Supporting Information

Multiplexed Profiling of Breast Cancer Exosomes Based on an Asymmetric Bipolar Electrode Electrochemiluminescence Immunochip

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Experimental Section

Materials and Reagents

The electrochemical aptamer probes were designed based on previously reported sequences and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and purified by HPLC. Their sequences were listed in Table S4. The obtained lyophilized aptamer powders were dissolved to a concentration of 100 μM according to the specified ratio, annealed at 95 °C for 5 min, and then allowed to cool naturally to 4 °C. The annealed probes were aliquoted and stored at -20 °C for later use. Laser-etched ITO-coated aluminosilicate glass slides (ITO, with a thickness ~185 nm, sheet resistance ~6 Ω/m^2) were purchased from South China Xiangcheng Technology Co., Ltd. (Shenzhen, China). Photomask films were produced by Kunshan Kaisheng Electronics Co., Ltd. Polydimethylsiloxane (PDMS, RTV 615) base (part A) and curing agent (part B) were obtained from Sylgard 184 (Dow Corning, Midland, MI, USA). Single-side polished silicon wafers were purchased from Shanghai Xiangjing Electronic Technology Co., Ltd. (Shanghai, China). Negative photoresist (SU-8 2050) and the corresponding developer were purchased from MicroChem Corp. (USA). (3-Aminopropyl) triethoxysilane (APTES), sodium cyanoborohydride ($NaCNBH_3$), and glutaraldehyde (25 % aqueous solution) were all purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Luminol-L012 was obtained from Wako Pure Chemical Industries, Ltd. (Japan). Potassium ferrocyanide trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$), potassium ferricyanide ($K_3[Fe(CN)_6]$), hydrogen peroxide (H_2O_2, 30 %), and ammonium hydroxide (NH_4OH, 25 %) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Other reagents, including acetone, anhydrous sodium sulfite (Na_2SO_3), disodium hydrogen phosphate (Na_2HPO_4), and sodium dihydrogen phosphate (NaH_2PO_4), were also obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Solarbio Life Sciences Co., Ltd. (Beijing, China). A 0.01 M phosphate-buffered saline (PBS) solution was prepared by mixing appropriate amounts of 0.01 M Na_2HPO_4 and NaH_2PO_4, and the pH was adjusted to 7.0 using a

pH meter (Mettler-Toledo SevenCompact, Switzerland). PBST was prepared by adding 0.05 % (v/v) Tween-20 to the as-prepared PBS solution. A 1 % (w/v) BSA solution in PBS was used as a blocking agent during antigen/antibody immobilization steps. Mucin 1 (MUC1) and casein were purchased from Sigma-Aldrich. A-Chymotrypsin (α-Chymase) was purchased from Macklin Biochemical Co., Ltd. Transferrin (TRF) was obtained from Solarbio Life Sciences Co., Ltd. (Beijing, China). Cell culture medium (DMEM), fetal bovine serum (FBS), and trypsin digestion solution were purchased from KeyGEN Biotech Co., Ltd. (Nanjing, China). Uranyl acetate (1 %) was purchased from Beijing Zhongjing Keyi Co., Ltd. Deionized water (resistivity 18.2 MΩ·cm) used throughout the experiments was produced by a Thermo Scientific Smart2 Pure Water System (USA). Mouse anti-human CD63 monoclonal antibody was purchased from Thermo Fisher Scientific Inc.

Instrumentation

High-sensitivity imaging was performed using an Andor iXon Ultra 888 EMCCD camera (Andor Technology, UK). Electrochemical measurements were carried out with a CHI 660E electrochemical workstation (CH Instruments, Shanghai, China). ECL analysis was conducted on an MPI-E ECL analyzer (Remax Analytical, Xi'an, China). The microscopic morphology of exosomes was characterized by a JEM-2100 transmission electron microscope (JEOL, Japan). Centrifugation during sample preparation was performed using a Thermo Scientific Multifuge X1R centrifuge and a Thermo Scientific refrigerated centrifuge. Cells were cultured in a Thermo Scientific Steri-Cycle i160 CO_2 incubator. Aseptic operations were conducted in a BHC-1300IIA2 biological safety cabinet (Beijing Airtest Scientific Equipment Co., Ltd., China).

Cell Culture and Processing

All cell lines used in this study were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Triple-negative breast cancer cells (MDA-MB-231) and low-invasive breast cancer cells (MCF-7) were cultured in complete DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Normal mammary epithelial cells (MCF-10A) were cultured in MCF-

10A-specific medium. All cells were maintained in a humidified sterile incubator at 37 °C with 5% CO_2. The culture medium was changed every other day, and subculture was performed when the cell confluency reached 80-90%.

Isolation and Characterization of Exosomes

After culturing the cells for 48 h, exosomes were isolated from the cell culture supernatant using ultracentrifugation. The specific procedure was as follows: First, the supernatant was centrifuged at 300 × g for 10 min to remove cells. Then, it was centrifuged at 2,000 × g for 10 min to pellet cell debris, followed by centrifugation at 10,000 × g for 30 min to remove large vesicles. Finally, exosomes were pelleted via ultracentrifugation at 100,000 × g for 70 min. The resulting pellet was resuspended in PBS and subjected to a second ultracentrifugation step at 100,000 × g for 70 min to obtain purified exosomes. The purified exosome pellet was resuspended in PBS and stored at -80 °C.

The morphology of the isolated exosomes was characterized by transmission electron microscopy (TEM). A 10 μL aliquot of the purified exosome suspension was applied onto a copper grid and allowed to adsorb for 10 min, after which excess liquid was removed using filter paper. The grid was then rinsed with 10 μL of ultrapure water for 10 min, stained with 10 μL of 1% uranyl acetate solution for 5 min, and blotted dry with filter paper. After air-drying, images were acquired using a TEM operated at 80 kV. The concentration and size distribution of exosomes were determined by nanoparticle tracking analysis (NTA). The sample was appropriately diluted with PBS to achieve an optimal concentration for measurement. Then, 3–5 mL of the diluted sample was injected into the detection chamber, and the exosome concentration as well as the mean particle size were analyzed using the accompanying software (ZetaView 8.03.04.01). Transmission electron microscopy (TEM) imaging (Figure S1) revealed that the isolated vesicles exhibited the characteristic cup-shaped morphology typical of exosomes. Nanoparticle tracking analysis (NTA) indicated that the size distribution of these vesicles peaked at approximately 110 nm, with the vast majority of particles ranging between 50 and 150 nm in diameter. These morphological and size-distribution characteristics are in strong agreement with the

defining criteria for exosomes outlined in the guidelines of the International Society for Extracellular Vesicles (ISEV).

Serum collection and processing

Clinical blood samples of healthy donors and patients were collected with informed consent according to the Institutional Review Board (IRB) protocol at Taizhou People 's Hospital. A total of 20 patients and 10 healthy donors were included in this study. C linical information of all samples was listed in Table S3. All blood samples were centr ifuged at 3000 rpm for 15 min to obtain serum samples. The serum samples were stor ed at -80 °C for later processing.

Exosome Morphology and NTA Characterization

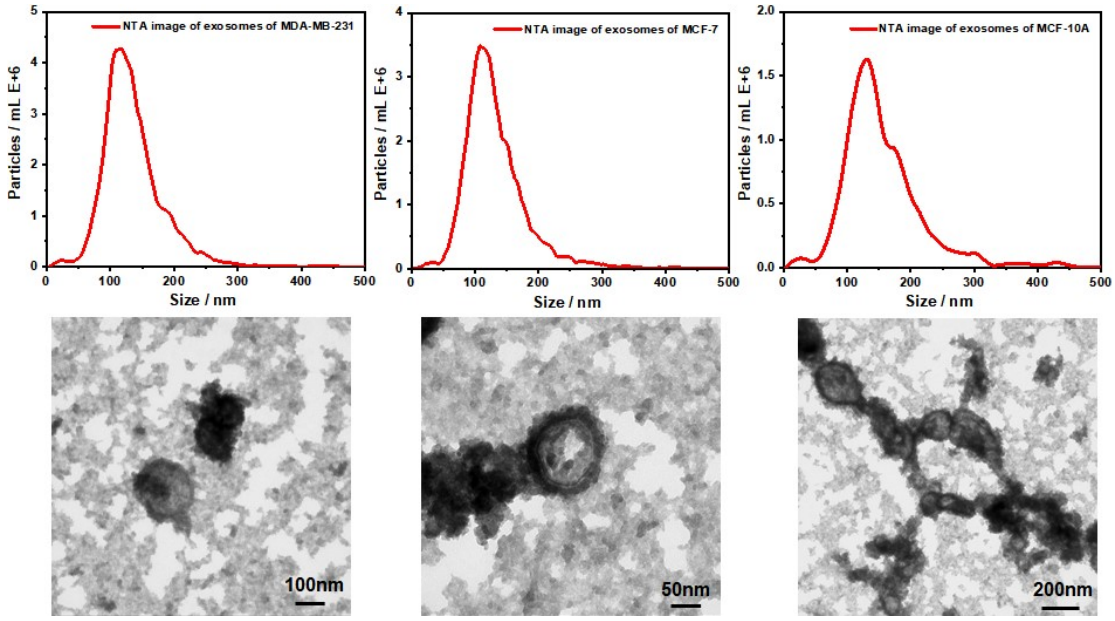

Figure S1. TEM images and NTA size distribution profile of isolated exosomes. Scale bars are 100 nm, 50 nm, and 200 nm, respectively.

Table S1 Intra- and inter-assay precision of the BPE-ECL immunosensing platform at different exosome concentration levels. (n=5)

Level	Conc (particles/μL)	Intra-assay RSD	Inter-assay RSD
Low (near LOD)	14	4.66%	7.09%
High	140	2.21%	2.44%

Table S2 Comparison of different sensing methods for exosome detection.

Method	Linear range (particles/μL)	LOD (particles/μL)	Time (h)	Ref
Colorimetry	10^3 to 10^5	394	1.0	[1]
Colorimetry	1.4×10^3 to 2.8×10^5	160	6.0	[2]
Electrochemistry	4.0×10^3 to 8.0×10^7	920	2.5	[3]
Electrochemistry	10^4 to 10^7	9961	3.0	[4]
Electrochemistry	0.5 to 5.0×10^3	482	4.0	[5]

Electrochemistry	10^2 to 10^7	43	4.5	[6]
Electrochemistry	1.1×10^2 to 1.1×10^8	96	6.5	[7]
Fluorescence	1.5×10^3 to 5.0×10^6	880	1.2	[8]
Fluorescence	8.5×10^3 to 8.5×10^5	4500	4.0	[9]
Fluorescence	1.7×10^4 to 1.7×10^{10}	180	10	[10]
Fluorescence	1.66×10^3 to 1.66×10^6	480	4.0	[11]
Digital ECL	10^2–10^5	10	1.5	[12]
CRISPR-enhanced assay	10–10^5	0.98	3.0	[13]
ECL	1.0×10^4 to 3.16×10^6	9080	1.4	[14]
ECL	5.0×10^2 to 1.0×10^5	195	4.0	[15]
ECL	5.0×10^2 to 1.0×10^6	125	4.0	[16]
ECL	10^2 to 10^5	30	6.0	[17]
ECL	10^2 to 10^4	11	0.6	[18]
ECL	14 to 1.4×10^4	1.39	2	This work

Table S3 Detailed Information of Clinical Serum Samples.

Sample ID	Status	Gender	Age	Subtype	Pathology
1	BC	Female	50	TNBC	Malignant
2	BC	Female	54	TNBC	Malignant
3	BC	Female	42	TNBC	Malignant
4	BC	Female	74	TNBC	Malignant
5	BC	Female	48	TNBC	Malignant
6	BC	Female	54	TNBC	Malignant
7	BC	Female	52	TNBC	Malignant

8	BC	Female	55	TNBC	Malignant
9	BC	Female	63	TNBC	Malignant
10	BC	Female	54	HR+/HER2-	Malignant
11	BC	Female	41	HR+/HER2-	Malignant
12	BC	Female	49	HR+/HER2-	Malignant
13	BC	Female	51	HR+/HER2-	Malignant
14	BC	Female	59	HR+/HER2-	Malignant
15	BC	Female	57	HR+/HER2-	Malignant
16	BC	Female	58	HR+/HER2-	Malignant
17	BC	Female	60	HR+/HER2-	Malignant
18	BC	Female	39	HR+/HER2-	Malignant
19	BC	Female	63	HR+/HER2-	Malignant
20	BC	Female	58	HR+/HER2-	Malignant
21	HD	Female	54	No history of cancer	Control
22	HD	Female	48	No history of cancer	Control
23	HD	Female	46	No history of cancer	Control
24	HD	Female	36	No history of cancer	Control
25	HD	Female	31	No history of cancer	Control
26	HD	Female	23	No history of cancer	Control
27	HD	Female	22	No history of cancer	Control
28	HD	Female	29	No history of cancer	Control
29	HD	Female	22	No history of cancer	Control
30	HD	Female	28	No history of cancer	Control

HD: healthy donor

Table S4 DNA sequences used in this work

DNA	Sequences and modification (5'-3')	Ref
Fc-Apt_{EpCAM}	Fc-CACTACAGAGGTTGCGTCTGT	[1]
Fc-Apt_{CD61}	Fc-TCCACGATGTAGGATCCACATGAGCGCTCCTGTTACCCTG	[19]
Fc-Apt_{CD44}	Fc-GAGATTCATCACGCGCATAGTCCCAAGGCCTGCAAGGGAACCAAG GACACAGCGACTATGCGATGATGTCTTC	[20]

Fc-Apt_{CD63}	Fc- CACCCCACCTCGCTCCCGTGACACTAATGCTA	[1]

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