Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Materials

Non-invasive isolation of rare circulating tumor cells with a DNA mimic of double-sided tape using multimeric aptamers

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The supporting materials contain two sections:

1. The experimental details.
2. The supporting data.

Experimental Section

Materials

T47D and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (ATCC). The cell culture media DMEM and RPMI-1640 were obtained from Corning Cellgro Inc. (Herndon, VA, USA). Fetal bovine serum (FBS) was obtained from GIBCO (Grand Island, NY, USA). The avidin-coated magnetic nanoparticles (MNPs) with the diameters of 130, 250, and 500 nm were obtained from Micromod Partikeltechnologie GmbH (Rostock, Germany). The streptavidin-coated magnetic particles (MPs) with diameters of 1 and 4 μm were obtained from Spherotech Inc. (Lake Forest, IL, USA). The neutravidin-coated fluorescent nanoparticles (FNPs, 250 nm) were obtained from Invitrogen Inc. (Carlsbad, CA, USA). Recombinant DNase I was obtained from TaKaRa Bio Inc. (Dalian, China). A LIVE/DEAD Cell Stain kit was obtained from Invitrogen (Grand Island, NY, USA). The cell staining dyes DIO, DID, and Hoechst 33258 were obtained from ThermoScientific (Waltham, MA, USA). Phi29 DNA polymerase, T4 DNA ligase, and dNTP mixture were also obtained from ThermoScientific. The biotinylated anti-human CD326 (EpCAM) antibody was purchased from Biolegend (San Diego, CA, USA). Anti-rabbit cytokeratin 19 (anti-CK19) polyclonal antibody was purchased from Abcam Inc. (Cambridge, CA, USA). Anti-mouse CD45 monoclonal antibody, anti-rabbit EpCAM monoclonal antibody, anti-mouse IgG F (ab')2 fragment (Alexa Fluor® 488 conjugate) and anti-rabbit IgG F(ab')2 fragment (Alexa Fluor® 594 conjugate) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). All DNA sequences were purchased from Integrated DNA Technologies (San Diego, CA, USA) and Sangon Biotech Co., Ltd. (Shanghai, China). CellROX® Green reagent was purchased from Life Technologies Inc. (Grand Island, NY, USA). A lipid peroxidation malondialdehyde (MDA) assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

DNA sequences:

Bio-anti-EpCAM aptamer: 5’-CAC TAC AGA GGT TGC GTC TGT CCC ACG TTG TCA TGG GGG GTT GGC CTG/3Bio/-3’;

Anchor DNA: 5’-TAG ATA AAA AAA AAA AAA AAA AAA A/36-Bio/-3’;

EpCAM ligation template: 5’-CTG CGC CGC CGG GAA AAT ACT G-3’;

EpCAM circular template: 5’-CGG CGG CGC AGC AGT TAG ATA AAA AAA AAA AAA AAA AAA ATC TAA CCG TAC AGT ATT TTC C-3’;

EpCAM primer: 5’-CTG CGC CGC CGG GAA AAT ACT G-3’

Rolling Circle Amplification Reaction

A rolling circle amplification (RCA) reaction was performed by following the reported method with slight modification.[1] It consists of two steps: first, circular DNA was prepared by annealing the circular RCA template and ligating with the ligation primer. Second, the prepared circularized DNA was used to perform the final RCA reaction. Briefly, to prepare circular DNA, 16.3 μL of circular template (500 pmol), 26.7 μL

milliQ water, 5 µL of 10X protein kinase buffer, and 1 µL of 100 mM ATP were added to a tube and vortexed gently. Before ligation, phosphorylation was performed by adding 1 µL (10U/µL) of T4 polynucleotide kinase (PNK) and placing the tube in a water bath at 37 ºC for 30 min. The reaction was quenched by heating the tube to 90 ºC for 5 min and followed by cooling to ambient temperature over 10 min. Then, 7.6 µL of ligation primer (600 pmol) was added into 209.4 µL milliQ water and the solution in the tube was heated to 90 ºC for 30 s followed by cooling to ambient temperature over 10 min. Finally, T4 DNA ligase buffer (30 µL) and T4 DNA ligase (3 µL) were added into the mixture and the reaction was allowed to proceed at ambient temperature for 30 min. The circular DNA was checked and purified using a 2% agarose gel extraction and purification method.

The final circularized DNA sample was used for the RCA reaction. Circular DNA (4 µL, 29 µM), 1 µL RCA primer (100 µM), 2 µL of RCA reaction buffer, and 13 µL milliQ water were mixed in a tube and placed in a heating block at 90 ºC for 5 min. Then the reaction mixture was cooled to ambient temperature for 30 min. Finally, 1 µL of dNTP mixture (10 mM) and 2 µL of Phi29 DNA polymerase were added and the reaction proceeded at 4 ºC for 6 h. The reaction was terminated by heating the tube to 90 ºC for 5 min. The final RCA product was characterized by 1% agarose gel electrophoresis and imaged on a blue-light transilluminator. DNA purification was performed using the phenol/chloroform method and the concentration of circularized DNA and the RCA product were determined by UV-Vis spectroscopy (HACH-DR 5000, Loveland, CO, USA).

Preparation of anchor DNA (biotin-labeled 20A) functionalized MNPs and FNPs

The anchor DNA were first denatured for 5 min at 95 ºC and then quickly cooled down on ice for 10 min. The avidin-coated MNPs or FNPs (1 mL, 5×10^6 particles/mL) as purchased were suspended in 1 mL PBS buffer (pH 7.4), then 2 µL of 100 µM anchor DNA (the molar ratio of anchor DNA and particles was 4.8×10^5:1) was added to the NPs solution, which was incubated in a shaker for 2 h at ambient temperature. The MNPs were isolated from the mixture using a magnetic stand and washed twice with PBS to obtain the anchor DNA functionalized MNPs and FNPs.

Preparation of antibody coated MNPs and FNPs

The avidin-coated MNPs or FNPs (1 mL, 5×10^6 particles/mL) as purchased were rinsed and re-suspended in 1 mL PBS buffer (pH 7.4); then 10 µL of biotinylated anti-EpCAM antibody (0.5 µg/mL) was added to the NP solutions and mixed thoroughly. The mixture was incubated on a shaker for 1 h at ambient temperature. After magnetic separation, the antibody-coated MNPs were washed twice with PBS to remove the free antibody molecules (non-adsorbed) and then re-suspended in 1 mL PBS prior to being kept at 4 ºC until use.

Analysis of EpCAM Protein Expression level

The T47D and MDA-MB-231 cell lines were cultured in DMEM and RPMI 1640 media, respectively. Both media were supplemented with 10% FBS. The cells were cultured in a humidified incubator at 37 ºC

containing 5% CO_2. Before performing the cell capture experiments, T47D and MDA-MB-231 with the cell number of 5×10^4 were spun down by centrifugation and rinsed with PBS. Afterwards, the cells were fixed in 4% (*w/v*) paraformaldehyde in microtubes for 10 min. After spinning down the cells, 3% bovine serum albumin (BSA) solution was introduced for surface blocking for 20 min. Afterwards, the cells were incubated with the primary antibody against EpCAM (1:100 in 3% BSA solution) for 1 h at ambient temperature. After rinsing twice with PBS, the cells were treated with Alexa Fluor® 488 labeled secondary antibodies (1:200 in 3% BSA solution) for 1 h. The cells were rinsed again with PBS and then analyzed using a FACS Calibur flow cytometer (Becton Dickinson, CA, USA).

Fabrication of the cell enumeration microchip

A PDMS microchip was fabricated with open microwells for rare cell enumeration. The diameter of the microwells was 5 mm, the same as the microscope field under a 4X lens, so that stained cells can be placed into the microwells for enumeration under the microscope. Briefly, a mixture of PDMS prepolymer and curing agent (10:1 by weight) was poured onto flat silicon wafer and cured in an oven (80 ºC) for 2 h. After cooling, the PDMS replicas (~4 mm thick) were peeled off from the silicon wafer and cut into the size of a regular microscope glass slide (76*26 mm); afterwards the round microwells (5 mm in diameter) were made using a hole punch before irreversibly sealing on glass slides after treatment with oxygen plasma for 90 s. The devices were cured at 60 ºC for 2 h to reinforce the bonding.

For cell enumeration, the captured cells were re-suspended in 20 µL PBS and transferred to the microwells on the microchip. They were then viewed under a Leica DMI 4000B fluorescence microscope (Wetzlar, Germany), and the cell numbers were analyzed using imageJ software (NIH).

Cell Capture in Buffer and Blood

To evaluate the efficacy of the methods for CTC capture in different matrices, both buffer and synthetic whole blood were employed.

Synthetic whole blood was prepared by mixing DID dye stained WBCs back into their original whole blood matrix, for which EDTA-anticoagulated whole blood samples from healthy donors were obtained from Shenzhen Second People's Hospital (Protocol # 2018-03-16 with Institutional approval by the Hospital Review Board). First, WBCs were separated from healthy blood samples using a density gradient centrifugation in Histopaque Ficoll medium following the reported method.[2] Second, WBCs were incubated together with DID dye for 10 min at 37 ºC for staining, before rinsing with PBS and being kept on ice until further dilution to the desired concentration for capture experiments. Third, to prepare whole blood matrix without WBCs, after a centrifugation treatment (1000 rpm, 5 min) of the whole blood to precipitate the WBCs, the supernatant, i.e., the whole blood matrix, was transferred to a fresh EDTA-anticoagulated micro-tube for storage. Fourth, the DID-stained WBCs were added into the whole blood matrix with the final concentration of 1×10^7 cells mL^{-1} to obtain synthetic whole blood, which was used to simulate whole blood. As a result, the CTC capture efficiency and purity by the three methods were evaluated in sample matrices that match real whole blood.

For the cell capture efficiency assay, DIO-stained T47D cells were diluted to about 20 cells mL^{-1}. The cell number was precisely counted using the cell enumeration chip as described above. The cells were spiked in either synthetic whole blood or PBS. Then, 10 μL of 0.12 μg mL^{-1} EpCAM poly-aptamer or 1 μL of 100 μM EpCAM unit aptamer were incubated with the cells either in 1 mL of PBS or synthetic whole blood for 10 min at ambient temperature. The cells were then treated with 1×10^5 MNPs/FNPs solution for 1 h. For comparison, the cells were also treated with the MNPs-antibody (final concentration 1×10^5 particles) for 1 h. The MNPs were separated from the mixture solutions using a magnetic stand and rinsed twice with PBS.

To evaluate the capture purity of the T47D cells in PBS, DIO stained T47D cells (final concentration 20 cells mL^{-1}) and DID stained MDA-MB-231 cells (final concentration 1000 cells mL^{-1}) were mixed in PBS buffer. To assess the purity of the captured cells in whole blood, DIO stained T47D cells were spiked at a final concentration 20 cells mL^{-1} into synthetic whole blood that contained DID stained WBCs with the concentration of 1×10^7 cells mL^{-1}. Then 10 μL of 0.12 μg mL^{-1} EpCAM poly-aptamer or unit aptamer were added into the cell mixtures and incubated for 10 min at ambient temperature. MNPs-antibody with final concentration of 1×10^5 particles were also incubated with the spiked cell solutions for comparison. After centrifugation, the cells were treated with 50 nM MNP solution for 60 min. The resulting solutions were separated using a magnetic stand and washed twice with PBS and resuspended in the wells of the cell enumeration microchip. Under the fluorescence microscope, the purity was calculated by dividing the number of T47 D cells (green) by the total number of the cells in each well.

Characterization of the treated T47D cells

The ζ-potential of unit aptamer, poly-aptamer, and antibody treated T47D cells suspended in PBS were measured using a Zetasizer Nano ZS90 (Malvern Instruments, Southborough, UK). Images of the MNPs-unit aptamer, MNPs-poly aptamer, and MNPs-antibody binding with T47D cells were also taken on a JEM-1230 transmission electron microscope (TEM) (JEOL, Tokyo, Japan).

The Viability and Proliferation of Released Cells

To release the captured cells from the DST device, DNase I with the final concentration of 100 U mL^{-1} was introduced into the captured cell solution. The mixture was incubated at 37 ºC for 20 min to ensure maximum enzyme efficiency. After magnetic separation of the MNPs using a magnetic stand, the released cell viability was tested using a LIVE/DEAD assay. Calcein AM and ethidium homodimer-1 were added to the cell culture medium to a final concentration of 1 mM. After incubation at 37 ºC for 15 min, the cells were imaged under the Leica DMI 4000B fluorescence microscope with the excitation channels at 488 and 561 nm, with green fluorescence for the live cells and red fluorescence for the dead. To evaluate the proliferative capacity of the isolated CTCs, after removal of MNPs via magnetic separation, the same number of cells isolated by each of the three methods were cultured in each individual well for 5 d before the final cell numbers were counted using the cell enumeration microchip.

Application of the DST device for clinical samples (whole blood) from breast cancer patients

Twenty-nine fresh whole-blood samples (~1 mL each stored in the EDTA-anticoagulated micro-tubes from BD vacutainer, San Jose, CA, USA) from breast cancer patients and 5 samples from healthy donors were obtained from Shenzhen Second People's Hospital (Protocol # 2018-03-16 with Institutional approval by the Hospital Review Board). The blood samples can be stored under refrigeration (4 ºC) for 1 month before use. During the cell capture experiments, 10 μL of 0.12 μg mL^{-1} poly-aptamer (against EpCAM) solution was added into every blood sample (1 mL of each) and incubated for 30 min at ambient temperature. After centrifugation at 1000 rpm for 5 min to remove the excess amount of poly-aptamer, the cells were treated with 1 mL PBS containing 1×10^5 MNPs for 60 min. The resulting solutions were separated using a magnetic stand and rinsed several times with PBS.

Immunostaining

The released cells captured from 1 mL of whole blood from leukemia patients and healthy donors were washed with PBS, then fixed in 4% (*w/v*) paraformaldehyde for 10 min. After blocking using 3% bovine serum albumin (BSA) for 20 min, the samples were incubated overnight at 4 ºC with both the anti-rabbit CK19 polyclonal antibody (1:100 in 3%BSA) and anti-mouse CD45 monoclonal antibody (1:100 in 3% BSA). The samples were then washed with PBS to remove excess antibody followed by incubation with either the Alexa 488–conjugated goat anti-rabbit IgG (1:200 in 3%BSA) or Alexa 594 conjugated goat anti-mouse IgG (1:200 in 3% BSA) for 1 h at ambient temperature. After washing with PBS twice, Hoechst 33258 staining solution with a final concentration of 5 μg/mL was added and incubated for 5 min. The cells were seeded on a 35 mm glass-bottomed dish (NEST, Shanghai, China), and the cellular images were taken using a Nikon A1R confocal laser scanning microscope system (CLSMS, Nikon, Japan) using excitation channels at 405, 488, and 561 nm. The merged confocal images were produced using NIS-Elements software version 4.0 (Nikon A1R).

Endocytosis Assay

Avidin-coated FNPs with the same size and surface functionalities were used as surrogates of MNPs to evaluate the endocytosis performance of the MNPs-poly aptamer, MNPs-unit aptamer, and MNPs-antibody. The FNPs were coated with anchor DNA by the methods described above. T47D cells were incubated with the poly-aptamer or unit aptamer for 10 min. After removing the excess poly-aptamer or unit aptamer, the cells were treated with FNPs-anchor DNA (1×10^5) for 60 min; Separately, for comparison purpose, T47D cells of the same concentration were treated with FNPs-antibody. Afterwards, the cells were rinsed twice with PBS. Then 4% paraformaldehyde was added for 10 min, the CLS and differential interference contrast (DIC) images were taken under a confocal laser scanning microscope (Nikon, Japan).

Detection of intracellular ROS levels

After the T47D cells were incubated with MNPs-unit aptamer, MNPs-poly aptamer, or MNPs-antibody, they were treated with CellROX® Green Reagent using the manufacturer's instructions. Then, the treated cells were fixed in 4% (*w/v*) paraformaldehyde for 10 min and the Hoechst 33258 staining solution with

the final concentration of 5 μg/mL was added and incubated for 5 min. After rinsing with PBS, the images were taken under a confocal laser scanning microscope with the excitation channels at 405 and 488 nm for the quantitative determination of ROS levels. The fluorescence intensity was analyzed using ImageJ software.

Lipid peroxidation assessment

The extent of lipid peroxidation of cell membranes was estimated using a lipid peroxidation MDA assay kit. The principle of the assay is that during peroxidation of lipids, the oxidation of polyunsaturated fatty acids produces malondialdehyde (MDA), which produces a chromophore when it reacts with thiobarbituric acid (TBA). Briefly, 0.1 mL of sample was mixed with 200 μL of MDA working solution, which was heated at 100 ºC for 15 min, and then cooled to ambient temperature. After centrifugation at 1000 g for 10 min, the supernatant was measured at a wavelength of 532 nm, and MDA level unit was expressed as μmol/mg of protein.

Supporting data

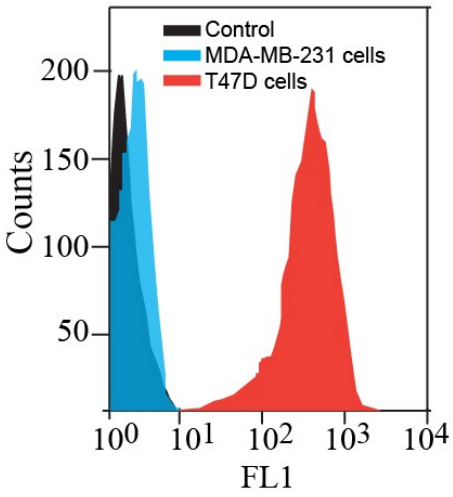

Figure S1. The expression level of the biomarker, epithelial cell adhesion molecule (EpCAM) of the two cell lines (T47D, the target breast cancer, and MDA-MB-231 cells, a triple negative breast cancer, which was used as the negative control). Untreated (non-fluorescently labelled) T47D cells served as a control group. The data show that the EpCAM expression by MDA-MB-231 is low; however, there is still a small fraction of it showing expression, which may contribute to the impurity during the isolation of T47D cells (Figure 2b).

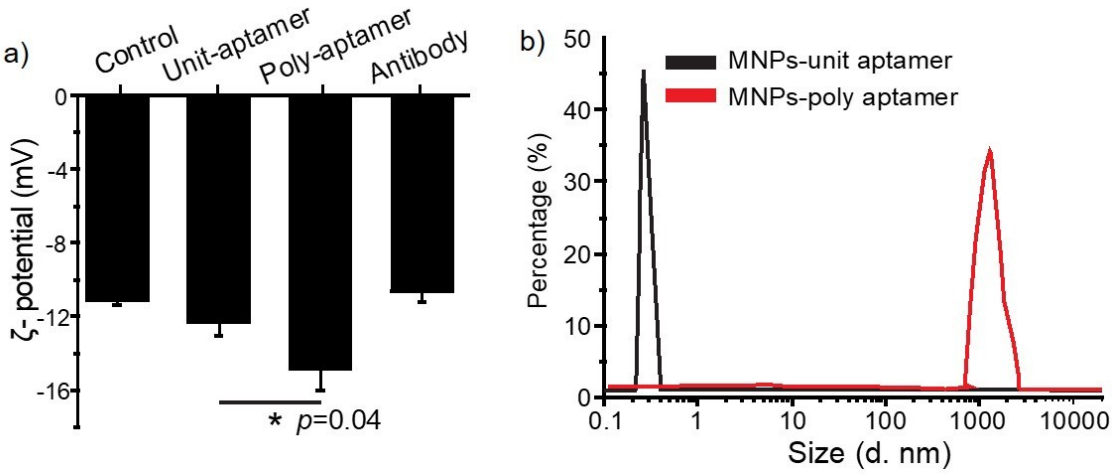

Figure S2. a) The surface charge indicated by ζ-potential of the cells bound with and without affinity ligands, including unit-aptamer, poly-aptamer, and EpCAM antibody. The medium was PBS buffer containing 220 mM sodium and potassium ions, thus the ζ-potential values were not high; nevertheless, the trend is observable and the numbers of negative charges on cell surface decreased in the following order: poly-aptamer-cells>unit-aptamer-cells>control cells (no ligands attached)>antibody-cells. The ζ-potential of poly-aptamer-cells is significantly greater than that of the unit-aptamer-cells. Antibody

attachment did not increase the negative charge on the cell surfaces, which may contribute to their approach, but which also increases their endocytosis. b) The size of the poly aptamer functionalized MNPs (~1.8 µm) is much larger than the unit-aptamer-MNPs (~ 260 nm). The larger size of the poly-aptamers and more negative charges protect the cells from direct interaction with the 20A-MNPs, which would induce MNP endocytosis.

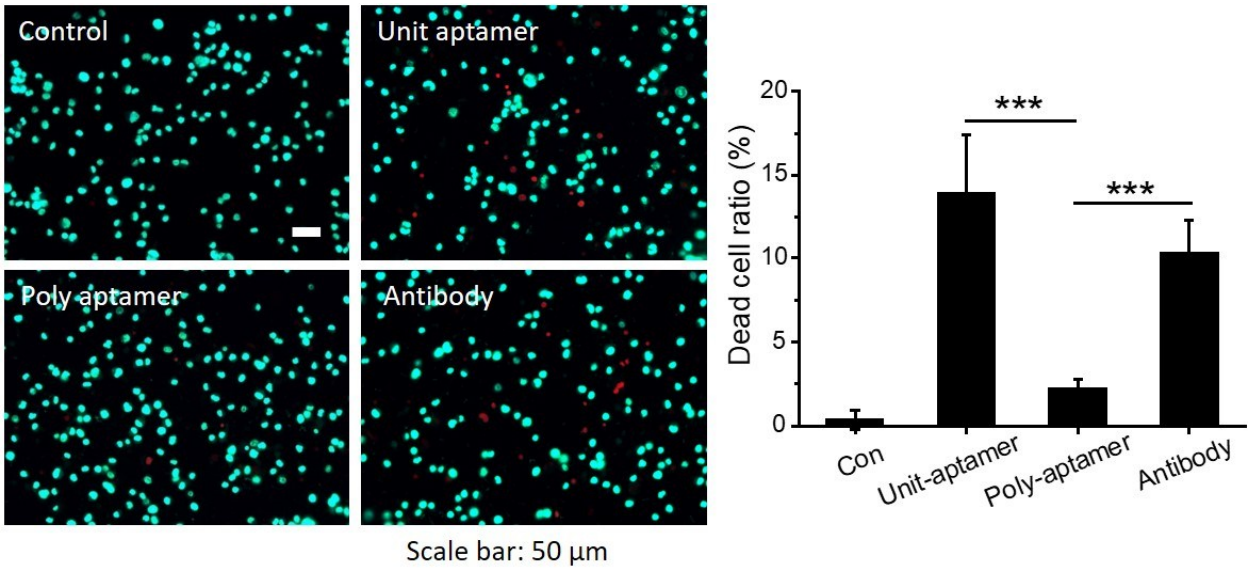

Figure S3. The viability of released cells, where the live cells were stained with green fluorescence while the dead cells were stained with red fluorescence. The results show that the cell death after treatment with either the unit-aptamer-MNPs or antibody-MNPs was much greater than those treated by the poly-aptamer-MNPs; there was no difference between the latter and the control.

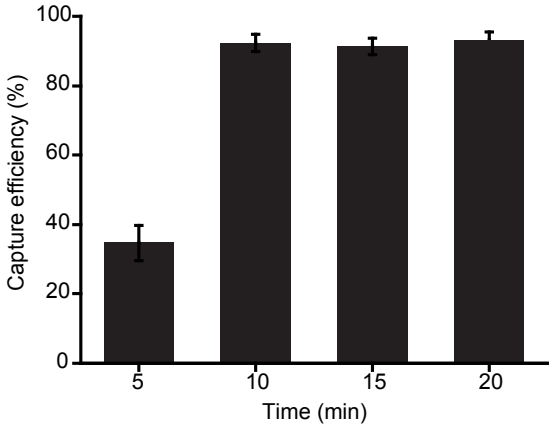

Figure S4. The capture efficiency of the target cells as a function of the incubation time with Multi-A DNA strands. 10 min is sufficient for ~100% recovery of 10 T47D cells spiked in 1 mL PBS buffer.

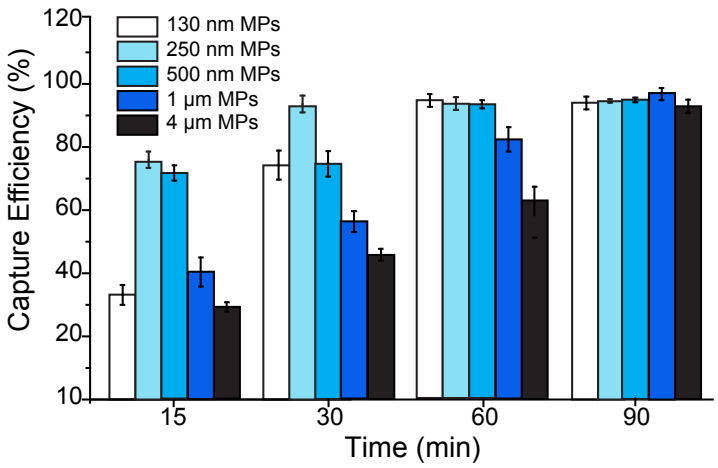

Figure S5. Capture efficiencies (CEs) of spiked T47D cells (10 cells/mL) as the function of the hybridization time of 20A-functionalized MNPs of different sizes. The hybridization of the 20As on MNP surfaces with the 15T spacer sequences within each Multi-A DNA strand is critical in determining the CEs. The results show it takes 30 min for the 250 nm MNPs to obtain ~100% CE, whereas it takes much longer, i.e., 90 min for larger MNPs (1-4 μm) to obtain 100% CE. Therefore, we used 250 nm MNPs in this work.

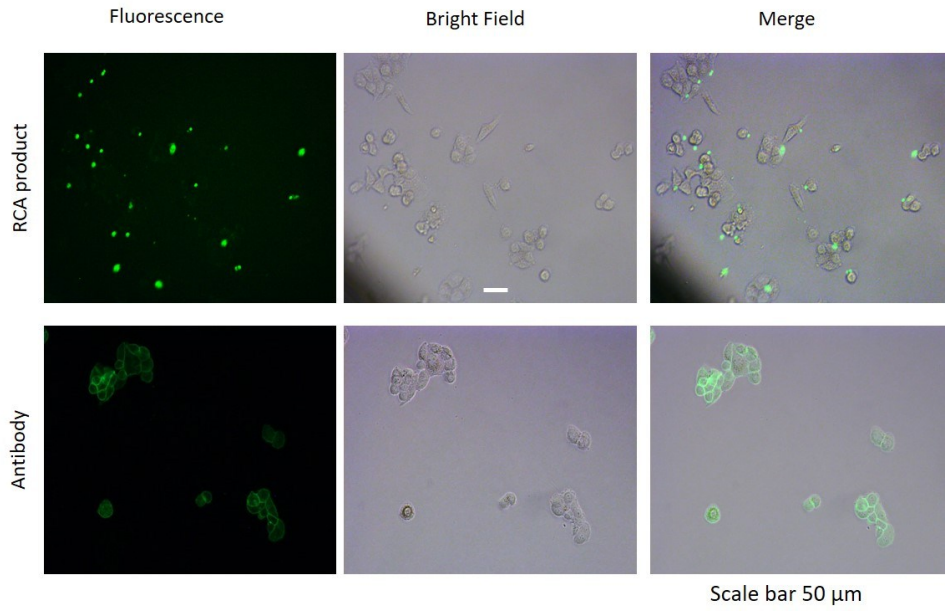

Figure S6. The built-in signal amplification mechanism of the DST-DNA devices is demonstrated by comparing the fluorescence intensity of the DST-DNA devices (stronger) with the EpCAM antibody (weaker) that bound the T47D cell surfaces. Because EpCAM receptor expresses not only on cell surfaces, but also in cytoplasm, the antibody-secondary antibody could enter cytoplasm, but DST-DNAs are not internalized; nevertheless, the surface bound DST-DNAs show much brighter fluorescence because each strand contains many copies of spacer sequences that can bind FAM-8A.

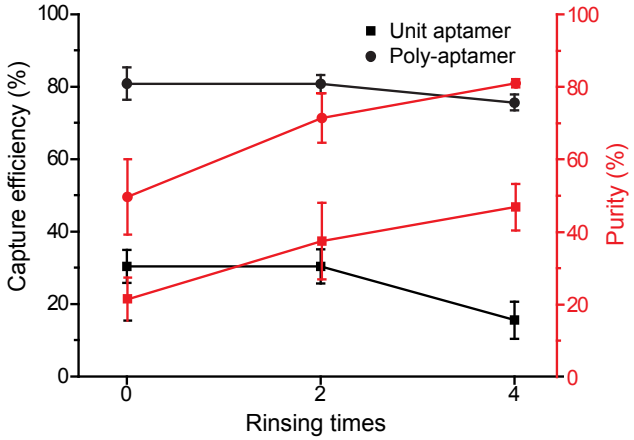

Figure S7. Capture efficiencies (CEs) and purity as the function of number of rinses of the captured cells. It is evident that multiple rinses increase the purity of the captured cells by removing the non-specifically bound cells, but they decrease the CE, especially for the unit-aptamer-functionalized MNPs. The Multi-A DNA (poly-aptamer) is bound tightly to both the cells and MNPs via multivalent binding, which maintained the CE during multiple rinses.

Video S1. Migration of the target CTCs under external magnetic field when they were captured by the DST device.

Video S2. No migration of the non-target cells in the external magnetic field, where they were not captured by the DST device.