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Capture and Stimulated Release of Circulating Tumor Cells on Polymer Grafted Silicon Nanostructures

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Keywords

Cancer Diagnosis; Circulating Tumor Cells; Nanostructured materials; Thermally responsive polymers; Cell Capture; Cell Release

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Circulating tumor cells (CTCs) are cancer cells that shed away from either primary tumors or metastatic sites.[1] They circulate in the peripheral blood as the cellular origin of metastases[2]. Comparing to biopsy, the gold standard of current cancer diagnosis, CTCs offer convenient and non-invasive access to tumor cells before fatal metastasis occurs. To exploit CTCs as a new cancer “biomarker” for disease progression and guided implementation of therapy, significant research endeavors[3] have been devoted to develop diagnostic assays capable of detecting and enumerating CTCs in cancer patients’ blood. For example, immunomagnetic separation approaches[4] utilize magnetic beads coated with CTC-specific capture agents (e.g., antibodies or aptamers) in order to capture CTCs. Based on them, CellSearch™ Assay is currently the only FDA-cleared CTC enumeration method that can predict prognostic outcomes in metastatic breast, prostate and colorectal cancer. However, the low CTC-capture efficiency encountered by CellSearch™ Assay does not fully address the major technical challenge on efficiently and specifically capturing CTC with extremely low abundance (a few to hundreds cells/mL of CTCs among a high number (10^9 cells/mL) of hematologic cells[5] in blood samples). Recently, several microchip-based technologies[6] have been developed to address this concern.

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Different from the existing CTC enrichment technologies, we pioneered a NanoVelcro cell-affinity assay, by which anti-EpCAM[7] (epithelial cell adhesion molecule)-coated nanostructured substrates (i.e., vertically oriented silicon nanowire substrates, SiNWS) were utilized to capture CTCs in a stationary device setting[8] with a capture efficiency ranging from 40 to 70%. The uniqueness of our NanoVelcro CTC assay is the use of a nanostructured substrate: The enhanced local topographic interactions[9] between the SiNWS and nano-scaled cellular surface components (e.g., microvilli) are analogous to the working principle of a velcro in nanoscale, resulting in a vastly enhanced cell-capture affinity compared to that observed for non-structured (i.e., flat) substrates. The general applicability of the NanoVelcro concept is supported by our recent studies, where we demonstrated that other types of nanostructured substrates, e.g., electrochemically deposited conjugated polymer nano-features,[10] and horizontally packed ultra-long TiO_2 nanofibers[11], also exhibit synergistic effects in conjunction with capture agents to achieve enhanced CTC-capture performance. Moreover, recent studies by other groups[12] also reported the utilization of SiNWS coated with immune cell-specific capture agents in order to sort subpopulations of immune cells.

Although the capturing and enumeration of CTCs provide preliminary diagnostic-relevant information, it is conceivable that the CTC-derived molecular signatures and functional readouts provide more valuable and significant insight into tumor biology during the critical window where therapeutic intervention could make a significant difference. In order to conduct molecular and functional analyses of CTCs, there is a desperate need to develop a new CTC assay that can not only capture CTCs with high efficiency, but also release CTCs with minimum contamination of the surrounding white blood cells (WBCs) and negligible disruption to CTCs' viability and functions. Although NanoVelcro cell-affinity assay using SiNWS exhibited enhanced cell capture performance, it has been proved difficult to release the immobilized cells (via enzymatic treatment) from capture agent-coated substrates. Only 10% of viable cells were released, and poor cell viability was observed. Herein, we introduce a new generation nanomaterial platform for cell-affinity assay that is capable of not only capturing CTCs with high efficiency, but also releasing the nanosubstrate-immobilized CTCs upon the application of an external stimulus (temperature change).

The idea is to covalently graft thermally responsive polymer brushes, poly(*N*-isopropylacrylamide (PIPAAm), onto SiNWS (Figure 1) by a surface initiated atom-transfer radical polymerization (polymer grafted SiNWS will be abbreviated as P-SiNWS).[13] In these polymer brushes, we strategically introduced covalently-linked biotin group by polymerizing isopropylacrylamide containing a small portion (2.5-10 %) of methyl aminoethylmethacrylate. The amino groups on the polymer brushes were then conjugated directly with activated biotin (biotin-NHS) to form biotinylated P-SiNWS (biotin-P-SiNWS). At 37 °C, the biotin groups and hydrophobic domains of these polymers are present on the surfaces of biotin-P-SiNWS. Through biotin-streptavidin interaction, the capture agent (i.e., biotinylated anti-EpCAM) can be introduced onto the substrates, enabling a highly efficient CTC capture that is comparable to that observed for the NanoVelcro cell-affinity assay.[8] When the temperature is reduced to 4 °C, the backbones of substrate-grafted PIPAAm undergo conformational changes, leading to an internalization of anti-EpCAM embedded inside the elongated polymer brushes. As a result, the nanosubstrate-immobilized CTCs are effectively released. PIPAAm[14] is a well-established biocompatible polymer, which can reversibly bind and release cells due to the thermally responsive switch of its surface properties. One of the most powerful utilities of a PIPAAm-grafted substrate is "Cell-Sheet" technology, where cells adhere to the hydrophobic domains of PIPAAm at 37 °C, followed by growing into confluency.[15] The substrates are then cooled down to below PIPAAm's lower critical solution temperature (e.g., 4 °C) to induce its surface hydrophobic-to-hydrophilic switch, allowing detachment of confluently cultured

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cells from the substrates to produce a “cell sheet”.[16] Clearly, PIPAAm’s operation temperature ensures minimum disruption to cells’ viability and functions during their seeding and releasing process.[17] When using biotin-P-SiNWS to capture and release CTCs, we will demonstrate that the same advantages do apply.

The biotin-P-SiNWS were prepared as illustrated in Figure 2a. First, we fabricated densely packed SiNWS with diameters of 100–200 nm and lengths of 15-20 μm on silicon wafers using a wet chemical etching method. Onto these SiNWS, an aminosiloxane mono-layer was assembled and the initiator for atom transfer radical polymerization (ATRP) was introduced through covalent amide linkage. Surface initiated ATRP was carried out with a mixture of isopropylacrylamide and methyl aminoethylmethacrylate to yield grafted polymer brushes. The polymerization was carried out for 6 hours because it was necessary to obtain polymers across certain length threshold to confirm the thermal response. On the other hand, the polymerization could not take place too long because the nanostructures could be lost (see Figure S1 in supporting information for how polymerization times affect the surface morphologies,). The molecular weight of these polymer brushes was measured as 8800 g/mol and the thickness was estimated as 14 nm.[18] Biotin moieties were then conjugated to the free amino groups of these polymer brushes to yield the desired biotin-P-SiNWS. Three biotin-P-SiNWS with functional group densities of 2.5, 5, and 10% were prepared and temperature-dependent contact angle measurements of water droplets (see Figure S3 in supporting information) were utilized to examine the thermoresponsive surface properties of these biotin-P-SiNWS. As shown in Figure 2b, all three biotin-P-SiNWS underwent reversible switches between hydrophobic and hydrophilic surfaces at 37 and 4 °C, respectively. There were only minute differences observed with respect to their biotin densities. To examine the effect of biotin densities on the cell capture and release performance, biotinylated anti-EpCAM (10 μg/mL) was introduced onto biotin-P-SiNWS containing 2.5, 5, and 10% biotin moiety via streptavidin conjugation (40 μg/mL). After the antibody introduction, the surfaces became hydrophilic at 37 °C (Figure 2c) and the surfaces no longer switched between hydrophobic and hydrophilic when the temperature changed. This is likely due to the charged antibody on the surface. However, thermoresponsiveness of the polymer brushes should be the same as illustrated in Figure 1 because the backbone movement of PIPAAm remained.

To study cell capture performance of the thermoresponsive NanoVelcro cell-affinity assay, a cell suspension (10^5 cells mL^{-1}) containing an EpCAM-positive breast-cancer cell line (i.e., MCF7) in a DMEM medium was prepared and then introduced onto anti-EpCAM-coated biotin-P-SiNWS (1 × 2 cm, placed into a commercial cell chamber slide), followed by 30-min incubation (5% CO_2, 37 °C). After rinsing, the substrate-immobilized cells (pre-stained with DiO green fluorescent dye) were imaged and counted by a fluorescence microscope (Nikon, 90i). Successively, cell release studies were carried out by the aforementioned chamber slide (with immobilized MCF7 cells on anti-EpCAM coated substrates) in a 4°C refrigerator for 30 min. The remaining MCF7 cells on the substrates were then quantified. The results summarized in Figure 3a suggested that biotin-P-SiNWS containing 10% biotin displayed the highest cell-capture performance at 37 °C, whereas the lowest cell retention was also observed at 4 °C. Given the optimal cell capture/release performance, we therefore focused our further characterization and optimization studies on biotin-P-SiNWS with 10% biotin. To test this substrate for repeated capture and release of cells, we performed multiple cycles of studies in sequence using MCF7 cells again. We observed a gradually attenuated cell capture/release performance (see solid line in Figure 3b) with an increasing number of experimental cycles. We hypothesize that the capture agent, anti-EpCAM, could dissociate from the polymer brushes as a result of its thermally responsive conformation changes during the capture/release processes. To validate this hypothesis, we repeatedly conducted

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anti-EpCAM conjugation prior to cell capture/release studies. By doing so the cell capture/release performances in new cycles were restored (see dashed line in Figure 3b).

It is unique that our platform integrates three different features for capture and release of cells. To validate the individual contribution of anti-EpCAM-coated biotin-P-SiNWS (10% of biotin) to the performance, three control studies were conducted using (i) PIPAAm-SiNWS: no capture agent to examine how temperature-induced conformational changes of PIPAAm contribute to cell capture/release, (ii) anti-EpCAM-coated SiNWS: no thermal responsiveness as the original NanoVelcro cell-affinity assay, and (iii) anti-EpCAM-coated biotin-P on flat Si chips: no nanostructures. The results summarized in Figure 3c suggested that all three factors (capture agent, thermally responsive polymer brushes and nanostructures) play indispensible roles in achieving the enhanced cell capture/release performance, supporting our original conceptual design (Figure 1). In the case of PIPAAm-SiNWS and anti-EpCAM-coated biotin-P on flat Si chips, the capture efficiency was lower. On the other hand, established anti-EpCAM-coated SiNWS[8] showed similar capture efficiencies with anti-EpCAM-coated biotin-P-SiNWS. However, these bound cells remained attached to the surface when the surface was cooled to 4 °C. Finally, we tested the general applicability and specificity of biotin-P-SiNWs for capturing EpCAM-positive cancer cells. Three EpCAM-positive cancer-cell lines (MCF7 cells, LnCAP and PC3 prostate cancer cells) were studied in parallel with two EpCAM-negative cancer-cell lines (HeLa cervical cancer cells and Jurkat leukemia cells) and freshly isolated human white blood cells (WBCs). Summarized results in Figure 3d suggested that, anti-EpCAM-coated biotin-P-SiNWS were capable of specifically capturing and releasing EpCAM-positive cancer cells. On the contrary, relatively low cell numbers were observed for EpCAM-negative cells (i.e., HeLa and Jaket), as well as human WBCs.

To test the dynamic rage of the thermoresponsive NanoVelcro cell-affinity assay, a series of artificial CTC blood samples was prepared by spiking DMEM medium and heath donors' blood with DiO-stained MCF7 cells at densities of 10, 50, 100, 500 and 1,000 cells per mL of blood. The results are summarized in Figure 4a. Anti-EpCAM-coated biotin-P-SiNWS showed vastly improved capture yields (>70 %), similar to those observed for the previously demonstrated anti-EpCAM-coated SiNWS[8]. The captured MCF7 cells were also confirmed by the conventional immunostaining approach (see Figure S5 in supporting information). Most importantly, anti-EpCAM-coated biotin-P-SiNWS is capable of capture and release viable cancer cells. When 1,000 MCFs cells are subjected for CTC capture studies, more than 90% cell can be captured (at 37 °C) and released (at 4 °C) from the substrates (white bar, Figure 4b). Further, approximately 90% of the released cells remained viable (white bar, Figure 4b), and they can be further expanded in culture (Figure 4c). In contrast, cells can be captured efficiently on anti-EpCAM-coated SiNWS while trypsin-treated cell release led to moderate cell-release performance and poor cell viability (black bars, Figure 4b).

In conclusion, we develop an innovative cell capture and release platform with integrated features of capture-agent directed specific recognition, nanostructure amplified cell capturing, and stimulated cell release based on thermally responsive polymer brushes. This platform demonstrates superior performances in (1) capturing cancer cells with high efficiency at 37 °C, and (2) releasing the captured cancer cells with great viability and retained functionality at 4 °C. Both 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 the CTC-derived molecular signatures and functional readouts obtained from such platform will provide valuable insight into tumor biology. Eventually, therapeutic intervention is properly guided

to make a significant difference during the critical window of tumor progression and metathesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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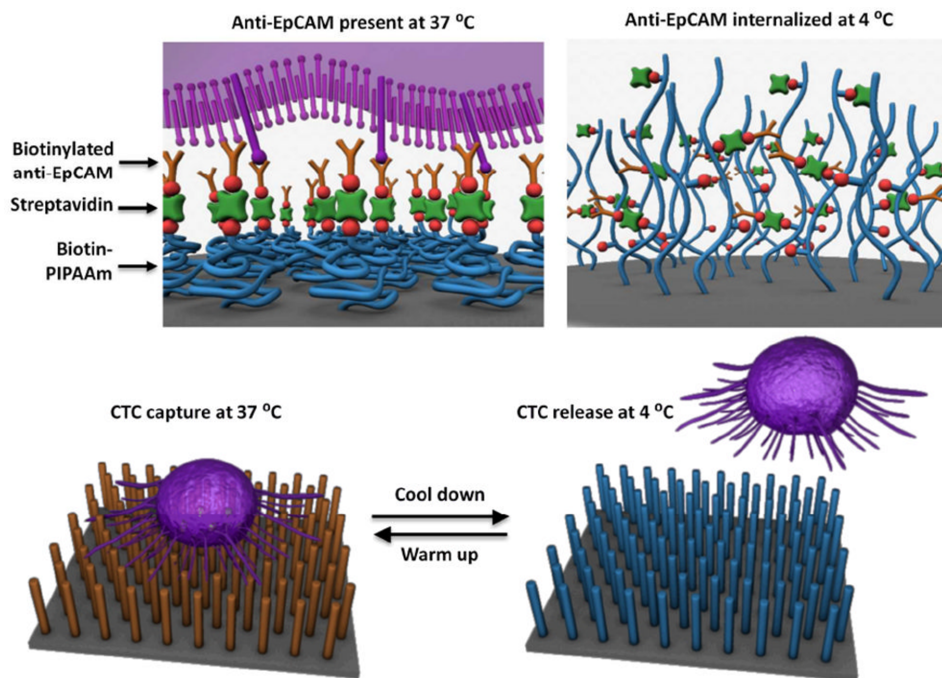

Figure 1.
Conceptual illustration of a new-generation nanomaterial-based platform for cell-affinity assay capable of not only capturing CTCs with superb efficiency, but also releasing upon stimulation these captured CTCs at a reduced temperature. In order to confer thermal responsiveness onto NanoVelcro CTC assay based on silicon nanowire substrate (SiNWS), biotin-functionalized polymer brushes (i.e., PIPAAm) are covalently grafted onto SiNWS. At 37 °C, the biotin groups and hydrophobic domains of biotinylated PIPAAm are present on the surfaces. *Via* biotin-streptavidin interaction, biotinylated anti-EpCAM can be introduced, enabling highly efficient CTC capture. When the temperature is lowered to 4 °C, the backbones of surface-grafted biotinylated PIPAAm expand, resulting the internalization of anti-EpCAM and releasing CTCs from the substrates.

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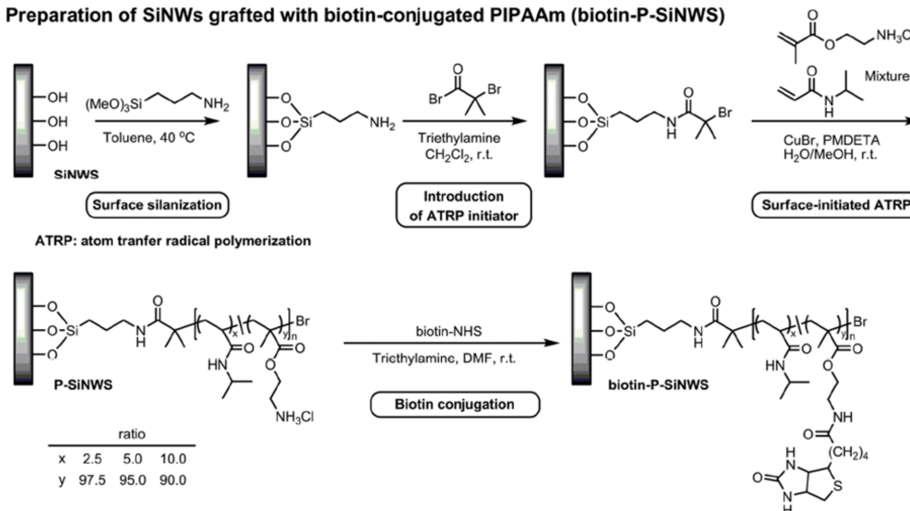

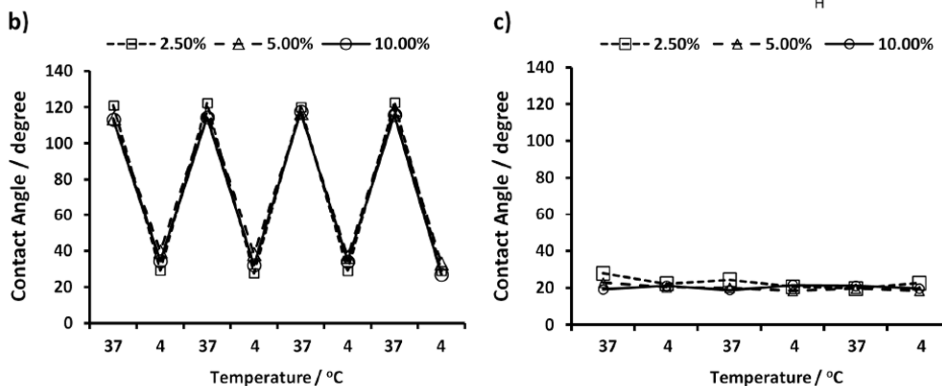

Figure 2.

a) Synthetic approach employed to covalently graft biotinylated PIPAAm onto SiNWS (biotin-P-SiNWS). By altering the ratios of the two monomeric precursors, three different densities (2.5, 5 and 10%) of biotin groups were incorporated onto the resulting biotin-P-SiNWS. b) Contact angle measurements were employed to examine thermal responsiveness of the three biotin-P-SiNWS. c) After introducing anti-EpCAM, the surfaces of all three biotin-P-SiNWS become hydrophilic at both 37 °C and 4 °C.

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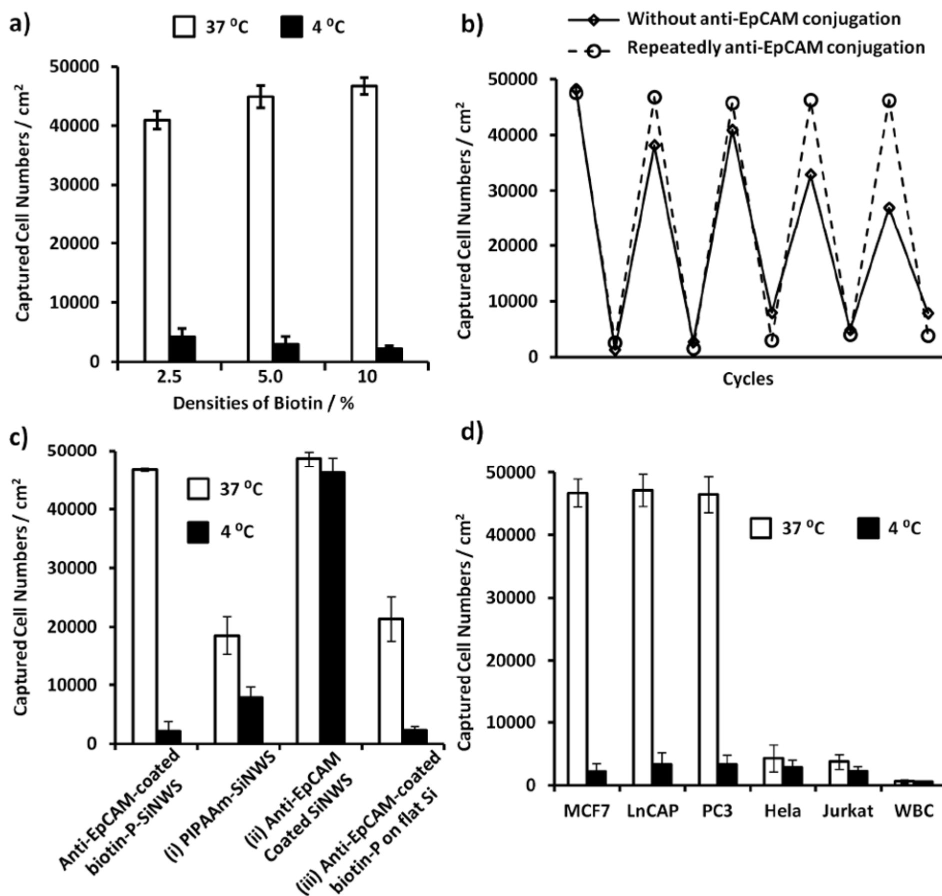

Figure 3.

a) Quantitative evaluations of cell capture/release performance of three biotin-P-SiNWS with different densities (2.5, 5 and 10%) of biotin group. b) Cell capture/release performance of biotin-P-SiNWS in multiple/sequential rounds of studies with and without repeated anti-EpCAM conjugation. c) Comparison of cell capture/release studies of anti-EpCAM-coated biotin-P-SiNWS with three control samples in parallel: (i) PIPAAm-SiNWS (no anti-EpCAM), (ii) anti-EpCAM-coated SiNWS (no PIPAAm), and (iii) anti-EpCAM-coated biotin-P on flat Si chips (no nanostructures). d) Quantitative evaluations of general applicability and specificity of biotin-P-SiNWS using three EpCAM-positive cancer-cell lines (i.e., MCF7, LnCAP and PC3 cancer cell lines), two EpCAM-negative cancer-cell lines (i.e., HeLa and Jurkat cell lines) and freshly isolated human white blood cells. The biotin-P-SiNWS used contains 10% biotin groups.

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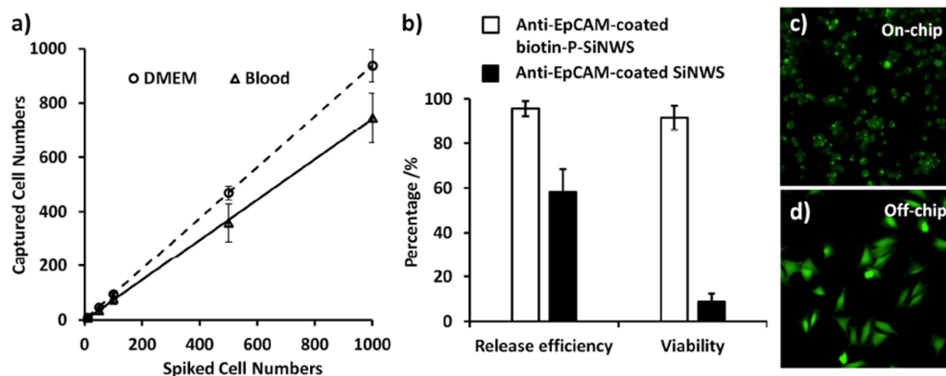

Figure 4.

a) Cell-capture efficiency at different cell numbers ranging from 10–1000 cells mL^{-1} in two different types of samples: DMEM medium (○) and whole blood (Δ). b) Cell-release performance and the viability of released cells observed for MCF7 cell capture/release studies using anti-EpCAM-coated biotin-P-SiNWS (white bars) and SiNWS (black bars). c) and d) DiO-stained MCF7 cells were successfully cultured after capture and released from anti-EpCAM-coated biotin-P-SiNWS.