Supporting Information

Precise Selection of Aptamers Targeting Small Extracellular Vesicle PD-L1 on Magnetism-Controlled Chips

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Table of contents

EXPERIMENTAL SECTION

Reagents and Instruments.

Human cervical cancer cells (HeLa cells) and Human tongue squamous carcinoma cells (Cal27 cells) were purchased from the China Center for Type Culture Collection. DSPE-PEG (2000)-Biotin was obtained from Avanti polar lipids (Beijing, China). Plasmids carrying PD-L1 and PD-L1-GFP fusion protein genes were constructed based on H102 pLenti-CMV-EGFP-3FLAG-PGK-Puro. PD-L1 expressing HeLa and PD-L1-GFP fusion protein expressing Cal27 cells were obtained by lentiviral infection of normal Hela and Cal27 cells, respectively. The vectors were purchased from Wogen Biotechnology (Shanghai, China).

Streptavidin (SA) was purchased from Biosciences (Shanghai, China). Biotin was purchased from Thermo Fisher Scientific (Shanghai, China). PD-L1 was obtained from Sinobiological (Shanghai, China). Phycoerythrin-modified streptavidin (LEGENDplex™ SA-PE) and PD-L1 antibody modified with phaeohemoglobin (PE anti-human CD274 (B7-H1, PD-L1) Antibody) were purchased from biolegend (USA). PCR reagents were purchased from TransGen (Beijing TransGen Biotech Co., Ltd., China). ssDNA marker was got from cnbioruler.cn Co., Ltd. All of the nucleic acid sequences in the experiments were synthesized by Sangon Biotechnology (Shanghai) Co., Ltd., China. AZ50XT and AZ9260 photoresists (PRs) were obtained from AZ Electronic Materials (AZ Electronic Materials Corp., USA). Poly-(dimethylsiloxane) (PDMS) was bought from GE (Toshiba Silicones Co., Ltd., Japan). Ultrapure water was got from Millipore water purification system (18.2 MΩ cm^{-1}, Milli-Q, Millipore). A quantum dots 605 streptavidin conjugate (SA-QDs) was obtained from Wuhan Jiayuan Quantum Dots Co., Ltd. Indium tin oxide (ITO) glass was obtained from LaiBao (LaiBao Hi-Tech Co., Ltd., China).

Construction of PD-L1 Positive and Negative Cell Lines.

To construct the PD-L1 positive cell line, 293FT cells were used as tool cells to produce lentivirus carrying the PD-L1 gene. The virus-containing supernatant from 293FT cells was collected and incubated with the HeLa cells, which were negative for endogenous PD-L1 expression. The virus inserted the PD-L1 gene into the genomic

sequence of the cells by infecting them, thus giving HeLa cells the ability to stably express PD-L1 genetically (PD-L1$^+$ cell). Normal cultured HeLa cells were used as a cell line of PD-L1 negative (PD-L1$^-$ cell).

Preparation of Biotinylated PD-L1 Positive and Negative sEVs.

After the above two groups of cells were cultured to 90% in a normal medium, they were cultured in an sEVs-free medium containing biotin-functionalized phosphatidylethanolamine (DSPE-PEG-biotin). After incubation for 48 h, cell culture supernatants were collected and centrifuged at 25°C (2,000 g for 20 min) twice. The supernatants were centrifuged at 4°C (16,500 g for 70 min). Further, the supernatants were centrifuged at 120,000 g for 70 min at 4°C to pellet sEVs. After resuspending sEVs in PBS, sEVs were washed once under ultra-centrifugation at 100,000 g for 2 h. SEVs were later characterised by transmitted electron microscopy (TEM, Hitachi, HT7700) and nanoparticle tracking analysis (NTA, Particlemetrix, Zetaview).

Flow cytometry of cells and sEVs.

The cultured cells were digested by trypsin, followed by centrifugation at 4°C 400 g for 5 min, removal of the medium and resuspension of the cells in PBS. The cells were washed by PBS and the single cell suspensions were prepared with PBS (containing 3% BSA) at a concentration of 10^6-10^7 cells/mL. The cell suspension was homogenously mixed with antibody at recommended concentration and reacted at 4°C for 1 h. The reaction solution was removed by centrifugation and the precipitate was washed and resuspended by PBS. The resuspended cells were detected by flow cytometer (A50 micro plus, Apogee).

Biotinylated small extracellular vesicles (sEVs) resuspended in PBS were labeled with SA-modified fluorescent antibodies at a volume ratio of 10:1 and incubated for 30 min at 37°C. Replenishing the volume to 400 μL using PBS and detected by flow cytometer. To select sEVs correctly, a mixture of standard microspheres of 110, 180, 240, 300, 500, 550, 880 and 1300 nm in size was tested under the same conditions as the extracellular vesicles, and the particles smaller than 180 nm were considered as small extracellular vesicles (Figure S11).

Fabrication of Magnetism-Controlled Chips.

The design of the magnetism-controlled chips was mainly based on our previous work[11]. AZ50XT photoresist and silicon wafer were used for the production of microfluidic channels through soft lithography technology. The nickel array was constructed by electroplating nickel technology. After the microchannel model was made, the PDMS prepolymer was poured into the silicon wafer to cure. The nickel array was encapsulated with a thin layer of PDMS, and the cured PDMS microchannel and the nickel array were treated in a plasma cleaner for 1 min, and then bonded.

Preparation of Magnetic Streptavidin-Modified Magnetic Beads.

The magnetic beads with the magnetic response and good dispersion were prepared by previous assembly method[15]. 50 μL of magnetic beads were washed three times with a buffer solution of pH = 7.2, and then the magnetic beads and 10 μg of streptavidin was added to a total volume of 400 μL of 10 mg/mL EDC and reacted for 4 hours. At the end of the reaction, the magnetic beads were washed three times by PBS, streptavidin-modified magnetic beads (MNs-SA) successfully were prepared.

Preparation of sEVs Streptavidin Magnetic Beads.

The content of protein expressed by sEVs was used as a quantitative target. The streptavidin-modified magnetic beads prepared above were washed three times with PBS, the sEVs and magnetic beads were incubated for half an hour, and the clear liquid was collected by magnetic separation for protein quantification. Further, the magnetic beads were washed three times with PBS and dispersed in PBS for later use. The modification of sEVs was judged by DLS measurement of particle size and Zeta potential changes on a Malvern Zetasizer (Nano ZS).

Selection Steps.

The original ssDNA library was composed of 60 nt random region in the center and 20 nt specific primer binding sites at both ends (5′-Alexa-AGCAGCACA-GAGGTCAGATG (N60) CCTATGCGTGCTACCGTGAA-3′). The front primer (FP) was labeled with Alexa Fluor and the rear primer (RP) was labeled with biotin. In the first, 150 pmol of the initial ssDNA library was diluted into 200 μL 1× binding buffer (20 mM HEPES, 150 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$). To make aptamers form a specific three-dimensional structure, the ssDNA library was heated at

95 °C for 10 min, immediately cooled to 0 °C for 10 min, and slowly restored to 25 °C for 5 min. The library was incubated with magnetic beads that modified negative PD-L1 sEVs (MNs-PD-L1$^-$) at 37 °C for 2 h. After incubation, the library and the magnetic bead complex were separated in the chip. The separated library was collected in the reservoir and reacted with magnetic beads that modified high-expressing PD-L1 sEVs (MNs-PD-L1$^+$) for 2 h. Then it was pumped into the positive selection area, and then the area was strictly washed with 2 μL/min washing buffer. The selection process was real-time monitored by a fiber optic spectrometer (Ocean Optics, QE65000) and a microscope (Nikon, TiU). Finally, the collected magnetic beads that enrich the positively selected aptamers were subjected to PCR amplification. The PCR amplification conditions were on our previous work[11]. After the PCR amplification, the successful amplification of the DNA fragment was further confirmed by a 20% denaturing gel. The amplified product was incubated with MNs-SA for 0.5 h for magnetic separation. The product was then treated with 0.1 M NaOH to denature the double-stranded DNA, followed by ultrafiltration using a 10K ultrafiltration tube (Amicon Ultra). The resulting library was used in the next step.

Evaluation of the Effectiveness of Each Round of Selection.

To assess the selection validity, 40 pmol of the ssDNA library of each round was incubated with 5 μL of the MNs-PD-L1$^-$ and MNs-PD-L1$^+$ for 1.5 h, separately. After incubation, the complexes of the magnetic beads that modified sEVs and nucleic acids were pumped into the magnetism-controlled chips, respectively. The trapped arrays were injected into a washing buffer to wash the unbound ssDNA, the fluorescence intensities of the array were captured by a fiber optical spectrometer.

Evaluation of Aptamers.

After four rounds of selection, the amplified library that used the unlabeled forward and reverses primers were cloned and sequenced, and the colonies of all sequences were sequenced and analyzed to choose the candidate aptamers. The candidate aptamers that were labeled with FAM were diluted to a variety of different concentrations and heat treatment method for aptamers according to the above selection method, the aptamer solution which treated and MNs-PD-L1$^+$ was incubated for 1 h at

25 °C, then it was washed with a washing buffer. Further, the dissociation constant (K_d) of the candidate aptamers was calculated.

Specificity Experiment.

The specificity of aptamers was analyzed by measuring the binding ability of aptamers to different targets. The method of protein modification to magnetic beads was based on our previous work[11]. The candidate aptamers with FAM-labeled was incubated with different proteins and sEVs magnetic beads complexes. The binding fluorescence intensity values were used to compare the specific binding ability of aptamers. Further, the secondary structure was simulated by Mfold.

The Capture of sEVs.

Two kinds of sEVs, derived from Cal27 cells (Cal27 Low) and transfected Cal27 cells (Cal27 High), were separated by ultra-centrifugation, then their PD-L1 contents were characterized by western blot. The biotin-modified aptamers synthesized by Sangon Biotechnology were incubated with MNs-SA to prepare capture magnetic beads (MNs-aptamer). Five μL of MNs-aptamer were incubated with two kinds of sEVs for 30 min, separately. After magnetic separation, the complex was washed three times with PBS. Further, it was stained with membrane probe 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), and fluorescence imaging of the complexes was got under an inverted fluorescence microscope (Nikon, TE2000-U). The capture efficiency was calculated by BCA protein quantification.

Detection of sEVs.

The selected aptamers can be further used for the detection of sEVs in the chip. Two hundred μL of serum samples containing PD-L1 positive sEVs (Cal27 High) were incubated with 200 μL of MNs-aptamer in EP tubes and then separated in the chip. The complexes were washed three times with PBS at a rate of 2 μL/min, then the aptamers were passed through the capture array and incubated with the complexes for 20 min. After the array was washed three times by PBS at a rate of 2 μL/min, streptavidin-modified quantum dots (SA-QDs) was passed through to label the complexes, and the signal was detected with a fiber optic spectrometer and a microscope.

RESULTS AND DISCUSSION

S.1 Biotinylation of sEVs.

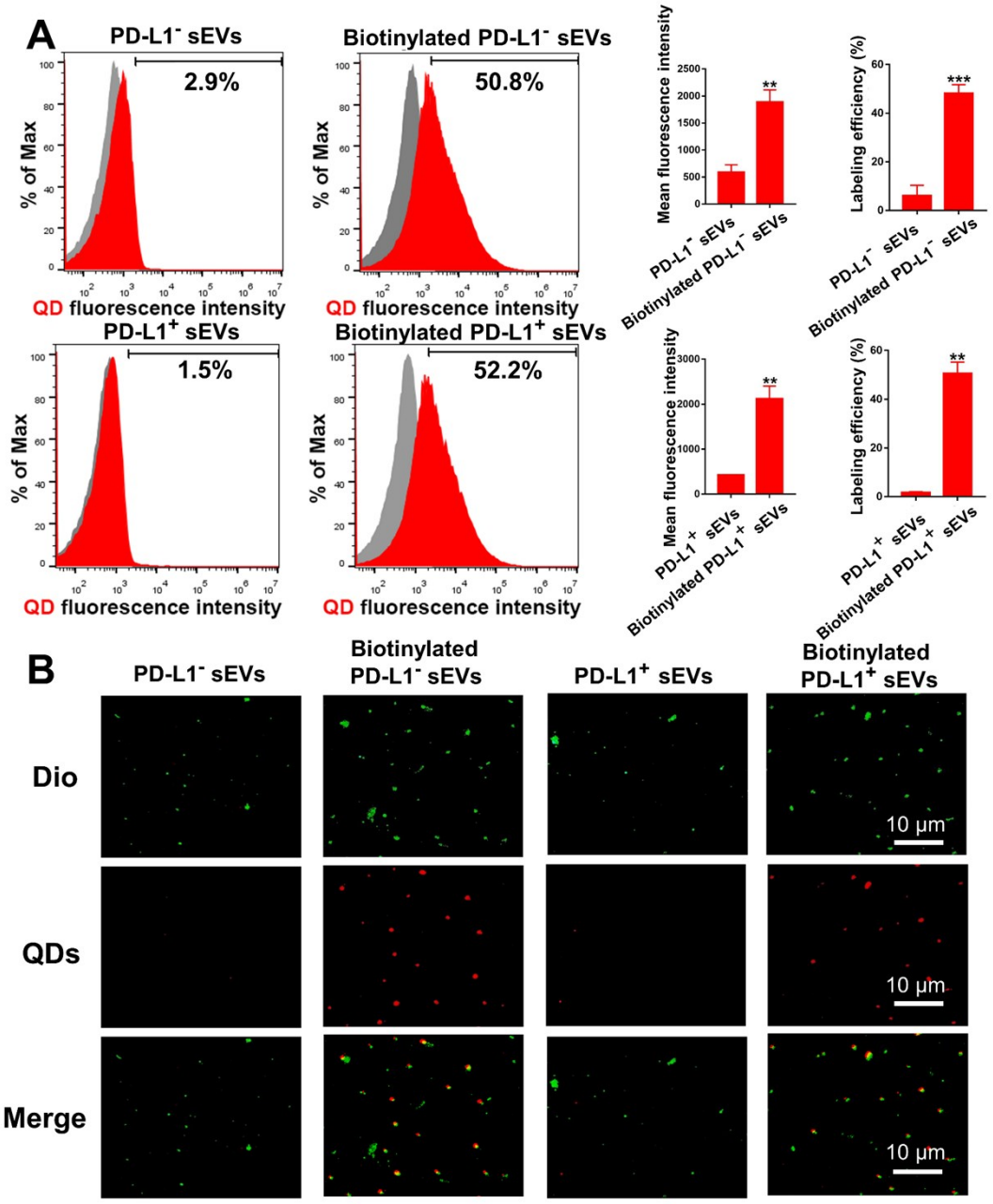

Figure S1. (A) Flow cytometry analysis of sEVs or biotinylated sEVs after labeling with SA-QDs. The errors indicate the standard deviation of three experiments. (B) Fluorescence images of sEVs or biotinylated sEVs after dual-labeling with SA-QDs and Dio.

S.2 Characterization of Magnetic Beads.

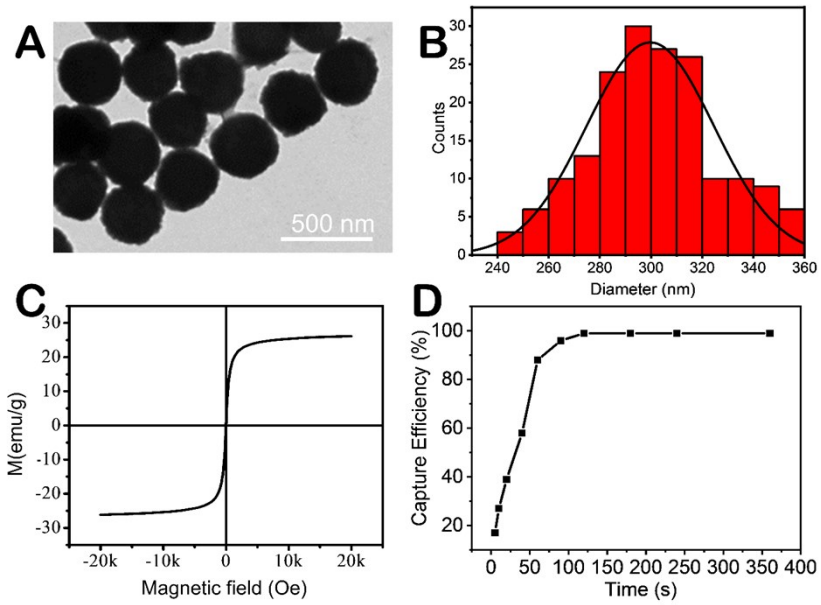

Figure S2. (A) TEM image of MNs. (B) Diameter distribution of MNs. (C) Magnetic hysteresis loop of MNs. (D) Capture efficiencies of MNs at different attraction times with a commercial magnetic scaffold.

S.3 Characterization of Streptavidin and sEVs Modification on the Magnetic Beads.

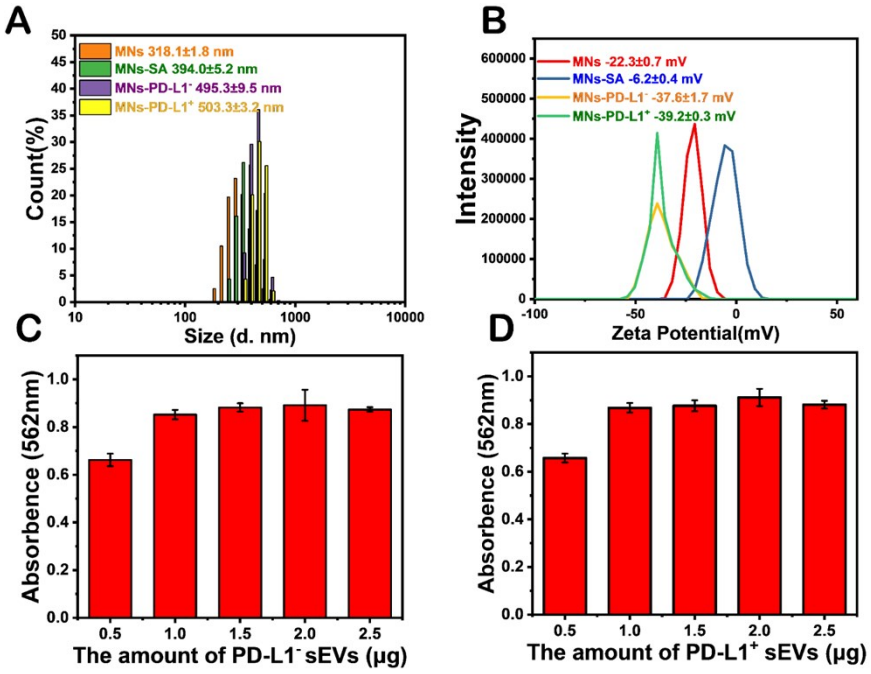

Figure S3. (A) Hydrodynamic diameter distribution of MNs, MNs-SA, MNs-PD-L1$^-$, MNs-PD-L1$^+$. (B) Zeta potential characterization of MNs, MNs-SA, MNs-PD-L1$^-$, MNs-PD-L1$^+$. (C) The amount of PD-L1$^-$ sEVs coupled with magnetic beads. (D) The amount of PD-L1$^+$ sEVs coupled with magnetic beads.

S.4 Overview of the entire selection process.

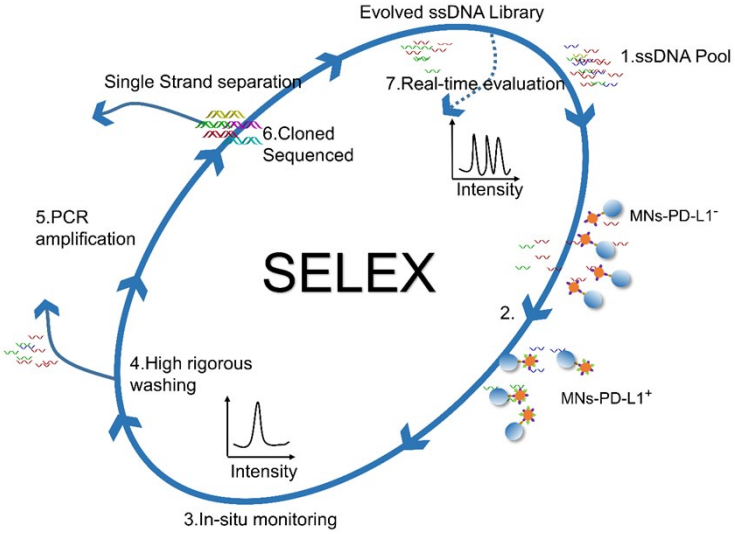

Figure S4. Overview of the entire selection process.

S.5 In-Situ Monitoring of Selection Areas.

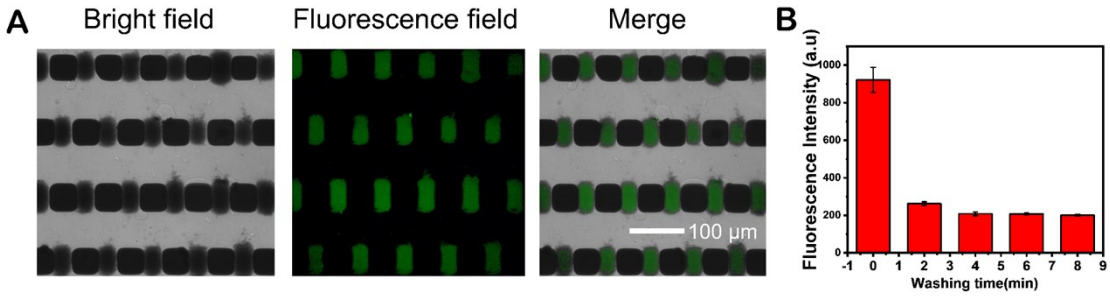

Figure S5. (A) Microscopic images of positive selection unit. (B) Histogram of fluorescence intensity with different washing times in the selection unit.

S.6 Characterization and denaturation of the PCR products.

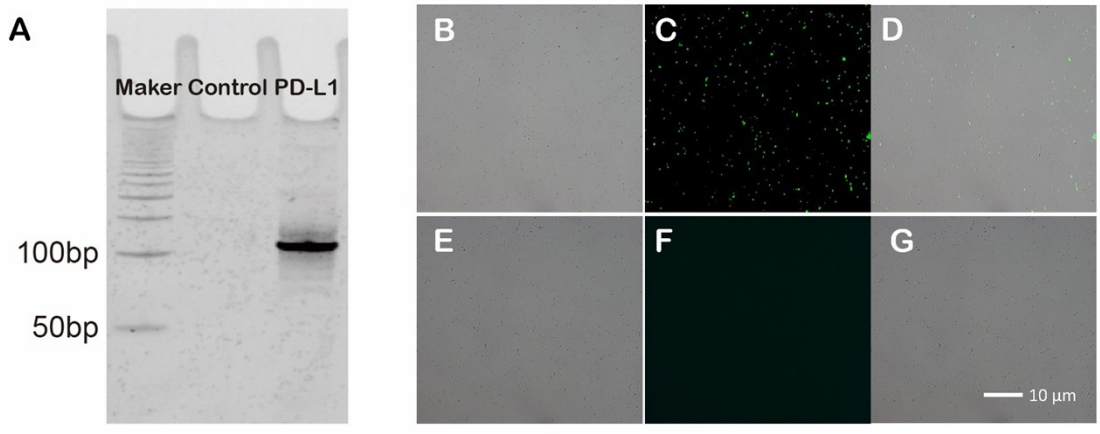

Figure S6. (A) Amplification products with denaturating PAGE analysis. Amplification products captured by MNs-SA. (B-D) Bright field, Fluorescence field, Merge. MNs-SA-biotin-DNA was treated with NaOH. (E-G) Bright field, Fluorescence field, Merge.

S.7 Real-time Assessment of Selection Progress.

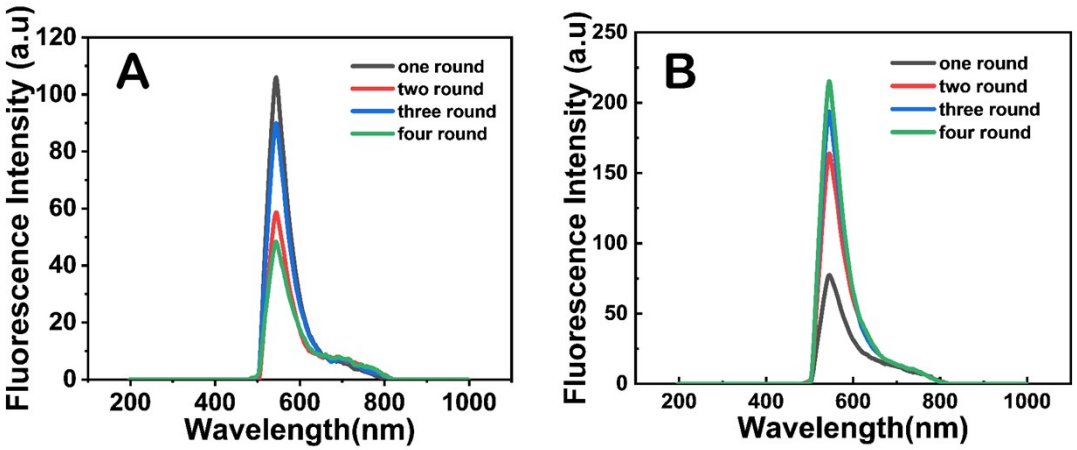

Figure S7. Fluorescence intensity of each round of negative selection toward PD-L1^{-} sEVs in area N (A) and of positive selection toward PD-L1^{+} sEVs in area P (B).

S.8 Secondary Structure Analysis.

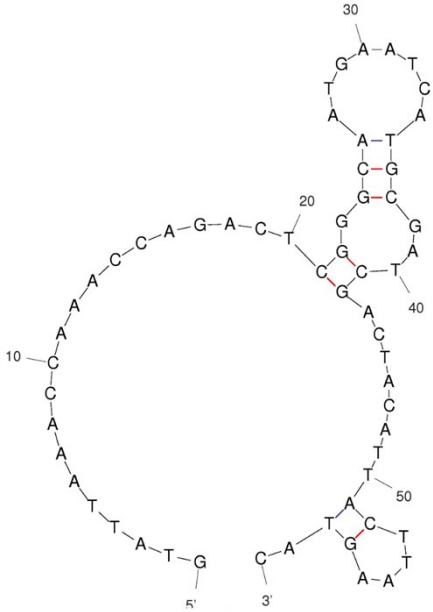

Figure S8. Secondary structure analysis of the PD-L1-11S aptamer after truncating flanked primer sites using Mfold software.

S.9 Determination of Specificity for the PD-L1-11S Aptamer.

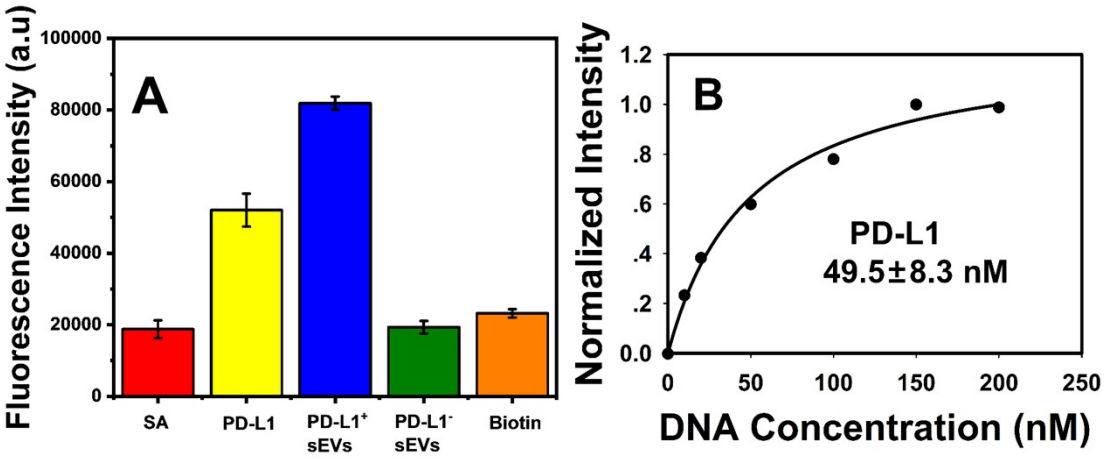

Figure S9. (A) Determination of specificity for the PD-L1-11S aptamer. (B) Determination of the dissociation constant for PD-L1 with PD-L1-11S.

S.10 The Capture of Cal27 PD-L1-High and PD-L1-Low sEVs and Western blot of the Cal27 PD-L1-High and PD-L1-Low cells and sEVs.

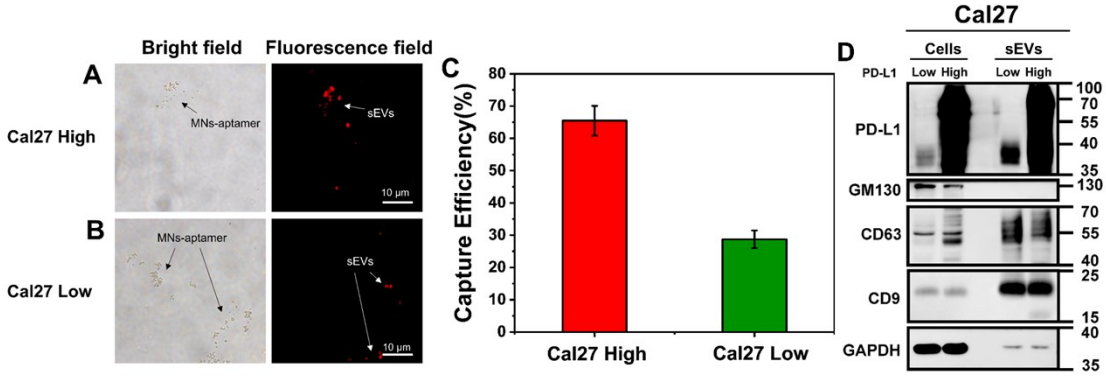

Figure S10. Inverted fluorescence microscope images after (A) Cal27 High sEVs (B) Cal27 Low sEVs captured by aptamer-coated MNs and stained with DiI. (C) Capture efficiency of the two sEVs. (D) Western blot of the Cal27 High and Low cells and sEVs.

S.11 The general gating strategy used to identify the sEVs by flow cytometer.

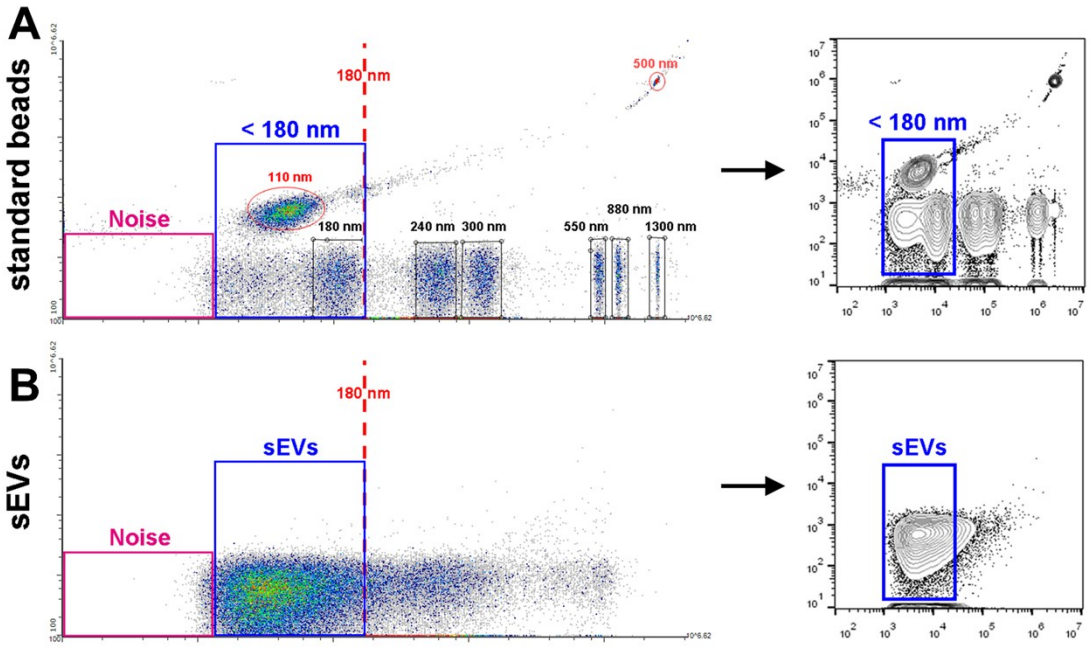

Figure S11. (A) Flow cytometry analysis of the mixture of the green fluorescent polystyrene (PS) standard beads of 110 nm, 500 nm and non-fluorescent silica (Si) beads of 180 nm, 240 nm, 300 nm, 550 nm, 880 nm, 1300 nm particle sizes. (B) Flow cytometry analysis of the sEVs.

S.12 Sequences in this work.

Table S1. Sequences in this work.

Group	Name	Sequence
Group 1	PD-L1-11	5'-**AGCAGCACAGAGGTCAGATG**GTATTAAACCAAACCAGACTCGGGCAATGA ATCATGCGATCGACTACATTACTTAAGTAC**CCTATGCGTGCTACCGTGAA**-3'
	PD-L1-32	5'-**AGCAGCACAGAGGTCAGATG**GGAATGGTACTTCAACTTCTCCAGAGGTCT CCTATTCGAGCACTGCTTTAACAAAATACC**CCTATGCGTGCTACCGTGAA**-3'
	PD-L1-19	5'-**AGCAGCACAGAGGTCAGATG**ACTGGTTCGAACCAACACTCTAAGAGTTTA TCGACGATATACTACCAAAGGTAGACCAAC**CCTATGCGTGCTACCGTGAA**-3'
Group 2	PD-L1-22	5'-**AGCAGCACAGAGGTCAGATG**GAGACTCAGTACCTATTGAGCGTACGATTC GACAATGCAGACATGCAACCTTGGAATATA**CCTATGCGTGCTACCGTGAA**-3'
	PD-L1-13	5'-**AGCAGCACAGAGGTCAGATG**TTTATGAACTCTCATATGGGGAACTCAATG CACAAGCCCGGATCAGCCCGCCTGTTTTAT**CCTATGCGTGCTACCGTGAA**-3'
Group 3	PD-L1-1	5'-**AGCAGCACAGAGGTCAGATG**TCACATCCGAACGCCCCCATGCAGCCTCA TAAAAGCCGCTTCTCATTACCAGTTCCTTTG**CCTATGCGTGCTACCGTGAA**-3'
	PD-L1-30	5'-**AGCAGCACAGAGGTCAGATG**ATAGTAGGTTGCTTACCATCAGTAGGGAG GCCACAATCCAGTAGGAGATAATTACACCAT**CCTATGCGTGCTACCGTGAA**-3'
	PD-L1-11S	5'-GTATTAAACCAAACCAGACTCGGGCAATGA ATCATGCGATCGACTACATTACTTAAGTAC-3'

S.13 Standard Addition Recovery in Complex Sample System.

Table S2. Recovery of standard addition in complex samples. RSD (%) indicates the standard deviation of three experiments.

Added（/μL）	Found（/μL）	Recovery （%）	RSD（%）
7.2×10^4	6.7×10^4	93.6	2.5
14.4×10^4	15.3×10^4	106.3	3.6
28.8×10^4	29.6×10^4	103.8	7.5