Programming Thermoresponsiveness of NanoVelcro Substrates Enables Effective Purification of Circulating Tumor Cells in Lung Cancer Patients

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## Side-by-side comparison between Thermoresponsive NanoVelcro CTC purification system and other NanoVelcro CTC assays

In contrast to the other NanoVelcro CTC assays demonstrated by our research team, the overall improvement of this Thermoresponsive NanoVelcro CTC purification system is explicitly illustrated in Table S1.

| Nanoveclo CTC assays | Thermoresponsive NanoVelcro CTC purification system | Side-by-side comparison between Thermoresponsive NanoVelcro CTC purification system and other NanoVelcro CTC assays |
|----------------------|-----------------------------------------------------|-------------------------------------------------------------------------------------------------|
| Automation           | N/A                                                 | N/A                                                                                             |
| Clinical utility     | Breast cancer (no clinical samples)                 | Breast cancer (no clinical samples)                                                             |
| CTC viability        | 85%                                                 | 90%                                                                                             |
| Overall    | Poor (not quantified)                              | 100%                                                                                           |
| Recovery            | 88-89% (2 rounds)                                  | 88-89% (2 rounds)                                                                              |
| Efficiency           | ~90%                                                | ~95%                                                                                           |
| CTC capture          | ~70%                                                | ~90-95%                                                                                         |
| Application          | microscopic Stain                                  | microscopic Stain                                                                              |
| Specificity          | Stain                                               | Stain                                                                                            |
| Mutational analysis  | CTC capture and release                            | CTC enrichment                                                                                 |
| Platforms            | Thermoresponsive NanoVelcro CTC purification system | Other NanoVelcro CTC assays                                                                   |

**Table S1**: Side-by-side comparison between Thermoresponsive NanoVelcro CTC purification system and other NanoVelcro CTC assays.
2. Development of Thermoresponsive NanoVelcro CTC purification system

Thermoresponsive NanoVelcro CTC purification system is composed of two individual components, including (i) a custom-designed chip holder for assembling of an overlaid PDMS chaotic mixer onto a lithographically patterned Thermoresponsive NanoVelcro substrate and integrating a thermo-electric Peltier cooling/heating system with a built-in thermocouple sensor (Figure 1a), as well as (ii) a digital fluidic handler for introducing blood and reagents into the assembled Thermoresponsive NanoVelcro Chips.

2.1 Custom-designed chip holder

The lower piece of the chip holder is designed to house both the Thermoresponsive NanoVelcro substrate and PDMS chaotic mixer. There are multiple alignment markers on the lower piece to allow instant assembly of the individual components. The center area of the lower piece is made of copper (good thermal conductivity), and the thermoelectric Peltier cooling/heating system and thermocouple sensor are located underneath the lower piece. The upper piece is designed to hold the individual functional components together and to connect the Thermoresponsive NanoVelcro Chip to the fluidic handler. We anticipate that it will take less than 1 min to assemble the device prior to conducting CTC purification studies. Using this embedded temperate control/monitor module, we were able to precisely control the temperature of Thermoresponsive NanoVelcro substrates at 4±2°C and 37±2°C within a short equilibrium time of 30 sec (see device temperature profile in Figure S1).

![Figure S1](image_url)
2.2 Digital fluidic handler

We have developed a fully automated fluidic handler and a user interface for controlling automated loading of blood samples and reagents. The system is composed of a pair of syringe/syringe pumps and rotary valves for controlling blood/reagent loading at variable flow rates (0.5-5.0 mL/h).

3. Preparation of Thermoresponsive NanoVelcro substrates

Thermoresponsive NanoVelcro substrates were prepared through two continuous steps, including (i) photolithography and wet etching to introduce vertically-aligned SiNWS onto a silicon wafer, and (ii) covalently grafting PIPAAm polymer brushes that confer “thermoresponsiveness” to the devices. Biotin-streptavidin mediated conjugation was employed to introduce NSCLC CTC-specific capture agent (i.e., anti-EpCAM) onto Thermoresponsive NanoVelcro substrates.

3.1 Fabrication of lithographically patterned SiNWS

Lithographically patterned SiNWS were prepared by a standard photolithography and a chemical wet etching process. The dimension of these patterned SiNWS is the same as the earlier design. Photoresist (AZ 5214, AZ Electronic Materials USA Corp., Branchburg, NY, USA.) was spin-coated onto a silicon wafer (Silicon Quest, San Jose, CA, USA) with 100 μm thickness. After exposure of UV light and development, the silicon wafer was kept in etching solution containing deionized water, HF (4.6 M), and silver nitrate (0.2 M). The length of nanowires was determined based on the duration of etching process. The optimal etching conditions in this study afforded ca. 10-μm silicon nanowires on the silicon substrate. Then, the substrate was treated with boiling aquaregia (3:1 (v/v) HCl/HNO₃) for 15 min. The patterned photoresist on the silicon substrate was removed by rinsing with acetone and ethanol. After washing with deionized water and then drying with nitrogen, the patterned SiNWS were obtained.

3.2 Surface silanization with 3-aminopropyltriethoxysilane (APTES)

The surfaces of the lithographically patterned SiNWS were cleaned using piranha solution (mixture of 2:1 concentrated H₂SO₄ to 30 % H₂O₂ solutions) for 10 min, rinsed with deionized water for three times, and dried with N₂. Then, the chips were baked at 50°C in a vacuum oven for an hour. The surfaces of the patterned SiNWS were modified with 1 % (v/v) APTES in dried toluene at 40°C for an hour (Figure S2), rinsed with methanol, and dried with N₂. The APTES-modified SiNWS were stored in a vacuum oven before the subsequent polymerization steps.
3.3 Introduction of atom transfer radical polymerization (ATRP) initiator

The APTES-grafted SiNWS was immersed in a reaction apparatus containing dichloromethane (200 mL) and triethylamine (10 mL, 72 mmol). The reaction apparatus was evacuated and back-filled with N₂ for three times. 2-Bromo-2-methylpropionyl bromide (9.1 mL, 72 mmol) was added dropwise to the solution at 0°C (Figure S3). The reaction apparatus was shaken overnight at room temperature. The SiNWS was then washed with dichloromethane, deionized water, and dried with N₂.

3.4 Surface-initiated ATRP of PIPAAm

By controlling the mixing ratios of copolymer precursors, PIPAAm containing three different amine group densities (i.e., 2.5, 5.0, and 10.0%) were obtained. Using the synthesis of PIPAAm2.5% amine group density as an example, a reactor containing the initiator-anchored SiNWS and CuBr (0.84 g, 60 mmol) was sealed, evacuated and back-filled with N₂ for three times. NIPAM (22 g, 195 mmol) and 2-aminoethyl methacrylate hydrochloride (0.84 g, 5 mmol), were dissolved in 200 mL of methanol/H₂O (1/1) solvent mixture and purged with N₂ for 30 min. Then, the monomer solution was transferred into the tube using cannula. The reactor was shaken for 6 h at room temperature (Figure S4). After the reaction, silicon wafers were washed with deionized water 3 times, and dried in vacuo to afford the poly (N-isopropylacrylamide) (PIPAAm)-grafted SiNWS with 2.5% amine group density. PIPAAm-grafted SiNWS with 5% amine group density were obtained by using the mixture of 190 mmol NIPAM and 10 mmol 2-Aminoethyl methacrylate hydrochloride. In addition, 180 mmol...
NiPAM and 20 mmol 2-Aminoethyl methacrylate hydrochloride were reacted with SiNWS to achieve 10% amine group grafted PIPAAm-grafted SiNWS.

**Figure S4.** Surface-initiated ATRP to form PIPAAm brushes on initiator-anchored SiNWS

3.5 Conjugation of biotin to (PIPAAm)-grafted SiNWS

To a reactor were added (PIPAAm)-grafted SiNWS, biotin (0.48 g, 1.9 mmol), EDC (0.35 g, 1.9 mmol), and DMF (200 ml). The system was shaken overnight (**Figure S5**). After washing thoroughly with DMF for several times and drying *in vacuo*, the biotin grafted SiNWS were obtained.

**Figure S5.** Conjugation of biotin onto (PIPAAm)-grafted SiNWS

4. Characterization of the biotin-grafted SiNWS

SEM surface characterizations (**Figure S6**) and temperature-dependent contact angle measurements of water droplets (**Figure S7**) were utilized to examine the thermoresponsive surface properties of these biotin grafted SiNWS, providing quality assurance for the resulting devices.

4.1 Scanning electron microscopy (SEM) observation

The biotin grafted SiNWS were confirmed with SEM (JSM-6330F, JEOL, 10 keV). The samples were coated with gold (< 3 nm) prior to examination with a FE-SEM. (This part was copied to method section) As shown in **Figure S6**, thick polymer coating on biotin grafted SiNWS was clearly observed compared to the original SiNWS.
4.2 Measurement of contact angles

To confirm the thermo-responsive property of biotin grafted SiNWS, the contact angle of water droplet on biotin grafted SiNWS was measured at 37 °C and 4 °C using contact angle measurement system (SImage mini, Excimer, Inc.) containing a heating/freezing stage (Linkam Scientific Instrument). The contact angle of 1.5-μL deionized water was measured 3 times at each temperature. (This part was copied in method section) As shown in Figure S7, a reversible change of the contact angle was clearly observed with different temperatures, indicating surface property of biotin grafted SiNWS was changed from hydrophobic (at 37 °C) to hydrophilic (at 4 °C).

5. Optimization of Thermoresponsive NanoVelcro CTC purification system

In order to optimize the operation parameters for conducting NSCLC CTC purification using Thermoresponsive NanoVelcro CTC purification system, the 1st-type artificial CTC sample containing EpCAM-positive H1975 NSCLC cells (200 cells mL⁻¹) and freshly purified human WBCs (5x10⁵ WBCs mL⁻¹) in a RPMI medium was prepared. For the convenience of cell counting, the H1975 cells were pre-stained with DiO green fluorescent dye. (This part was copied to method section) Besides the investigation of parameters including
flow rate, anti-EpCAM coverages and temperature alternating cycles, the distribution of immobilized CTCs in microfluidic channels and the limit for CTC capture performance in the presented system were also studied. Under the optimal capture conditions, the 1st-type artificial sample was introduced into Thermoresponsive NanoVelcro CTC purification system. After counting the substrate-immobilized cells under a fluorescence microscope (Nikon, 90i), the results summarized in Figure S8a suggest that 78% of the cells were captured in the first 4 channels of the anti-EpCAM modified Thermoresponsive NanoVelcro Chips. Additionally, we tested the dynamic range of the anti-EpCAM modified Thermoresponsive NanoVelcro Chips using a series of artificial NSCLC CTC samples that were prepared by adding DiO-stained H1975 cells into healthy donors’ blood at densities of 10, 50, 100, 200, 500, 800, and 1,000 cells mL⁻¹. The results indicate that Thermoresponsive NanoVelcro CTC purification system exhibited sufficient performance (Figure S8b) for clinical samples that normally have CTC density ranging from a few to hundreds of CTCs mL⁻¹.

![Figure S8](image_url)

**Figure S8.** Distribution of captured CTCs in the channels and the dynamic range for CTC capture.

6. **Viability and purity study on purified CTCs**

For testing the viability of CTCs over the purification and the feasibility of performing downstream molecular analysis, the 2nd-type artificial CTC samples was prepared by spiking EpCAM-positive H1975 NSCLC cells (approximately 200 cells mL⁻¹) into freshly collected human blood. (This part was copied to method section) Using these artificial samples (H1975 cells in 1.0-mL WBCs), we conducted CTC purification/viability studies according to an optimal CTC purification protocol. In a general workflow (Figure 3a), the 2nd-type of artificial blood samples were introduced into Thermoresponsive NanoVelcro Chips at 37°C at a flow rate of 0.5 mL h⁻¹. After 3 rounds of heating/cooling cycles, the specifically captured H1975 cells were released at 4°C under a flow rate of 0.5 mL h⁻¹ for 15 min. After parallel staining of FITC-labeled anti-CK, TRITC-labeled anti-
CD45 (a surface marker for WBCs) and DAPI, the purified cells were counted in a 96-well plate by a fluorescence microscope. We employed scatter plots (Figure 3b,c) to summarize one of the experimental outcomes. By repeating the CTC purification twice, the purity of CTCs can be further improved to 88-99%. Meanwhile, Acridine Orange/Ethidium Bromide (AO/EB, Cat# A3568/Cat# E1374) was obtained from Invitrogen and used for dual-fluorescent cell viability assay. The results suggested that the purified cells exhibited ca. 85% viability. In parallel, the culture expansion of purified cells was conducted in a RPMI medium. We were able to successfully expand the released H1975 cells up to two weeks (Figure 3d).

7. Performed mutational analysis (on EGFR gene) using the 2nd-type artificial blood samples

1 mL of the 2nd-type artificial blood sample (see Section 6) was prepared from freshly collected human blood for testing the feasibility of the mutational analyses on EGFR gene in the purified CTCs. Three 1.0-mL 2nd-type artificial blood samples were subjected to the 2-round purification protocol (Figure S9) to obtain purified CTCs. The purified CTC samples were subjected to DNA amplification by whole genome amplification (WGA) kit (REPLI-g, Qiagen). Subsequently, primers (sequences provided in Table S2) spanning EGFR exon 20 (covering codon 790) and 21 (covering codon 858) were used to amplify the resulting WGA DNA by PCR. The amplified DNA was then sent for Sanger sequencing. Either L858R or T790M mutation was detected in these purified CTCs (Figure S9).

| Table S2. EGFR Primers for point mutation analysis. |
|---------------------------------|------------------|
|                                | L858R            | T790M            |
| **Forward**                    | 5’-CTCAGAGCCTGGCATGAAC-3’ | 5’-CATTCTGCCTCCTACCTG-3’ |
| **Reverse**                    | 5’-ATCCTGCCCTGCATGTGTA-3’ | 5’-TTATCTCCCTCCCCGTATC-3’ |
8. Enumeration and molecular analysis of CTCs in NSCLC patients’ blood samples

From January to May of 2014, a protocol approved by Sun Yat-sen University’s Medical Ethics Committee (IRB, [2013] C-084 was adopted to recruit patients, who were diagnosed with advanced NSCLC (stages III and IV). The 7 NSCLC patients participated in our studies have signed the informed consent forms to release their disease classifications and diagnostic information for the purpose of validation. The tumors identified in these patients were then collected by bronchoscopy, and the tumors also displayed standard adenocarcinomatous traits when they underwent H&E staining. The immunohistochemistry (IHC) staining results of tumor tissue showed positively on M-CEA (carcinoembryonic antigen), TIF-1 (transcription factor-1), and CK-7 (cytokeratin-7) proteins expression. The patients’ treatment with gefitinib was completed in the Affiliated First Hospital, Sun Yat-sen University, and their clinical responses were evaluated according to Response Evaluation Criteria in Solid Tumors. From these patients, patient 1, 2, 3, 4 and 5 had stable disease upon the treatment, while patients 6 and 7 experienced cancer recurrence in their disease progression. Our Thermoresponsive NanoVelcro CTC purification system was applied on the blood samples from all seven patients. Furthermore, the mutational analysis for EGFR mutations was conducted on the purified CTC cells from their blood. The Sanger Sequencing results for mutation analysis are summarized in Table S2.

8.1 CTC Enumeration studies

1.0-mL Patient blood samples were introduced into Thermoresponsive NanoVelcro Chips at 37°C at a flow rate of 0.5 mL h⁻¹. Following the operation protocol summarized in Figure 4a (similar to those described in Section 6), the purified CTCs were collected in a 96-well plate. After parallel staining of FITC-labeled anti-CK,
TRITC-labeled anti-CD45 (a surface marker for WBCs) and DAPI, total numbers of CTCs per 1-mL blood were obtained by counting the CTC events (CK+/CD45-/DAPI+) under a fluorescence microscope (see Table 1).

### 8.2 CTC-based molecular analysis

Similar to CTC enumeration studies, separate batches of CTCs purified by Thermoresponsive NanoVelcro CTC system were subjected to molecular analysis. The purified CTCs were subjected to DNA extraction and whole genome amplification. Similar to the cell line studies (Section 7), the purified CTC samples were subjected to DNA amplification by whole genome amplification (WGA) kit (REPLI-g, Qiagen). Subsequently, primers (sequences provided in Table S1) spanning EGFR exon 20 (covering codon 790) and 21 (covering codon 858) were used to amplify the resulting WGA DNA by PCR. The amplified DNA was then sent for Sanger sequencing. The Sanger sequencing results on the CTCs and their matching NSCLC tissues are summarized in Table S3.
**Table S3.** Summary of Sanger Sequencing analysis for EGFR point mutations between NSCLC CTCs and tissues.

| Patient  | CTCs (EGFR point mutation status) | Tissue (EGFR point mutation status) |
|----------|-----------------------------------|--------------------------------------|
| Patient1 | ![CTC Graph](image1)              | ![Tissue Graph](image2)              |
| Patient2 | ![CTC Graph](image3)              | ![Tissue Graph](image4)              |
| Patient3 | ![CTC Graph](image5)              | ![Tissue Graph](image6)              |
| Patient4 | ![CTC Graph](image7)              | ![Tissue Graph](image8)              |
| Patient5 | ![CTC Graph](image9)              | ![Tissue Graph](image10)             |
| Patient6 (BT) | ![CTC Graph](image11) | ![Tissue Graph](image12) |
| Patient6 (AR) | ![CTC Graph](image13) | ![Tissue Graph](image14) |
| Patient7 | ![CTC Graph](image15)              | ![Tissue Graph](image16)              |

**Abbreviations:** CTC, circulating tumor cell; L858R, a single amino acid substitution from Leucine to Argine at codon 858; T790M, a single amino acid substitution from Threonine to Methionine at codon 790; BT, before treatment; and AR, at relapse.
9. Reference

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