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Supplementary data

Nanopore-based enzyme-linked immunosorbent assay for exosome detection

Xinyao Liu[1,2], Yakun Yi[1,*], Lei Liu[3] and Haichen Wu[1,2,*]

[1] Beijing National Laboratory for Molecular Sciences, Key Laboratory of Analytical Chemistry for Living Biosystems, Institute of Chemistry, Chinese Academy of Sciences, Beijing, China
[2] University of Chinese Academy of Sciences, Beijing, China
[3] College of Food and Bioengineering, Xihua University, Chengdu, China

* Correspondence authors; E-mails: yiyakun@iccas.ac.cn (Y.Y.); haichenwu@iccas.ac.cn (H.W.).

1. Supplementary methods

1.1. Materials and characterization

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin–streptomycin antibiotics, and phosphate-buffered saline (PBS, 1×) were purchased from Gibco, Thermo Fisher Scientific (Waltham, MA, USA). The exosome isolation reagent (for cell culture media) was purchased from TCI (Japan). Human embryonic kidney 293 (HEK293) cells and human cervical adenocarcinoma (HeLa) cells were obtained from Meisen Cell Technology Co., Ltd. (Zhejiang, China).

1.2. Cell culture

Cells were recovered and passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HEK293 and HeLa cells were cultured in complete DMEM containing 10% FBS until the fourth passage. When cells reached approximately 40%–50% confluency, the culture medium was replaced with DMEM containing 5% FBS and incubated for 6 h, followed by incubation with DMEM containing 2% FBS for another 6 h. Subsequently, the cells were cultured in serum-free DMEM. When the cells reached approximately 90% confluency, the conditioned medium was collected for exosome isolation. All cell culture procedures were performed in a humidified incubator at 37 °C with 5% CO_2.

1.3. Exosome isolation and characterization

After the aforementioned cell culture procedure, a total of 40.0 mL of conditioned medium was collected and centrifuged at 3000 *g* for 15 min at 4 °C to remove cells and large debris. The collected supernatant was transferred into a new tube, and 20.0 mL of exosome isolation reagent was added. The mixture was gently pipetted until homogeneous and incubated overnight at 4 °C. Subsequently, the mixture was centrifuged at 10,000 *g* for 1 h at 4 °C. After centrifugation, the supernatant was discarded, and the precipitate (typically invisible) was resuspended in 2.0 mL of PBS. The obtained exosome suspension was stored at −80 °C for subsequent experiments. The concentration of isolated exosomes was determined by nanoparticle tracking analysis (NTA), which was used as the reference for establishing the calibration curve in subsequent quantitative experiments. NTA measurements and data analysis were performed by Shiyanjia Lab (www.shiyanjia.com).

2. Supplementary figures

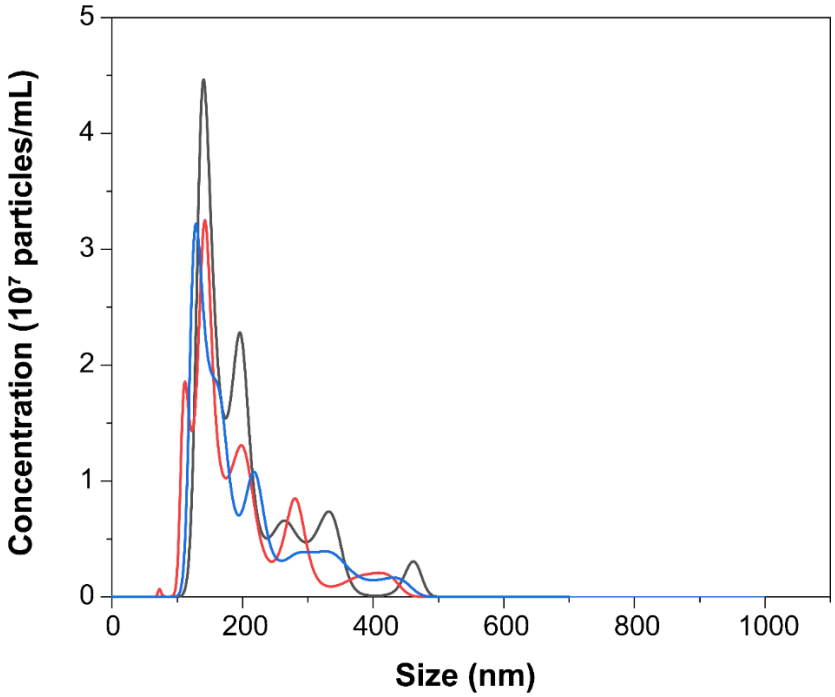

Figure S1. NTA analysis of the first batch of HeLa cell-derived exosomes. the isolated exosomes exhibited an average particle size of 140 nm with a concentration of 2.76×10^9 particles/mL, as determined by NTA. Three independent measurements were performed, and representative size distribution profiles are shown.

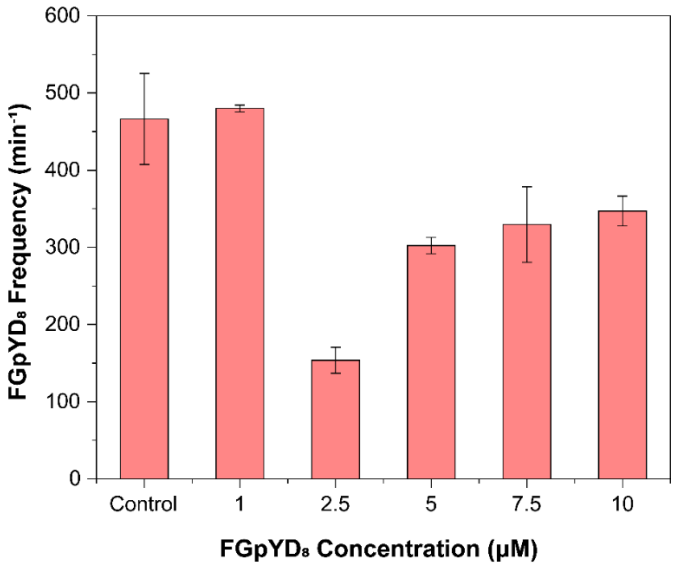

Figure S2. Effect of probe concentration on the NELISA for the detection of exosomes. to determine the optimal peptide probe concentration for the detection, the enzyme immunoassay was tested with 2.76×10^9 particles/mL of HeLa-derived exosomes at different $FGpYD_8$ concentrations. The samples were then added to the buffer in the cis compartment together with 5.0 μL CB [7] (5.0 mM), to achieve the same final peptide probe concentration (100.0 nM) in the nanopore testing system. To ensure the highest efficiency of probe cleavage, it was necessary to select the peptide probe concentration with the lowest event frequency of $FGpYD_8$ after the reaction, which was 2.5 μM. The $FGpYD_8$ concentration for the enzymatic reaction in subsequent experiments was set to 2.5 μM. All data were acquired in a buffer comprising 3.6 M KCl, 10.0 mM PBS and pH 5.0 in *trans* compartment and 1.0 M KCl, 10.0 mM PBS and pH 5.0 in *cis* compartment, with the transmembrane potential held at +200 mV. Number of individual experiments, $n = 3$.

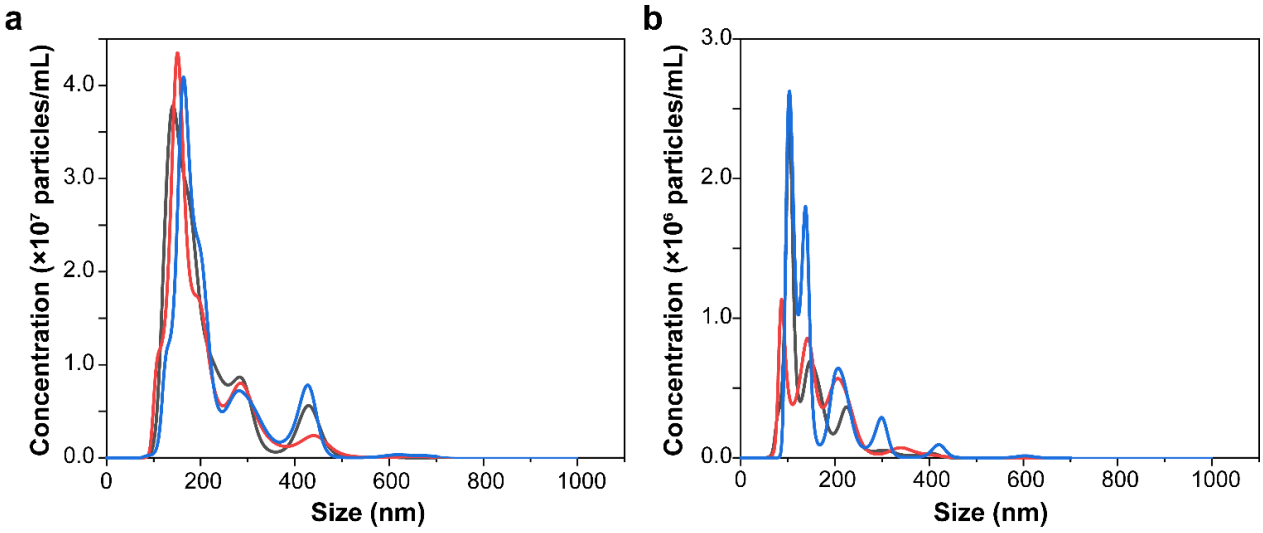

Figure S3. Nanoparticle tracking analysis (NTA) characterization of the second batch of cell-derived exosomes. **(a)** NTA analysis of HeLa cell-derived exosomes. The isolated exosomes exhibited an average particle size of 156.4 nm with a concentration of 3.72×10^9 particles/mL, as determined by NTA; **(b)** NTA analysis of HEK293 cell-derived exosomes. The isolated exosomes exhibited an average particle size of 102.7 nm with a concentration of 1.18×10^8 particles/mL. Three independent measurements were performed, and representative size distribution profiles are shown.

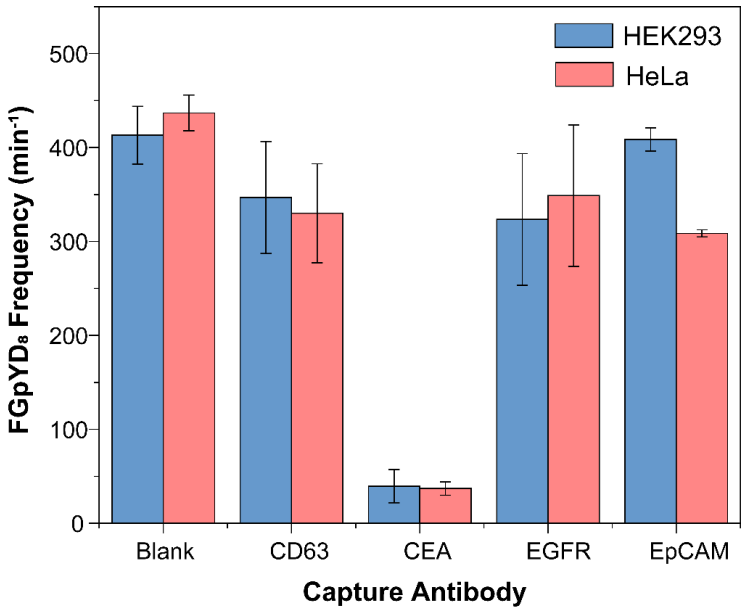

Figure S4. Screening of capture antibodies for exosome detection. HEK293- and HeLa-derived exosomes (10^8 particles/mL) were analyzed using the NELISA platform with different capture antibodies, including anti-CD63, anti-CEA, anti-EGFR, and anti-EpCAM. Alkaline phosphatase-conjugated anti-CD63 was used as the detection antibody. Blank represents the control group without capture antibody immobilization on the microplate. All data were acquired in a buffer comprising 3.6 M KCl, 10.0 mM PBS and pH 5.0 in *trans* compartment and 1.0 M KCl, 10.0 mM PBS and pH 5.0 in *cis* compartment, with the transmembrane potential held at +200 mV.

Table 1. Comparison of LOD with literature methods for the detection of exosomes.

Detection Method	Liner Range (particles/mL)	LOD (particles/mL)	Reference
Hybridization chain reaction (HCR)-based electrochemical aptamer sensor	44–8.8×10^9	1.8×10^4	[1]
Enzyme-driven DNA motor-based fluorescent biosensors	2×10^4–2×10^9	8.2×10^3	[2]
DNA tetrahedron assisted enzyme amplification electrochemical aptamer sensor	2.16×10^4–7.5×10^7	1.66×10^4	[3]
CRISPR/Cas12a-based fluorescent sensor	0–5×10^8	9.84×10^2	[4]
Dual-target magnetic immunoassay nanohybrid platform	1×10^3–1×10^7	3.7×10^4	[5]
pH-responsive colorimetric biosensor	2.0×10^6–5.0×10^8	1.2×10^6	[6]
Nanopore-based enzyme-linked immunosorbent assay	10^2–10^9	10^2	This work

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