Electroactive Platforms

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An Electroactive Biotin-Doped Polypyrrole Substrate That Immobilizes and Releases EpCAM-Positive Cancer Cells**

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Abstract: *The specific capture and remotely controlled release of the EpCAM-positive cancer cells from biotin-doped polypyrrole (Ppy) films in response to an electrical potential is presented. As Ppy allows the direct incorporation of biotin molecules during the electrochemical process, densely packed biotin molecules can serve as the binding sites for streptavidin-tagged biomolecular complexes. This study demonstrates not only the enhanced capture and enrichment of EpCAM-positive cancer cells but also "on-demand" release of the viable cells from conductive Ppy in an electrical-potential-dependent way. This novel approach is of great importance in a diverse range of applications, and in particular in cancer diagnostics and screening.*

Significant efforts have been directed toward the development of novel strategies for sorting, characterizing, and subsequently releasing desired pure cells from complex cell mixtures.[1–3] Cell isolation and detailed analysis of purified cells is essential for research in a variety of fields such as fundamental biology and for the development of new clinical diagnostics and therapeutic modalities. The isolation of rare cells (for example, cancer stem cells and circulating tumor cells) from various biological sources is of great importance because the study of rare types of cancer cells is critical to unraveling previously inaccessible mechanisms that might be associated with cancer development and progression. In particular, because circulating tumor cells play an important role in the metastasis of cancer, their detection could have an impact on establishing the presence of metastasis, which could be useful in point-of-care medical tests.[4–6] Approaches that rely primarily on antigen–antibody affinity by recognizing biomarkers found on target cell membranes with high affinity and specificity have been developed. These include immune-magnetic beads, micro- and nano-structured surfaces, and microfluidic devices.[7–13] Compared with traditional bench-top methods (for example, flow cytometers and isolation by size of epithelial tumor cells), current platform-based technologies have demonstrated improved cell recovery and purity and enhanced enrichment of target cells from blood samples. However, although recent findings have typically focused on enhancing capture yield and sensitivity, techniques for demonstrating the feasibility of non-destructive release of captured cells and subsequent characterization of retrieval cells have not been actively developed.

This study is an initial attempt to develop a novel biotin-doped conductive polypyrrole (Ppy) platform ideally suited for the specific capture and enrichment of epithelial-cell adhesion molecule (EpCAM)-positive cancer cells and their subsequent non-destructive, weak electrical-potential-mediated release. Conducting polymers, such as Ppy, have widely been used in novel polymeric implants or even as drug carriers because they can accommodate a variety of anions and cations, including growth factors, anti-inflammatory drugs, ATP, glutamate, and protonated dopamine through simple electro-polymerization.[14–22] The dopants embedded in the Ppy membrane are specifically released when triggered by electric fields. In parallel with advances in drug delivery systems, we applied this strategy to capture and release EpCAM-positive cancer cells efficiently. Biotin, as a counter-anion for Ppy formation, offers significant affinity interactions with targets through biotin-streptavidin coupling, ultimately yielding a surface that is readily available for the sequential absorption of biotinylated anti-EpCAM (Scheme 1, upper inset). Given that most surfaces are limited to inducing suitable functionalities with various biomolecules, incorporation of the biotin moiety into Ppy can provide strong binding or tethering sites for sequential reactions. Such biotin-doped Ppy surfaces would ultimately not only make these surfaces compatible with a variety of conjugation strategies, but also greatly enhance structural and functional stability. More recent findings have indicated that Ppy-based platforms specifically respond to external electrical stimuli through repetitive oxidation and reduction processes of Ppy that ultimately provide valuable insights into "smart" drug-delivery systems.[23–27] We expanded on this concept to establish an efficient alternative for the non-destructive detachment of captured cells. Toward this end, we plan to demonstrate "on-demand" release of captured cells depending on the magnitude of electric field (Scheme 1, bottom). The reduction of the polymeric backbone of biotin-doped Ppy occurs in response to the electrical stimulation, consequently causing the release of the biotin and attached biomolecules. To demonstrate the feasibility of this approach, we initially characterized the effect of anti-EpCAM on the preferential adsorption of a cell suspension (2×10^6 cells mL^{-1}) of EpCAM-positive cancer cells (MCF7 breast cancer and PC3 prostate cancer cells) and EpCAM-negative cancer cells (HeLa cervical cancer cells) on Ppy substrates.

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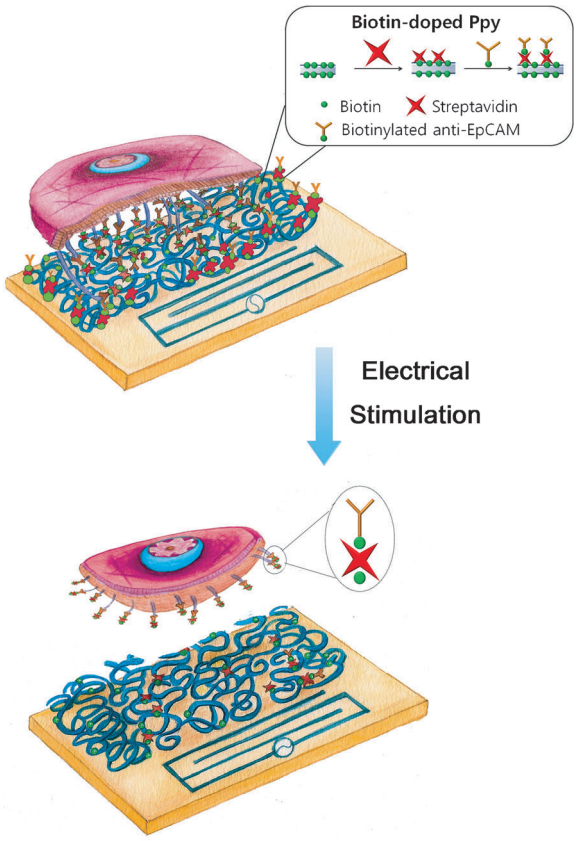

Scheme 1. Depiction of the specific capture of epithelial cell adhesion molecule (EpCAM)-positive cancer cells using an electroactive anti-EpCAM-immobilized, biotin-doped polypyrrole (Ppy) platform (upper), and "on-demand" release of captured cells via application of a direct current electric field (bottom). By integrating biotin as a doping compound, electrical potential can modulate the release of the biotin and attached cells by inducing the reduction of the Ppy polymeric backbone.

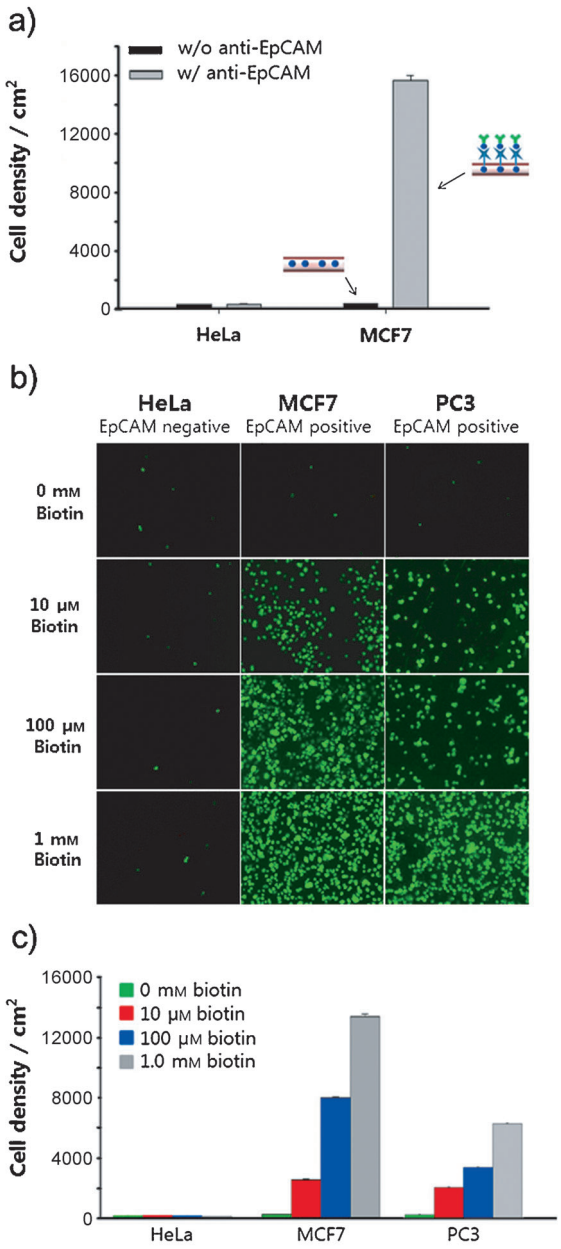

Figure 1. a) Evaluation of the cell-capture yield of the biotin-doped Ppy platform with or without subsequent anti-EpCAM conjugation on EpCAM-negative (HeLa) and EpCAM-positive (MCF7) cancer cells. b) Fluorescence images of HeLa, MCF7, and PC3 cells captured on an anti-EpCAM immobilized, biotin-doped Ppy platform as a function of biotin concentration. c) Quantitative analysis of capture yield of various cell lines on anti-EpCAM-immobilized, biotin-doped Ppy surfaces labeled at different densities of biotin.

As can be readily observed in Figure 1a, no relationship was apparent between the presence of anti-EpCAM attached on the Ppy substrate and the adherence of HeLa cells. Conversely, MCF7 cells appeared to be strongly correlated with the existence of anti-EpCAM. Such behavior most likely relied on the targeting capability of anti-EpCAM immobilized biotin-doped Ppy substrate to MCF7 cells bearing the highly expressed membrane antigen, EpCAM. We next attempted to elucidate the effect of cell density on the concentration of biotin doped within Ppy (Figure 1b,c). Indeed, the biotin amount incorporated in the Ppy substrate seems to be strongly correlated with the number of anti-EpCAM immobilized on the surface. In case of EpCAM-negative HeLa cells, no apparent difference in capturing efficiency occurred regardless of the concentration of biotin applied. However, the obtained MCF7 and PC3 cell density was linearly proportional to the amount of biotin bound to Ppy. A high density of biotin receptors leads to increased absorption of anti-EpCAM, which increases the number of available sites for EpCAM-positive cells. Figure 1c shows more details with regard to the number of cells bound depending on the amount of biotin incorporated within Ppy.

With the increased concentration of biotin, the greater would be the buildup of EpCAM-positive cancer cells. Another intriguing approach contained in this study is to demonstrate the spontaneous redox behavior of the conducting polymer. In other words, a weak electric potential can modulate the unique interface between captured cells and biotin-doped Ppy, thus efficiently releasing the biotin entity in a controlled manner.

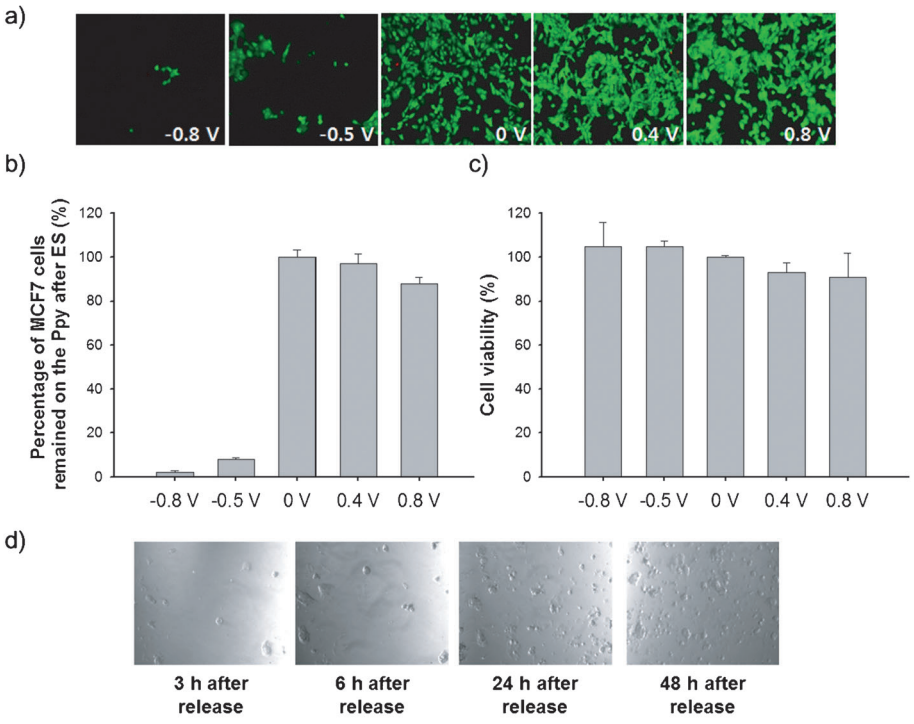

Figure 2. a) Release profiles of MCF7 cells captured on the anti-EpCAM immobilized, biotin-doped Ppy platform in response to applied electric fields ranging from +0.8 V to −0.8 V for 15 s. b) Quantitation of the percentage of MCF7 cells remaining on the surfaces after electric stimulation (ES) for 15 s. c) Viability of MCF7 cells released from Ppy surfaces after a 15 sec exposure to various electrical potentials. Released cells were washed twice with PBS and incubated at 37 °C and 5 % CO_2 for 48 h. d) Optical microscope images showing time-dependent spread and proliferation of the cells released after electrical stimulation at −0.8 V for 15 s.

We attempted to develop a method for non-destructively releasing captured cancer cells from anti-EpCAM-immobilized, biotin-doped Ppy (Figure 2). Before the experiment, MCF7 cells (2×10^6 mL) were captured on the Ppy surface with 1 mM biotin doping, and unbound or nonspecifically bound cells were removed by repeated washing. To investigate the effect of the electrical fields on cells, we applied a variety of potentials to MCF7 cells on Ppy surfaces. After 15 seconds of electric stimulation ranging from −0.8 V to +0.8 V, the captured cells were detached from the surface with gentle agitation of the cell-laden Ppy surfaces in 1× phosphate-buffered saline (PBS). Then, the substrate-immobilized cells were rinsed, fixed, and stained with a live/dead cell marker. Interestingly, the release pattern showed no sensitivity to those of positively stimulated Ppy surfaces; resulting in similarity to the control with no stimulation. Consistently showing programmable release of gold nanoparticles from biotin-doped Ppy film, preferential cell detachment and release was observed only when the surfaces were subjected to negative electrical potential.[26] As seen in Figure 2b, only 10 % of the cells remained on the surface after exposure to −0.5 V, and most of the cells were released from the Ppy surfaces after electrical stimulation with −0.8 V. A voltage higher than −0.8 V induces characteristic morphological changes of cells while lower voltage than −0.1 V leads to statically insignificance in the amount of released cells compared to controls. These results support the variation in the binding affinity between biotin-doped Ppy and streptavidin-tagged cell complexes that might arise from charge transfer coupled to the reduction–oxidation behavior of this engineered surface. In detail, the major factors affecting the release of captured cells would be associated with the reversible volume change occurring as a result of the electrochemical reaction in conducting polymer chains during reduction–oxidation cycles. According to a previous study, the repetitive reduced and oxidized state results in an large volume change, up to 35 %, in response to the applied voltage.[28] This process readily accounts for the release of large numbers of captured cells at negative potentials by altering the chemical strength of the Ppy-biotin interaction. Consequently, the released cells were collected and re-cultured to test viability. In Figure 2c, weak electric fields did not affect cell viability with no cytotoxic effect and retrieved cells were successfully cultured for more than 5 passages (3 days/passage), emphasizing that the biotin-doped Ppy platform appears to be most promising as an active cell capture/release platform. The optical microscopic images in Figure 2d give more details on the morphological changes of the released cells as a function of time after plating on a Petri dish. Soon after electrical stimulation, cells have a more round or flattened appearance, but they progressively spread normally with time, returning to fully viable biological function. On the other hand, elucidating the electrochemical behaviors of biotin-doped Ppy films is essential for evaluating the electron-transfer capability of electroactive ferricyanide species throughout Ppy surfaces. As seen in the Supporting Information, Figure S1a, the biotin-doped Ppy surface shows obvious peak potentials for oxidation and reduction current, whereas the Ppy surfaces with biomolecular layers exhibit significant changes in redox peaks. This behavior is most likely caused by the immobilization of anti-EpCAM and MCF7 cells on the Ppy surfaces, which readily blocks the free transfer of electrolytes, thereby varying current intensity and peak positions. However, the application of electrical stimulation of −0.8 V for 15 sec was sufficient to restore the peak intensity by preferentially removing the biomolecular assemblies from the Ppy surfaces. The total peak currents obtained by cyclic voltammetry conducted using ferricyanide solutions as sensitive redox indicators are depicted in the Supporting Information, Figure S1b. This observation reflects the fact that such electrochemical responses would be direct evidence of the adsorption and desorption process of the anti-EpCAM/MCF7 cells

from the Ppy surfaces. On the other hand, it is crucial to note that detailed molecular characterization of recovered cancer cells provides important insights into tumor biology. To obtain the molecular expression profile of cells released using an electric-field-mediated approach, we initially evaluate the magnitude of EpCAM antigen expression by assessing the immunofluorescence and quantifying by a Fujifilm Multigauge 3.0 (Figure 3a).

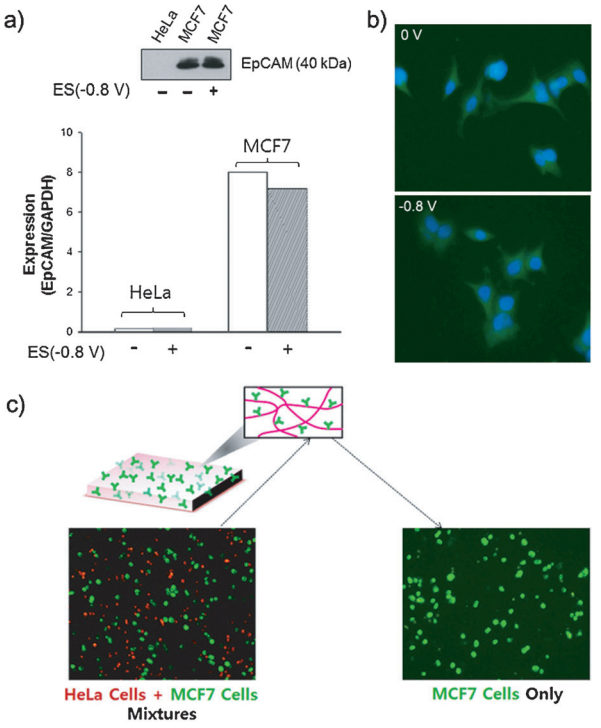

Figure 3. EpCAM protein expression. a) upper: western blot analysis to measure the effect of electrical stimulation (presence, +; absence, −) on EpCAM expression in HeLa and MCF7 cells. The electrical stimulation (ES) was carried out at −0.8 V for 15 s; lower: western blotting results summarized in a histogram in which protein expression was calculated using a Fujifilm Multigauge 3.0. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. b) Detection of EpCAM expression in MCF7 cells in the absence and presence of an electric field at −0.8 V for 15 s. The cells were stained using anti-mouse immunoglobulin G secondary antibody (green) and counterstained with Hoechst 33342 (blue). c) Specific capture of EpCAM-positive MCF7 cells from a mixture of cells using anti-EpCAM immobilized, biotin-doped Ppy surfaces. The proposed platform demonstrated 93% capture efficiency with 95% purity.

Interestingly, negligible effect on the EpCAM protein band in MCF7 cells was observed even when using a negative potential of −0.8 V for 15 s. It is apparent, therefore, that the applied electric field must be chosen to untangle the biotin moieties from Ppy backbone, which would reliably ensure that cell structure and biological functions are not compromised. We also visualized EpCAM expression in MCF7 cells after electrical stimulation by staining the cells with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G secondary antibody (green) and counterstaining the nuclei with Hoechst 33342 (blue; Figure 3b). Similar to results in the control cells (0 V), EpCAM protein was abundantly expressed in green and mostly localized in the cytoplasm and membrane of the released cells. To demonstrate the specific recognition of EpCAM-positive cells by the biotin-doped Ppy platform, we applied a 1:1 mixture of MCF7 cells (green; 2×10^6 cells mL^{-1}) and HeLa cells (red; 2×10^6 cells mL^{-1}) to the EpCAM-labeled platform (Figure 3c). As we had hypothesized, the majority of the cells captured by the biotin-doped Ppy surfaces were green (that is, MCF7 cells) with approximately 95% purity. Overall, the proposed approach appears to be a valuable method for cell capture and non-destructive cell release with high efficiency and purity.

Finally, to evaluate the potential use of our approach in a clinical setting, we examined the cell capture and release performance using artificial blood samples that were prepared by ex vivo spiking MCF7 cells (10–100) into 1.0 mL of whole blood from healthy donors. Whole blood was collected in Vacutainer tubes containing the anti-coagulant EDTA under NCC Institutional Review Board Approval. As shown in Figure 4b, a series of cell spiking experiments demonstrated high capture efficiency regardless of the number of cells spiked. Additionally, the electrically stimulated Ppy platform enabled the selective and non-detrimental release of cancer cells, consequently yielding the recovery rates of more than 85% (Figure 4c). After being recovered, MCF7 cells could be continuously sub-cultured with multiple passage number. On the other hand, the number of captured and released white blood cells (WBCs) was negligible compared to the original amount in the sample (5×10^6 mL). As our findings show, the selective release of MCF7 cells from biotin-doped Ppy polymer was triggered by applying a potential (from −0.8 to −0.1 V) for 2 seconds, whereas nonspecifically trapped blood cells (WBCs and RBCs) have no immediate effect on the electrical stimulation, resulting in the release of statistically insignificant numbers of these cells (Supporting Information, Figure S2). We also performed the capture and release studies using whole blood from healthy donors ($n=5$). Very few WBCs (<0.004%) were captured on Ppy-based platform, indicating very low probability of giving a false positive in a real experiment. To identify and enumerate, the cells captured on biotin-doped Ppy and released after electrical stimulation were further characterized by staining cell specific phenotype markers, such as Hoechst for DNA content, anti-cytokeratin (CK) antibodies for epithelial cells, and anti-CD45 antibodies for haematologic cells (Figure 4d). MCF7 cells captured and subsequently released displayed strong cytokeratin positive and CD45 negative expression. Furthermore, Hoechst staining exhibits nuclear integrity of the captured and released MCF7 cells. Indeed, when considering several attributes such as 1) simple assembly of cell-specific antibody via biotin moiety embedded into Ppy film, 2) redox-responsive features of conductive polymer, and 3) a weak electric-field triggered programmable release of dopants, developing and validating such versatile conductive architecture is crucial for enabling subsequent culture of the rare types of cancer cells and downstream cell molecular analysis.

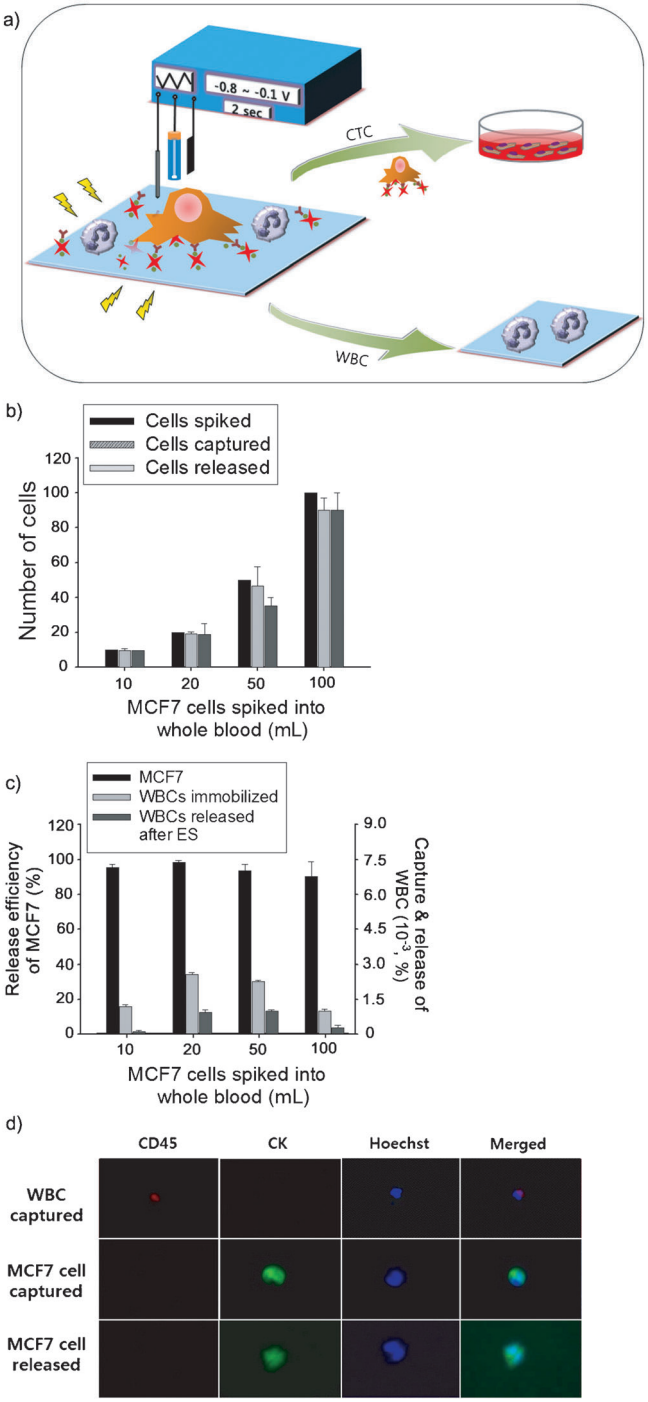

Figure 4. a) Electrically stimulated release of EpCAM-positive MCF7 cells from human blood samples. Negative voltage applied across the polymer induces the release of the biotin and the attached MCF7 cells from Ppy. b) The number of MCF7 cancer cells spiked, captured on anti-EpCAM-immobilized, biotin-doped Ppy platform from spiked whole blood, and released by applying an electric field at −0.8 V for 2 s. c) The capture and release efficiency of spiked MCF7 cells and WBCs under various numbers of spiked target cells. The number of immobilized and released WBCs is substantially lower than to the initial number of WBC (5×10^6 cells mL^{-1}), indicating that such biotin-doped Ppy provides excellent cell purities. d) Immunofluorescence images of captured MCF7 and WBCs and released MCF7 cells. Cells were stained with Hoechst33342, cytokeratin, and CD45. All of the studies were performed in quintuplicate.

In conclusion, we have demonstrated the use of our novel biotin-doped conductive Ppy platform capable of capturing EpCAM-specific cancer cells and non-detrimentally releasing the captured cells following the application of a weak negative potential. Our results indicate that this approach will be a valuable and flexible method in both basic and clinical cancer-related applications because the early detection of malignant cells is critical for cancer diagnosis. In the future, this novel system will be combined with conventional microfluidic devices in which stimuli-responsive release of captured cells will be used to separate and sort different types of cells by taking advantage of their intrinsic phenotype and function in the microdevices, ultimately enabling cell-based functional assays.

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