Electronic Supplementary Material (ESI) for Nanoscale Horizons.

Electronic Supplementary Information (ESI)

for

Methylation dependent gold adsorption behaviour of extracellular vesicular DNA enables liquid biopsy test for cancer

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Methods

1. Cell culture and isolation of Extracellular Vesicles (EV):

BT-474 Breast cancer cell line was maintained in microvesicles depleted serum free Media 171 (Gibco, UK) supplemented with Mammary Epithelial supplement (Gibco, UK), 1% Penicillin/streptomycin and grown in 5% CO_2 at 37ºC. The conditioned medium from 10^6 cells were collected after 60 h and centrifuged at 2000×g for 30 min to eliminate cell contamination (*e.g.*, cells and debris). EV were isolated using Total Exosome isolation reagent (Life Technologies) as per the manufacturer's instructions. Briefly, the supernatant was transferred to a new tube and the isolation reagent was added to the tube in the ratio 2:1. The samples were incubated overnight at 4ºC followed by filtration using 0.22 µm filter and centrifugation at 10000 × g for 1h to obtain exosome pellets. Exosome pellets were then resuspended in 100 µL PBS (10 mM, pH 7.0) and stored at -20ºC for further use.

2. Cryo-transmission electron microscopy (cryo-TEM) and Nanosight analysis:

For cryo-TEM, 4 µL of EV preparations were directly adsorbed onto lacey carbon grids (Quantifoil, Germany) and plunged into liquid ethane, using an FEI Vitrobot Mark 3 (FEI Company, The Netherlands). Grids were blotted at 100% humidity at 4 ºC for about 3-4 s. Frozen/vitrified samples were imaged using Tecnai T12 Transmission Electron Microscope (FEI Company) operating at an acceleration voltage of 120 kV. Images were taken at 30,000x magnification, (approximate dose of 13.6 electrons/Å2), using an FEI Eagle 4k CCD (FEI Company), and Serial EM image acquisition software. For AFM analysis, 10 uL of evDNA was pipetted on an ultra-flat gold surface and incubated for 10 min at room temperature. After incubation, the gold surface with adsorbed DNA was washed three times with purified water to remove all the unbound DNA. Subsequently, 50 uL of 5X SSC buffer was added to the gold surface to perform in-solution AFM analysis. AFM was performed on a Cypher S AFM microscope (Oxford Instruments Asylum Research, U.S.A.) with a scan speed of 0.8 Hz (256 points per line) using a Mult75-G cantilever (BudgetSensors, Bulgaria) with a resonance frequency of 75 kHz and a force constant of 3 N/m. The surface was scanned in tapping mode. The AFM image processing was performed by using WSXM software.[1] Nanosight analysis was performed by using NanoSight® LM10 (Nanosight Ltd., Minton Park, Amesbury, UK).

3. EVs isolation (from serum):

Invitrogen Total Exosome Isolation Kit was used to extract EVs from serum samples according to the manufacturer's instructions. Firstly, after thawing, the serum samples were centrifuged at 2000 rcf for 30 minutes to remove debris and cells. Then the supernatant containing EV were transferred is to a new 1.5 mL eppendorf and 0.2 volumes of the Total Exosome Isolation reagent was added, mixed with vortex and then incubated in ice for 30 minutes. After incubation, the samples were centrifuged at 10000 rcf for 10 minutes, and the EVs pallets were obtained. Suparnatant solutions were removed and the isolated EV were resuspended in 1X PBS and stored at -20°C for further use.

4. External DNA digestion:

Optimized digestion reaction protocol of cfDNA by DNase I (RNase-free) is: 80 µ L of EV (1x1000 particles/mL), 110 µ L of 1X DNase I reaction buffer, and 10 µL of DNase I enzyme. Add 110 µL of 1X DNase I reaction buffer (10 mM Tris-HCl, 2.5 mM MgCl2, 0.5 mM CaCl2, pH 7.6 @ 25°C) to 80 µL of diluted liposomes/EV (1x1000 particle/mL). Add 10 µL DNase I enzyme (Biolab.Inc.M0303S) and incubate reaction at 37°C for 20 minutes. Add 10 µL of 0.5 M EDTA, and heat at 75°C for 10 minutes.

5. DNA extraction (from EVs):

After digesting external cfDNA, EV-DNA was extracted by the DNeasy Blood & Tissue Kit (Cat. No. 69506, Qiagen, Germany) according to the instructions. Firstly, transfer EV to 2 mL Eppendorf and add 1X PBS until 200 µ L. Add 20 µ L of proteinase K and 200 µ L of buffer AL Blood and tissue DNeasy Kit), vortex and incubate at 56°C for 10 minutes. Add an equal volume (same as sample) of phenol: chloroform (pH 8.0, AMRESCO. 0833.) and vortex thoroughly until the two phases are mixed. Add a small amount of silicone grease (ACC Silicones Ltd, SGM494.), and centrifuge the sample at 5000 rcf for 10 minutes. When the two phases are separated, carefully transfer the aqueous (top) phase without disturbing the bottom layer (protein), which will be discarded. Take a known volume of DNA to be precipitated. Add 0.1 volume of 3 M sodium acetate (pH5.2), 1 volume of 100% isopropanol and 2 µ L of glycogen. Centrifuge at 13000 rcf for 15 min at 4°C. Discard the majority of isopropanol. Add 1 mL of cold 70% ethanol and gently invert, then put the tube on ice.

When DNA pallet is visible, remove the ethanol by pipetting and heating carefully without disturbing pellet. Add a suitable volume of ultrapure water (Depend on the size of the pellet) to dissolve DNA pellet fully.

6. DNA Concentration Quantification:

DNA concentrations are all quantified by Qubit high sensitive double strand DNA (HS dsDNA) Kit with sensitivity can be achieve to100 pg/µL of ds DNA. Each reaction contains 2 µL of sample and 198 µL of HS dsDNA dye and HS dsDNA reaction buffer mixture in proportion 1:200.

7. Electrochemistry:

All electrochemical experiments were carried out using a CH1040C potentiostat (CH Instruments) with a three electrode system consisting of a gold working electrode (2 mm in diameter), Pt counter electrode, and Ag/AgCl reference electrode (all electrodes are from CH Instruments, USA). Differential pulse voltammetric (DPV) experiments were conducted in 10 mM PBS solution containing 2.5mM $[K_3Fe(CN)_6]$ and 2.5mM $[K_4Fe(CN)_6]$ electrolyte solution. DPV signals were obtained with a potential step of 5 mV, pulse amplitude of 50 mV, pulse width of 50 ms, and pulse period of 100 ms. For DNA methylation detection, the gold electrodes were initially cleaned by polishing with Alumina polishing powder (CH Instruments) followed by ultrasonication with deionised water for 1 minutes and then dried under the flow of nitrogen. DPV signals of clean electrodes were measured in the electrolyte solution to get the baseline current. The tested samples (5 µL, 10 ng/µL diluted by SSC 5X buffer) were then incubated on the electrode for 10 min with 350 rpm shaking speed. The electrodes were then washed with 1 mL of 1XPBS. The relative DPV currents (i.e., %*i*r, percent difference of the DPV signals generated for DNA sample (*i*sample) with respect to the baseline current (*i*baseline)) due to the adsorption of DNA samples were then measured by using equation 1.

$$\text{Relative DPV signals } (\%i_r) = [(i_{baseline} - i_{sample})/i_{baseline}] \times 100 \quad \ldots\ldots (1)$$

8. Polymerase chain reaction (PCR):

The PCR was performed to confirm whether EV samples still contain eternal DNA. PCR was

performed in 20 µL reaction solution, containing 0.4 µL of exosome samples, 1 µM of each primer (forward primer: 5'-ACCTGTGTTCATTCACATGAGG-3', reverse primer: 5'-AGAGCTTGTTCTGGCCGTTT-3'), 9.34 µL of water and 4 µL of 5X Green go taq® Flexi buffer (Promega, Madison, USA), 1 µL of CES 5X, 4µL of 25 mM $MgCl_2$, 0.2 µL of 10 mM dNTP and 0.06 µL of 5U/µL Hot Start Taq. Amplification was carried out under following conditions: 95 ℃ for 7 min, 35 cycles of 94 ℃ for 20 s, 57 ℃ for 30 s, 72 ℃ for 30s, 72 ℃ for 7 min, and 10 ℃ for 10 min. Then, the amplified samples were analyzed through electrophoresis, using 2 % agarose gel (Sigma Ltd.) and running in 1X TAE buffer under 200 V for 20 min.

9. Global DNA Methylation Analysis:

Global methylation analysis of BT474 DNA was performed by using Imprint® Methylated DNA Quantification kit from Sigma Aldrich as per manufacturer instructions. Briefly, desired amount of DNA was diluted in 30 µl DNA Binding Solution and added to each well of the plate. The DNA Binding Solution alone was used as a blank. The wells were covered and the samples were incubated at 37 °C for 60 minutes. After incubation, 150 µL of Block Solution was directly added to each well and incubated again for 30 minutes. All the solution from each well was then removed and the wells were washed three times with 150 µL of 1x Wash Buffer. Methylation specific capture antibody was then diluted in 50 µL wash buffer, added to each well and incubated for 60 minutes. After that the capture antibody solution was removed from each well and the wells were washed four times with 150 µL wash buffer. Subsequently, the diluted Detection Antibody was added to each well and incubated, removed and washed. After that 100 µL of Developing Solution was added to each well and the wells were incubated at room temperature away from light for 1-10 minutes. When the solution turned blue, 50 µL of stop solution was added to each well and the solutions were turned yellow. The absorbance of the solutions in each well was then measured at 450 nm by using a plate reader. The global methylation level of all DNAs is calculated using the following equation.

Global Methylation level =

$[(A_{450}$ Sample $- A_{450}$ Blank$)/ (A_{450}$ Methylated Control DNA $- A_{450}$ Blank$)] \times 100$…….(1)

10. Fabrication of Microdevice:

The devices were designed using L-Edit Layout Editor V15 from Tanner Research (USA) and written to a 5-inch soda lime chrome mask (Shenzhen Qingyi Precision Mask Making, Singapore). The devices were then fabricated using 2-step standard photolithographic process. In the first step, negative photoresist AZnLOF 2020 (Microchemicals GmbH) was spincoated on 4-inch borofloat glass wafers (Bonda Technology Pte Ltd., Singapore) for 30 s at 2000 rpm prior a softbake for 2 min at 110 ˚C and UV exposure at a constant dose of 340 mJ/cm with using an EVG 620 mask aligner (Austria). After a post-exposure bake of 1 min at 110 ˚C, the wafers were developed for 40 s in AZ726 MIF Developer (Microchemicals GmbH, Germany), washed, dried, and oxygen plasma cleaned using a PlasmaPro 80 (Oxford Instruments, United Kingdom) to remove photoresist residues. Next, 10 nm Ti and 200 nm Au were deposited on the wafers using a Temescal FC-2000 electron beam evaporator (Ferrotec, USA). The gold structures of the microdevice were then revealed by overnight lift-off in Remover PG (Microchemicals GmbH). In the second step, the whole device except the sensing structures were was insulated with negative photoresist similar to the procedure described above. The device fabrication was completed by final cleaning using oxygen plasma. The inner circular electrodes were prepared with a diameter of 250 µm, a gap of 1000 µm, and outer ring width of 30 µm.

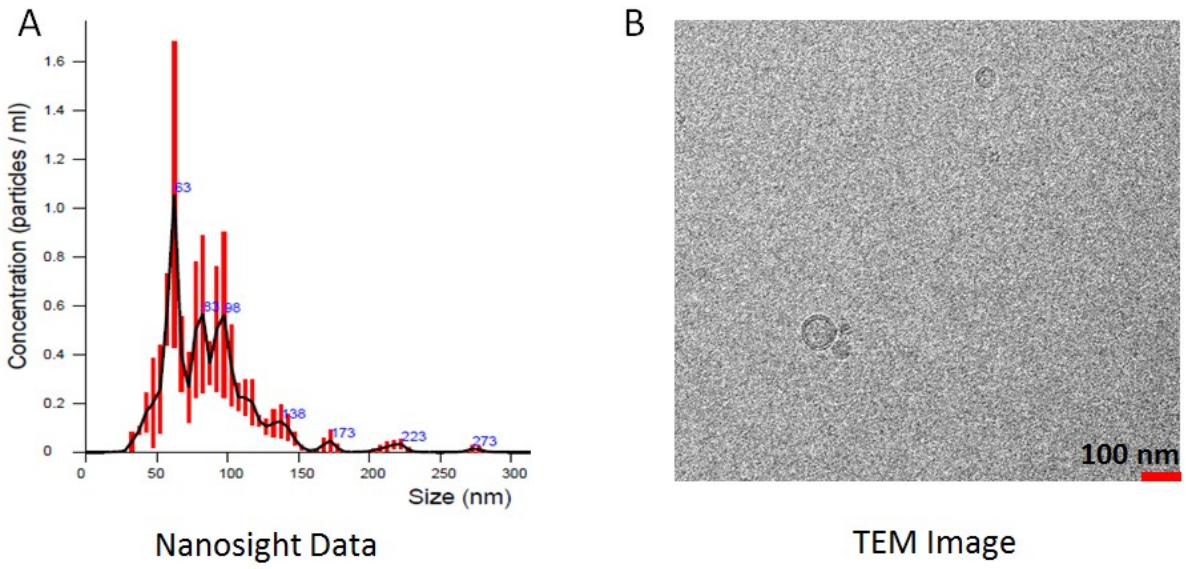

Figure S1. Characterization of isolated EV from BT474 cell culture media. A) Nanosight data

showing the size of EV ranging from approximately 30nm-100nm. B) TEM image showing the EV with lipid bilayer.

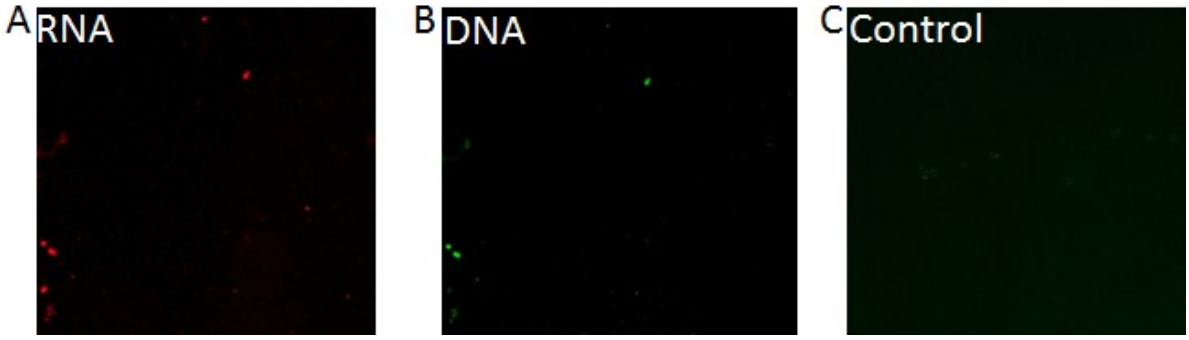

Figure S2. Fluorescence image of EV showing the presence of A) RNA and B) DNA in EV along with the C) control experiment with the dye without any EV.

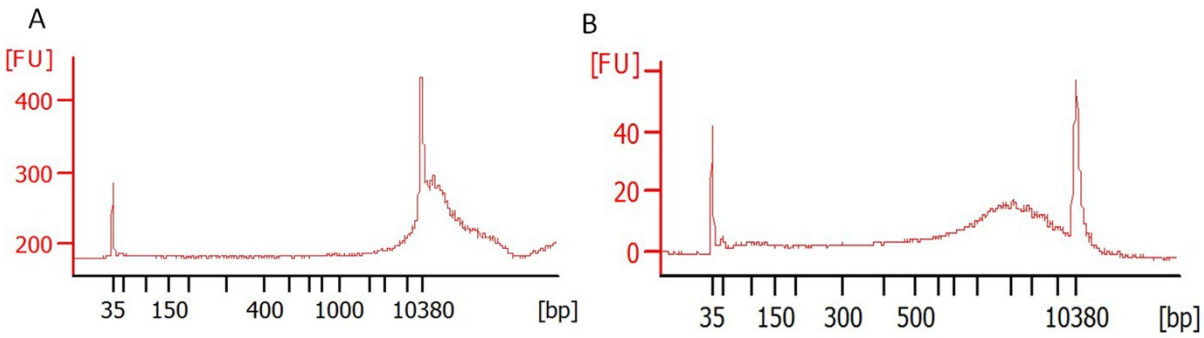

Figure S3. Agilent Bio-analyzer data showing the size of BT474 breast cancer cell derived A) gDNA and B) evDNA

Table S1. Global Methylation Analysis of cell derived evDNAs and gDNAs

Cell Line	DNA Type	Cancer Type	Global Methylation (%)
BT474	evDNA	Breast	26.75
MCF7	evDNA	Breast	43.49
SKMEL28	evDNA	Melanoma	37.84
BT474	gDNA	Breast	43.15
MCF7	gDNA	Breast	33.56
SKMEL28	gDNA	Melanoma	45.23

Table S2. Clinical Information of Cancer Patients. *N/A= Not Applicable, NA = Not available

Sample Number	Gender	Age (Yrs)	Cancer Type	Cancer Stage	Mean Relative Adsorption (%i_r)
N1	Female	43	N/A	N/A	11.65
N2	Female	41	N/A	N/A	17.76
N3	Female	25	N/A	N/A	15.98
N4	Female	25	N/A	N/A	22.78
N5	Female	27	N/A	N/A	19.64
N6	Female	21	N/A	N/A	17.02
N7	Female	56	N/A	N/A	15.29
P1	Female	66	Breast	Metastatic	23.52
P2	Female	58	Breast	Metastatic	39.72
P3	Female	64	Breast	Metastatic	36.19
P4	Female	59	Breast	Metastatic	33.08
P5	Female	76	Breast	Metastatic	38.58
P6	NA	NA	Melanoma	Metastatic	25.80
P7	NA	NA	Melanoma	Metastatic	31.43
P8	NA	NA	Melanoma	Metastatic	25.54
P9	NA	NA	Melanoma	Metastatic	30.60

References

1. I. Horcas, R. Fernández, J. Gomez-Rodriguez, J. Colchero, J. Gómez-Herrero and A. Baro, *Review of scientific instruments*, 2007, **78**, 013705.