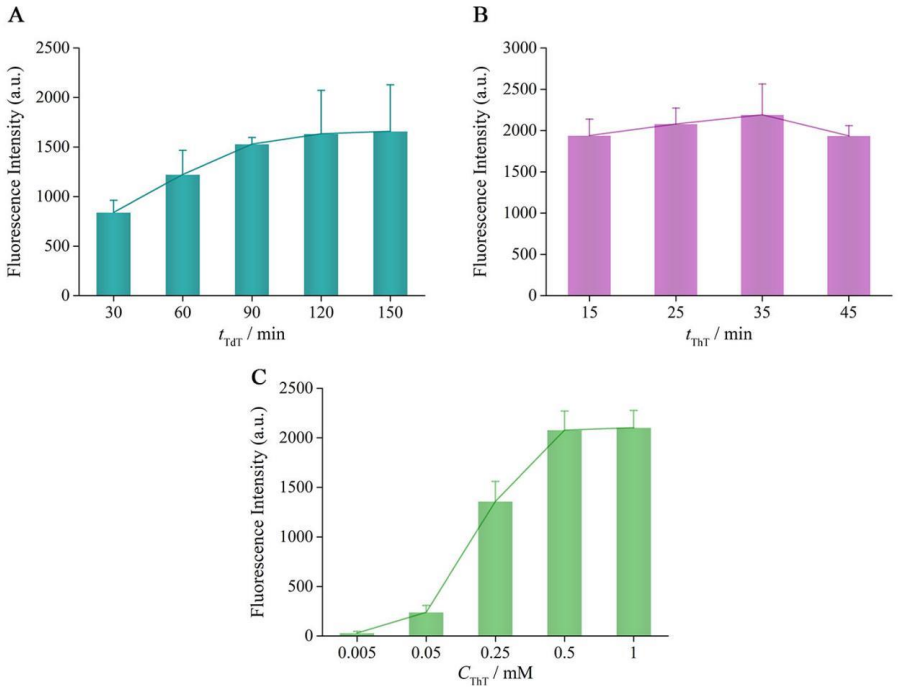

Fig. S3. Influence of TdT reaction time (A), the incubation time of ThT with G-quadruplexes (B) and ThT concentration (C) on the detection performance of the method.

Table S2. Comparison with some previous exosomes detection strategies.

Signal output mode	Linear range (particles/μL)	LOD (particles/μL)	References
Colorimetry	2.3×10^3 to 2.3×10^5	2.2×10^3	[5]
Colorimetry	1.84×10^6 to 2.2×10^7	5.2×10^5	[6]
Electrochemistry	1×10^3 to 1×10^5	1×10^3	[7]
Electrochemistry	1×10^2 to 5×10^4	17	[8]
Fluorescence	7.5×10^4 to 1.5×10^7	4.8×10^4	[9]
Fluorescence	1.0×10^5 to 1.0×10^9	1.0×10^5	[10]
Fluorescence	1×10^3 to 1×10^5	100	[11]
Fluorescence	1×10^4 to 5×10^5	3.12×10^3	[12]
Fluorescence	5×10^5 to 1×10^7	7.5×10^2	[13]
Fluorescence	1.66×10^3 to 1.66×10^6	480	This work

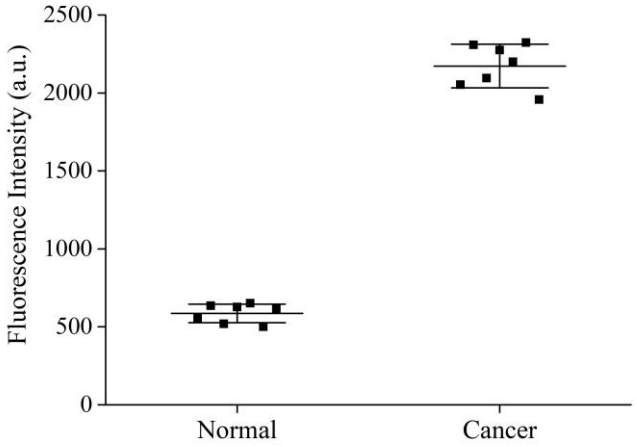

Fig. S4. Analysis of serum from normal people and cancer patients by the proposed method.

Table S3. Results for exosomes detection in 10 % human serum samples (n=3).

Samples	Coefficients of Variation (%)	Recovery (%)
a	9.60	106.37
b	13.78	98.42
c	5.54	107.79

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