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Electronic Supplementary Information

Ultrasensitive Profiling of Multiple Biomarkers from Single Cells by Signal Amplification Mass Spectrometry

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Experimental Section

Chemicals and Materials. Recombinant human epithelial cell adhesion molecule (EpCAM, >90%), recombinant human keratin 19 (CK19, >90%), mouse anti-EpCAM monoclonal antibody (mAb-EpCAM, >95%), mouse anti-CK19 monoclonal antibody (mAb-CK19, >95%), rabbit anti-EpCAM polyclonal antibody (pAb-EpCAM, >95%), rabbit anti-CK19 polyclonal antibody (pAb-CK19, >95%) and rabbit anti-MUC-1 polyclonal antibody (pAb-MUC-1, >95%) were purchased from Biosynthesis Biotechnology (Beijing, China). MCF-7 cell, DU145 cell and MG63 cell were obtained from Shanghai Institute of Biochemistry and Cell Biology. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Gibco. $SH(CH_2)_{11}(OCH_2CH_2)_6OCH_2COOH$ (antibody linker) was bought from SensoPath technologies (MT, USA). Three kinds of MLs, $[S(CH_2)_{11}(OCH_2CH_2)_nOH]_2$, n=4 for ML1 ($[M+Na]^+$ m/z=781), n=6 for ML2 ($[M+Na]^+$ m/z=957), n=3 for ML3 ($[M+Na]^+$ m/z=693), Human α-thrombin (Thrombin, ≥2000 NIH units/mg protein), bovine serum albumin (BSA, ≥98%), N-hydroxysuccinimide (NHS, 98%), 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochioride (EDC, >98%) and 2-(N-morpholino) ethanesulfonic acid (MES) were purchased from Sigma-Aldrich. 6-Mercapto-1-hexanol (MCH, >98%) was bought from TCICo. Ltd. (Shanghai, China). $HAuCl_4 \cdot 4H_2O$, NaH_2PO_4 and Na_2HPO_4 were bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ultrapure water (18.2MΩ/cm) used in all experiments was obtained from a Milli-Q system (Millipore, Bedford, MA). All solvents were used as received without further purification.

Apparatus. All MS data were acquired using a Bruker Microflex LRF MALDI-TOF MS (Bremen, Germany). Polarizing microscope images were obtained on a Leica DM2500 (Germany). Transmission electron microscopy (TEM) image was recorded by a FEI Tecnai G2 F20 STWIN electron microscopy (USA). Scanning electron microscopy (SEM) images were recorded by Zeiss Ultra 55 FE-SEM (Germany).

Preparation of Antibody-Coated Au Chip. Au plates in the size of 0.4 cm × 0.4 cm × 0.5 mm were cleaned with fresh piranha solution (30%H_2O_2/H_2SO_4 (v/v) = 1/3). Two μL of antibody linker (379.5 pmol) was deposited on a cleaned Au plate, and incubated overnight at room temperature (RT) in a humid environment. Then, the plate was washed three times with water and blocked by 1 mM MCH with 1 h of incubation. Subsequently, 2 μL of freshly prepared EDC (15 mg/mL) and NHS (15 mg/mL) in MES buffer (15 mM, pH 5) was added on the antibody linker modified spot. After incubation for 30 min at RT, a mixture of antibodies (2 μL of mAb-EpCAM and 2 μL of mAb-CK19, 1 mg/mL each) were dropped onto the spot and incubated for 3 h at 4 °C. Afterwards, the antibodies coated plate was blocked with 1% BSA in phosphate buffer (PB, 10 mM, pH 7.4) for 30 min to reduce nonspecific adsorption. The prepared antibody-coated Au chips were stored at 4 °C.

Preparation of ML-labeled AuNPs probe. Firstly, AuNPs with an average diameter of 13 nm were synthesized by reducing $HAuCl_4$ with sodium citrate. Then, 500 μL of the obtained AuNPs were incubated with diverse MLs (ML1, 2 or 3, 32 nmol each) and antibody linker (6 nmol) overnight at RT. Before being activated with 50 μL of freshly prepared EDC (15 mg/mL) and NHS (15 mg/mL) in MES buffer, the ML-labeled AuNPs were rinsed three times with water by successive centrifugation at 9400 *g* for 30 min. Then, the activated AuNPs were incubated with different antibodies (pAb-EpCAM, pAb-CK19 or pAb-MUC-1, 30 μL, 1 mg/mL each) for 3 h at 4 °C, respectively. Subsequently, the functionalized AuNPs (ML1/EpCAM, ML2/CK19 and ML3/MUC-1) were washed three times with 1% BSA in PB (10 mM, pH 7.4) in order to prevent nonspecific adsorption. The ML-labeled AuNPs probes were stored at 4 °C in 500 μL PB (10 mM, pH 7.4).

Multiplexed detection of protein biomarkers with LDI-MS. Protein biomarkers (2 μL EpCAM and 2 μL CK19) at different concentrations were deposited on the antibodies coated Au chips to form sample spots, and incubated for 45 min at 37 °C in a humid environment. After washing three times with PB (10 mM, pH 7.4), a mixture of ML-labeled AuNPs probes (2 μL of AuNPs@ML1/EpCAM and 2 μL

AuNPs@ML2/CK19) were added onto the same spots and incubated for another 45 min at 37 °C in humidity. Subsequently, the formed sandwich immunoassay chips were cleaned five times with water, followed by attaching to an MALDI plate for MS analysis.

Simultaneous detection of multiple biomarkers on cells with LDI-MS. Cells were introduced to the sandwich structure in order to detect multiple biomarkers on a single cell simultaneously. The procedure was similar to the protocol above for protein biomarker detection. 2 μL of MCF-7 cells or DU145 cells at various concentrations were added onto the antibodies coated Au chips and kept for 45 min at 37 °C in a humid atmosphere, followed with the addition of the ML-labeled AuNPs probes for MS detection.

Cell culture. MCF-7, DU145 and MG63 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were incubated in a 37 °C incubator with 5% CO_2. Cell solutions were obtained as follows: cells were detached by trypsin digestion, followed by collection *via* centrifugation and washing with PBS buffer (10 mM, PH 7.4) for three times.

LDI-TOF MS Analysis. The attached Au Chips were analyzed in the reflectron positive ion mode with 500 shots accumulation by a partial sample random walk function. The instrument was equipped with an accelerating voltage of 20 kV, a repetition rate of 60 Hz, the delayed extraction time of 120 ns and the laser energy was set as 50%. The instrumental parameters were kept consistent during all the analysis. MS data in each graph were normalized to the peak with the highest intensity.

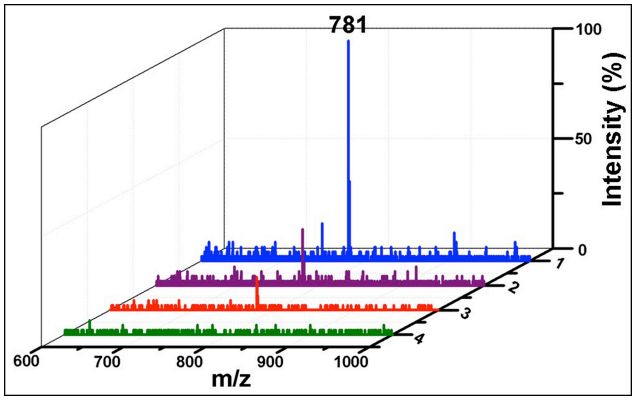

Fig. S1 EpCAM at different amounts detected by ML1/pAb-EpCAM labeled AuNPs with LDI-MS (Line 1-4: 2 amole, 200 zmole, 2 zmole, blank). MS data in the graph were normalized to the peak at m/z = 781 with the highest intensity.

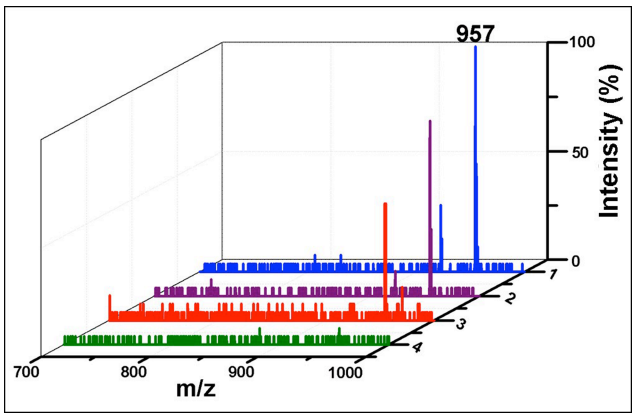

Fig. S2 CK19 at different amounts detected by ML2/pAb-CK19 labeled AuNPs with LDI-MS (Line 1-4: 2 amole, 200 zmole, 2 zmole, blank). MS data in the graph were normalized to the peak at m/z = 957 with the highest intensity.

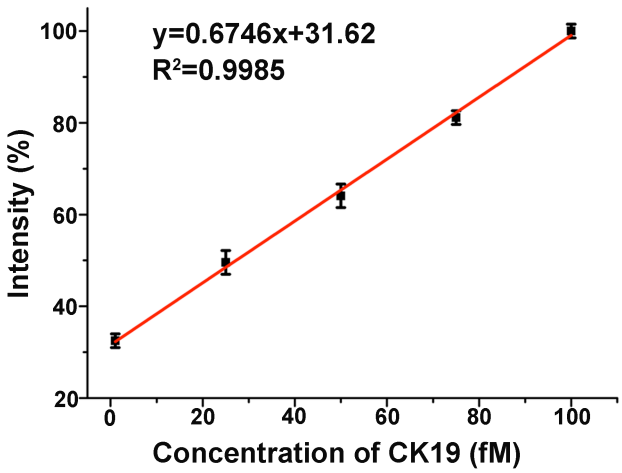

Fig. S3 Calibration curve between normalized MS peak intensity and concentration of CK19, corresponding to 1, 25, 50, 75, 100 fM. The MS peak intensity at m/z = 957 obtained from 100 fM CK19 was set as 100%. Error bar shows the standard deviation from three replicates.

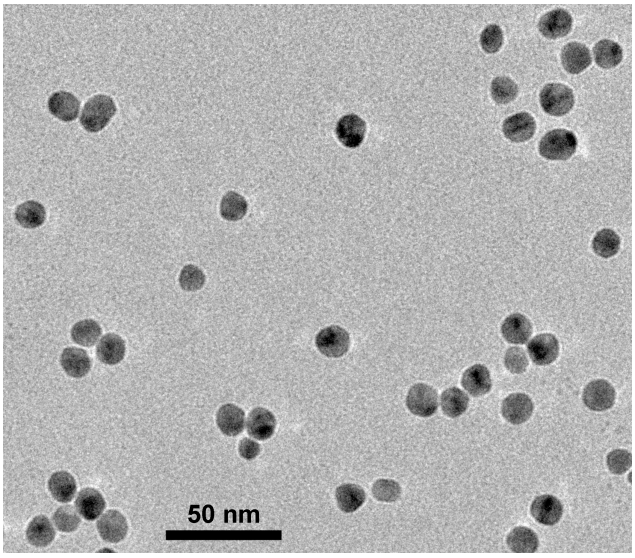

Fig. S4 TEM image of freshly synthesized AuNPs.

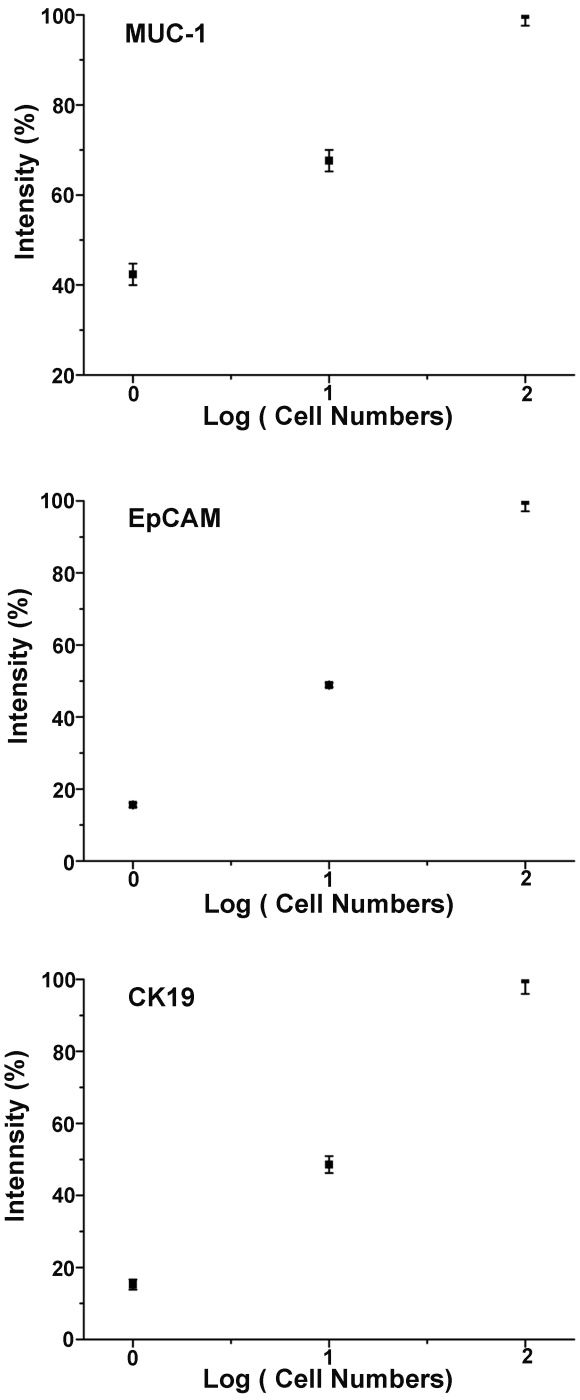

Fig. S5 Plots between the normalized intensity of MS peak corresponding to cell membrane biomarkers of MUC-1, EpCAM and CK19 and the logarithm value of cell number. The MS peak intensity for each biomarker obtained from 2 μL, 10^3 cells/μL MCF-7 cell was set as 100%. Error bar shows the standard deviation from three replicates.

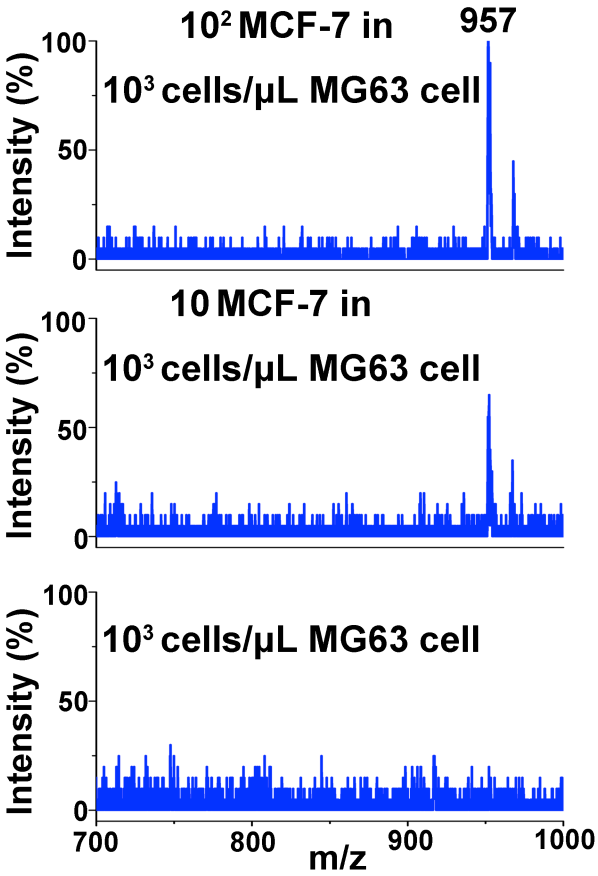

Fig. S6 CK19 detected from 2 μL MCF-7 cell with various concentrations (10^2, 10, 0 cells/μL) in the presence of 10^3 cells/μL MG63 cell using ML2/pAb-CK19 labeled AuNPs and LDI-MS. MS data in the graph were normalized to the peak at m/z = 957 with the highest intensity.