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Specific Capture and Release of Circulating Tumor Cells Using Aptamer Modified Nanosubstrates

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Circulating tumor cells (CTCs)^[1] are cancer cells that have propagated from tumors, spreading into the bloodstream as the cellular origin of fatal metastasis. Besides conventional diagnostic approaches (e.g., tumor biopsy, anatomical/molecular imaging and serum marker detection), detecting CTCs in peripheral blood is of prognostic value in different types of solid tumors, especially for predicting patient survival. The fact is that CTC detection have been technically challenging because of the extremely low abundance (a few to hundreds per mL) of CTCs among a high number (10^9 cells mL⁻¹) of hematologic cells.^[2] Over the past decade, a diversity of diagnostic technologies has been demonstrated for CTC detection using different working mechanisms. The current FDA-cleared CellSearch™ Assay is based on immunomagnetic separation of CTCs. Due to its

unsatisfactory efficiency and high cost, researchers have been exploiting new technologies,^[3] e.g., flow cytometry, size-based filtration systems and microfluidic devices that may offer improved sensitivity and reduced cost for CTC detection. In addition to the prognostic utility of CTC-based diagnostics, it is conceivable that the molecular signatures and functional readouts derived from CTCs will shed much valuable insight into tumor biology during the critical window where therapeutic intervention could make a significant difference.

Previously, we have demonstrated a highly efficient, inexpensive CTC assay capable of enriching, identifying and enumerating CTCs in whole-blood samples collected from prostate cancer patients. First, we pioneered a unique concept of “NanoVelcro” cell-affinity substrates,^[4] by which capture agent (anti-EpCAM)-coated silicon nanowire substrates (**SiNWS**) were utilized to immobilize CTCs in a stationary device setting. The general applicability of the NanoVelcro substrates is supported by our recent studies, where other types of nanostructured substrates, e.g., electrochemically deposited conjugated polymer nano-features,^[5] and horizontally packed TiO₂ nanofibers^[6], can also be deposited onto substrates, exhibiting enhanced affinity for CTC capturing. Further, recent studies by other groups^[7] also have confirmed the utility of **SiNWS** (grafted with immune cell-specific capture agents) for capturing subpopulations of immune cells. The uniqueness of our approach is the use of nanostructured substrates: There are enhanced local topographic interactions^[8] between the anti-EpCAM-coated nanosubstrates and nanoscaled cellular surface components (e.g., microvilli) on a CTC, which are analogous to the working principle of a velcro. Second, by integrating a NanoVelcro substrate with a polydimethylsiloxane (PDMS)-based chaotic mixer^[9] that enhances contact frequency between flow-through CTCs and the substrate, further improved CTC capture efficiency has been achieved.^[10] Although NanoVelcro devices allow efficient and reproducible enumeration of CTCs in clinical setting, one of the remaining challenges is to confer cell-release performance to the NanoVelcro devices. A diagnostic assay capable of highly effective capture and specific release of CTCs will pave the way for implementing subsequent molecular and functional analyses.^[11]

On the basis of our previously reported NanoVelcro Chips, we introduce a new-generation NanoVelcro Chip (Figure 1) that is capable of not only capturing non-small cell lung cancer (NSCLC) CTCs from blood with high efficiency, but also recovering the nanosubstrate-immobilized NSCLCCTCs upon treatment of a nuclease solution. To achieve such an inexpensive and sensitive CTC capture and recovery platform, we replaced the antibody-based capture agent (i.e., anti-EpCAM) employed in earlier NanoVelcro devices with two DNA-aptamers, **Ap-1** and **Ap-2**. Both **Ap-1** and **Ap-2** are single-stranded oligonucleotides^[12] generated by an in vitro cell-SELEX (systematic evolution of ligands by exponential enrichment) process (see Scheme S1 in supporting information) through a positive selection using A549 NSCLC cells. To allow streptavidin-mediated conjugation onto **SiNWS**, biotin groups were covalently connected to the 5'-terminus of these aptamers. Similar to other cell-specific aptamers, **Ap-1** and **Ap-2** can fold into unique secondary or tertiary structures to recognize surface ligands on A549 cells with low nM level of affinities. An earlier attempt,^[13] which combined the use of aptamer-based capture agents with a microfluidic device achieved highly efficient capture of CTCs, while subsequent cell-release study was not demonstrated. We first employed A549 NSCLC cells to optimize and validate the cell-capture and release performance of aptamer-coated NanoVelcro Chips. Here, a genetically engineered endonuclease (i.e., Benzonase Nuclease, EMD Millipore USA) capable of digesting all forms of DNA and RNA, was employed to specifically degrade the **SiNWS**-grafted DNA aptamer, allowing recovery of viable NSCLC cells. Finally, an artificial NSCLC CTC blood sample was prepared to examine the cell-capture/release performance of the aptamer-coated NanoVelcro Chip. Gradually improved CTC purities (the

ratios between CTCs vs. non-specifically captured WBCs) were observed over two rounds of cell capture/release processes. We noted that it was proven difficult to release target cells from the earlier NanoVelcro Chips via nonspecific enzymatic digestion by trypsin. Only 10% of substrate-immobilized cells were recovered, and poor cell purity and viability were observed.

Similar to the earlier NanoVelcro Chips, the aptamer-coated NanoVelcro Chip is composed of two functional components (Figure 1): 1) a patterned SiNWS with streptavidin coating for conjugation with either **Ap-1** or **Ap-2**, and 2) an overlaid PDMS chaotic mixer with a 22-cm-long microchannel that increases cell-substrate contact frequency (as a result of vertical flows generated by the embedded herringbone features). These two components were fabricated using previously reported procedures^[10] and can be assembled together by a chip holder (made of stainless steel and transparent plastic materials). A syringe pump (KD Scientific) was utilized to introduce 1) capture agents (**Ap-1** and **Ap-2**), 2) cell suspensions or blood samples, 3) fixation and permeabilization agents, 4) immuno- and nuclear staining agents, and 5) Benzonase Nuclease into the chips with defined flow rates ranging from 0.5 to 2.0 mL h⁻¹. Prior to the cell-capture studies, biotinylated aptamers (20 μM, see optimization in Figure S1) in 200 μL PBS was freshly grafted onto the streptavidin coated SiNWS.

The capture performance of the aptamer-coated NanoVelcro Chip was first characterized with a 1.0-mL suspension of A549 cells (100 cells mL⁻¹, in F-12K medium) at flow rates of 0.5, 1.0, 2.0, 4.0 and 8.0 mL h⁻¹. After rinsing and parallel staining of FITC-labeled anti-EpCAM (a surface marker for epithelial cells) and DAPI (nuclear), the substrate-immobilized cells were imaged and counted under an upright fluorescence microscope (Nikon 90i). The transparent PDMS chaotic mixer allows direct imaging analysis without disassembling the sandwiched devices. The results (Figure 2a) suggest that both of the **Ap-1** and **Ap-2**-coated NanoVelcro Chips exhibited similar cell-capture performances, and an optimal flow rate (1.0 mL h⁻¹) was determined for both aptamers based on the observed cell-capture efficiencies. We then analyzed the distribution of the substrate-immobilized cells at different locations of the 22-cm-long serpentine channel footprints. At a flow rate of 1.0 mL h⁻¹ (Figure 2b), 79% and 70% of the cells were captured in the first 8 cm of the **Ap-1** and **Ap-2**-coated NanoVelcro Chips, respectively. These results suggest that a 22-cm-long microchannel is sufficient to achieve the desired cell-capture performance. Further, five control studies using 1) **Ap-1**-coated Si-chips (flat, no nanofeature), 2) **Ap-2**-coated Si-chips, 3) random DNA-coated NanoVelcro Chip (no cell affinity), 4) NanoVelcro Chip (no aptamer) and 5) anti-EpCAM-coated NanoVelcro Chip, were carried out in parallel with the **Ap-1** and **Ap-2**-coated NanoVelcro Chips. The results shown in Figure 2c suggest that aptamer-based capture agents and the embedded nanostructures play indispensable role in achieving the superb cell capture performance. To test the specificity of **Ap-1** and **Ap-2**-coated NanoVelcro Chip for capturing NSCLC cells, two NSCLC cells (A549 and HCC827), four non-NSCLC cancer cells (i.e., Hela cervical cancer cells, PC3 prostate cancer cells, U87 glioblastoma cells, and Jurkat T cell leukemia), and WBCs obtained from healthy donors were examined in parallel. Summarized results in Figure 2d suggest that the aptamer-coated NanoVelcro Chips were capable of specifically capturing NSCLC cells. In contrast, there were less than 10% the non-NSCLC cells (i.e., Hela, PC3, U87, and Jurkat cells, and WBCs) non-specifically trapped in the devices, reflecting a device background signal that is consistent with our earlier observation.^[10] At this point, we found that **Ap-1** exhibited relatively better cell capture performance than that of **Ap-2**, thus we selected **Ap-1** for subsequent cell capture/release studies. Finally, we tested the dynamic range of the **Ap-1**-coated NanoVelcro Chips using a series of artificial NSCLC CTC samples that were prepared by spiking PBS and healthy donor's blood with DiO-stained A549 cells at densities of 10, 50, 200, 400, 600, 800, 1,000 cells mL⁻¹. The results indicate that the devices exhibit

sufficient performance (See Figure S2 in supporting information) for handling clinical samples that normally have CTC density ranging from a few to hundreds CTCs mL⁻¹.

To prepare the cell-release studies, cell suspensions containing both A549 cells (200 cells mL⁻¹) and human WBCs (5×10^6 cells mL⁻¹) were generated in F-12K medium. 1.0 mL of the cell suspensions were first introduced into **Ap-1**-coated NanoVelcro Chips to obtain model systems that mimics an **Ap-1**-coated NanoVelcro Chips with specifically captured CTCs and non-specifically captured WBCs. Parallel staining of FITC-labeled anti-EpCAM, Cy5-labeled anti-CD45 (a surface marker for WBCs) and DAPI allowed us to identify and enumerate CTCs and WBCs under the microscope. Among separate studies, we found that there were 179 ± 6 A549 cells (EpCAM+/CD45-/DAPI+) and 3000-8000 WBCs (EpCAM-/CD45+/DAPI+) immobilized on the substrates of the chips. These model systems were then subject to two categories of cell-release studies in order to determine a reasonable time required to achieve effective enzyme-induced cell release via enzyme treatment. Initially, these model systems were kept at 4°C, and 200-μL ice-cold PBS solutions containing Benzonase Nuclease (25 units mL⁻¹, EMD Millipore) were introduced to individual devices. Enzyme-induced cell-release studies were then conducted by placing the devices in an incubator (37 °C) for 5, 10, 20, 30 and 45 min. After the released cells were flushed out of the channels, the remaining cells in the devices were imaged and enumerated again. The data summarized in Figure 3a suggest that after 10-min enzyme digestion > 90% of immobilized A549 cells were specifically released, while <5% of immobilized WBCs were non-specifically released. A 15-min enzyme digestion time were selected at this point for the later portion of studies. To understand how the spiked A549 cell numbers affect the cell-release performance. Cell-release studies were conducted using cell suspensions (containing 10-500 A549 cells and 5×10^6 WBCs) with the 15-min enzyme digestion time. The results summarized in Figure 3b suggest that the cell-release performance is independent from the spiked cell numbers under the optimized experimental parameters.

To examine the clinical utility of the aptamer-coated NanoVelcro Chips, we performed cell capture and release studies using artificial blood samples, prepared by spiking 200 A549 NSCLC cells into 1.0 mL of healthy donor's blood (collected in BD EDTA Vacutainer tube, containing ca. $5\text{--}12 \times 10^6$ WBCs mL⁻¹). The artificial blood samples were introduced into **Ap-1**-coated NanoVelcro Chips at a flow rate of 1.0 mL h⁻¹. After rinsing, followed by parallel staining of anti-EpCAM, anti-CD45 and DAPI, the specifically captured A549 cells and non-specifically immobilized WBCs on the substrates of **Ap-1**-coated NanoVelcro Chips were identified. Here, the expression levels of EpCAM and CD45 in individual cells were quantified using image cytometry technique^[14] (Figure 4a). After the cell-capture process, there were 165 ± 12 A549 cells and 4,000-11,000 WBCs founded in the individual devices, reflecting >80% of A549-cell-capture efficiency and 0.04-0.3% background signals. The scatter plot shown in Figure 4b records the cell distribution in one of the capture studies (n=5), where 181 A549 cells and 8700 WBCs were identified on the substrates. The substrate-immobilized A549 cells were then released via 15-min enzyme treatment (Benzonase Nuclease, 25 units mL⁻¹). There were 150 ± 17 A549 cells (specifically released) and 300-1500 WBCs (non-specifically released) found in the resulting cell suspensions, suggesting >85% of A549 cell release efficiency and <15% WBC release performance. The scatter plots shown in Figure 4c and d represent the original data of remaining cells (12 A549 cells and 6930 WBCs) on the substrates and released cells (165 A549 cells and 1370 WBCs) in the media. In short, after the 1st round of capture/release process the final purities of A549 cells are in the rage of $15 \pm 5\%$ (n=5). The gradually improved cell purities over the 1st round of capture/release process are summarized in a flow chart (Scheme S3a in supporting information). Subsequently, we demonstrated that the purity of A549 cells could be further increased to >95% by repeating the capture/release process twice. The scatter plots in Figure 4e and f shows A549/WBC distributions of the cell suspensions obtained

from one of the 1st and 2nd rounds of cell capture/release studies (n=5). Again, the gradually improved cell purities over the two rounds of capture/release processes are summarized in a flow chart (Scheme S3b in supporting information).

It is crucial to note that such dramatically improved purities of A549 cells make molecular and functional analyses of the recovered cells possible. To test the feasibility of culturing the recovered A549 cells, we first performed viability studies on the A549 cells in the cell suspension recovered from the two rounds of capture/release processes. The results suggested that the recovered A 549 cells exhibited 78-83% viability. Further, these recovered A549 cells can be continuously passaged for five cycles. The viability and passage studies were concluded in Figure S3 in supporting information. To examine the feasibility of performing molecular analysis on the recovered A549 cells, we were able to identify *KRAS*^{G12S} mutation (a known oncogenic mutation present in A549 cells, see Figure S4 in supporting information) in the recovered cells by performing PCR amplifications, followed by Sanger sequencing. In contrast, only wild-type *KRAS* (present in WBCs) was detected from the initial artificial blood samples since the surrounding WBCs constitute the major cell population, making the *KRAS*^{G12S} mutation signal essentially invisible.

In conclusion, we have demonstrated a new-generation NanoVelcro Chip that is capable of not only capturing NSCLC CTCs from blood with high efficiency, but also recovering the nanosubstrate-immobilized NSCLC CTCs upon treatment of a nuclease solution. Two single-stranded DNA-aptamers (**Ap-1** and **Ap-2**) were generated via Cell-SELEX process to replace conventional anti-body based capture agents, allowing specific capture and release of NSCLC cells from whole-blood samples using aptamer-grafted NanoVelcro Chips. The capturing and releasing features enable isolation of circulating tumor cells (CTCs) with minimum contamination of the surrounding white blood cells (WBCs) and negligible disruption to CTCs' viability and functions, thus paving the way toward molecular and functional analyses of CTCs. It is conceivable that, by this new diagnostic platform, the CTC-derived molecular signatures and functional readouts will provide valuable insight into tumor biology during the critical window where therapeutic intervention could make a significant difference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) Image of an aptamer-coated NanoVelcro Chip for capturing and releasing NSCLC CTCs from blood samples. b) Cross-sectional view of the microchannel embedded in the aptamer-coated NanoVelcro Chip, where an aptamer-coated SiNWS is combined with an overlaid microfluidic chaotic mixer to enhance the contact frequency between the aptamer-coated SiNWS and flow-through NSCLC CTCs, leading to enhanced CTC capture efficiency. Upon enzymatic treatment, the surface-grafted aptamer can be specifically cleaved, resulting in specific release of nanosubstrate-immobilized NSCLC CTCs. c) Conceptual illustration of the molecular mechanism governing the capture and enzymatic release of NSCLC CTCs from the aptamer-coated SiNWS.

Figure 2.

a) Cell-capture efficiency of the aptamer-coated NanoVelcro Chip at flow rates of 0.5, 1, 2, 4, and 8 mL h⁻¹. Error bars show standard deviations (n=3). Cell suspensions (1.0 mL) containing A549 NSCLC cells (100 cells mL⁻¹) were employed as a model system. b) Spatial distribution of substrate-immobilized A549 cells along the serpentine microchannel at flow rates of 1.0 mL h⁻¹. c) In parallel with the **Ap-1** and **Ap-2**-coated NanoVelcro Chips, five control studies based on 1) **Ap-1**-coated Si-chips (flat, no nanofeature), 2) **Ap-2**-coated Si-chips, 3) random DNA-coated NanoVelcro Chip (no cell affinity), 4) NanoVelcro Chip (no aptamer) and 5) anti-EpCAM-coated NanoVelcro Chip were examined. d) Cell-capture efficiency of aptamer based microfluidic system using suspensions of lung (A549 and HCC827), cervical (Hela), prostate (PC3), brain (U87), and T-lymphocyte (Jurkat) cell lines, and WBC.

Figure 3. Specific release of captured A459 cells from artificial blood sample using Benzonase Nuclease. a) Time dependent release efficiencies for both A549 cells and WBCs. b) The release performance between spiked A549 cells and WBCs under different spiked A549 cell numbers.

Figure 4.

a) Conceptual illustration of identification of captured A549 cells spiked in the artificial patient blood sample. Fluorescent micrographs of CTCs captured from the artificial blood samples. Three-color immunocytochemistry method based on FITC-labeled anti-EpCAM, Cy5-labeled anti-CD45, and DAPI nuclear staining was applied to identify and enumerate CTCs from non-specifically trapped WBCs on the aptamer-grafted SiNWS. The scatter plots summarize the A549/WBC cell distribution b) on one of the **Ap-1**-coated NanoVelcro Chips after cell-capture process, c) on one of the **Ap-1**-coated NanoVelcro Chips after the enzyme-induced cell-release process, d) in one of the cell suspensions obtained from the cell-release process, e) in one of the cell suspensions obtained from the 1st-round capture/release process, and f) in one of the cell suspensions obtained from the 2nd-round capture/release process. All studies were performed in quintuplicate. Two flow charts that summarized the gradually improved cell purities over the 1st and 2nd rounds of capture/release processes can be found in supporting information (Scheme S3).