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Effective and Selective Cell Retention and Recovery from Whole Blood by Electroactive Thin Films

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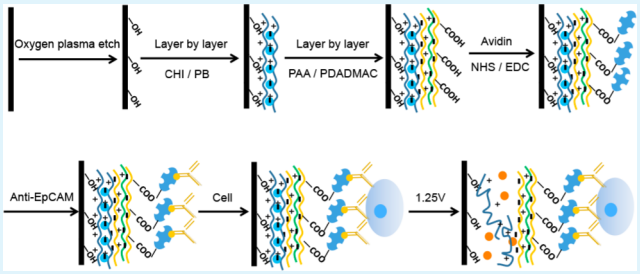
Supporting Information

ABSTRACT: Hematogenous metastatic spread causes most cancer patient deaths. Because circulating tumor cells (CTCs) are highly relevant to early metastatic spread, the capture or detection of these cells provides a diagnostic tool for patients with metastatic conditions. Herein, we demonstrate a programmable electroactive multilayered material platform with a smart electrically induced "switch" that captures CTCs from biological plasma with high efficiency and releases the captured cells flexibly. The released cells are still viable and proliferative, which facilitates the detection of trace levels of CTCs by amplification. Furthermore, the inherent rough characteristics of the nanoparticle-composed interface can promote capture efficiency and cell purification by integration with a simple microfluidic device. This elegant, inexpensive, and versatile platform for cell sorting and enrichment makes subsequent molecular and cell biological analysis achievable. The strategy has broad implications for favoring fundamental cancer biology research, for the diagnosis and monitoring of cancer individually, and for advanced intervention based on blood purification.



KEYWORDS: *cell sorting, circulating tumor cells, controlled release, electroactive, multilayer films*

INTRODUCTION

Globally, cancer constitutes the main cause of death,[1] ostensibly because of a limited access to timely diagnosis and accurate treatment.[2] The spread of cancer cells, also known as hematogenous metastatic spread, homing in on distant organs and subsequent formation of overt metastases, leads to the death of most cancer patients. The classical view is that the blood-borne circulation-mediated dissemination of primary tumor cells from original to distant sites occurs only during advanced (i.e., at very late stages of) malignancy; however, recently a wealth of evidence suggests that metastatic spread may well be a very early event.[3] It is proposed that detachment of cancer cells from primary solid tumors, and migration to distant organs, is mediated by blood circulation, creating what are known as circulating tumor cells (CTCs).[4] Detailed analyses of these cells may pave the way not only for gaining more insight into cancer problems but also for improving cancer diagnostics and treatment in patients with metastatic conditions.[5,6] When CTCs can survive existing chemotherapy treatments, that could suggest unsuccessful therapeutic interventions; the profiling of these cells should be investigated, and a potential switch in treatment modality is required in clinical trials.[4] Furthermore, molecular analysis of CTCs paves the way for information-gathering with regard to cancer dormancy as well as genomic heterogeneity and phenotypic diversity, which might lead to the identification of tumor-initiating (cancer stem) cells with important implications for precise therapies.[7]

Research on the capturing and enumeration of CTCs focuses on the development of new methods to provide information relevant to less-invasive and more-reliable diagnostics, which distinguishes itself from conventional methodologies such as clinical evaluation, radiographic screening,[8] and the analyses of serum tumor markers.[9] Several technological approaches for

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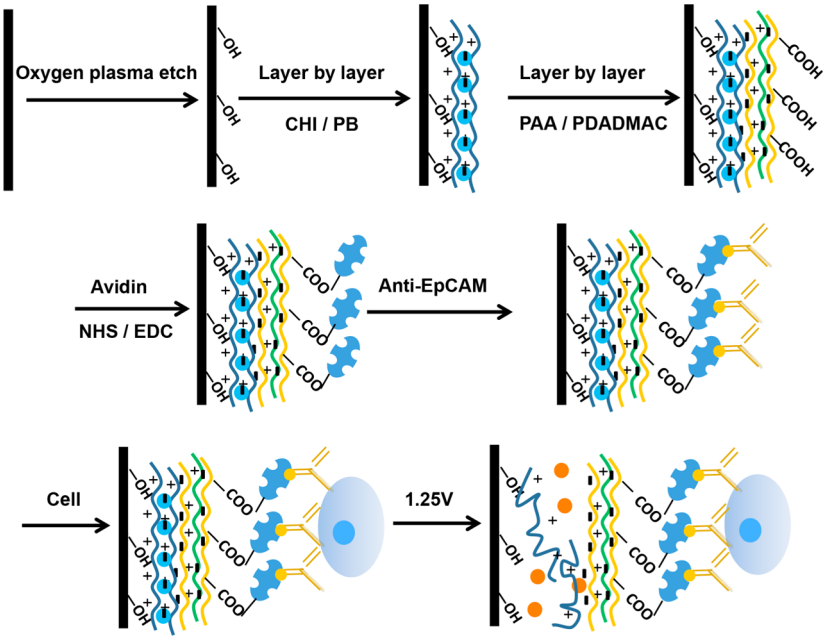

Figure 1. Schematic illustration of the fabrication of the electroactive thin films and their electrotriggered cell harvest.

sorting of CTC based on their physical characteristics such as cell size,[10−12] deformability, or density,[13] dielectrophoretic signature[14] or immune-separation with tools, such as magnetic beads,[15] ferrofluids,[16] or rosettes, have been developed.[17] The clinical assessment of CTCs is currently used for the assessment of prognosis and systemic therapy in some groups.[18] Our capability to detect, sort, and molecularly and functionally analyze CTCs could facilitate discovery of signatures specifically associated with cancer stem cells and broaden the horizon for understanding the biology of metastatic conditions.[19] Hence, there is an urgency to devise a strategy to not only capture CTCs with high efficacies and accuracies but also to recover cells with minimal disruption.

Materials equipped with high-affinity ligands are extensively utilized for specific cell retention with high efficacies by way of polyvalent ligand−receptor recognitions.[20] However, it remains strategically difficult to realize efficient cell release while maintaining structural and functional integrities of cells. Smart materials that are responsive to external stimuli that involve enzyme interaction,[21−23] pH, glucose levels,[24] temperature,[25] light, electricity,[26] compression, and so on, provide the opportunities for solving the problem. In the present work, we develop a programmable nanomaterial platform with a smart electrically induced "switch" that is able to not only selectively and effectively capture CTCs but also flexibly release/recover them. Such a switch effect is beneficial for further molecular characterization and biological analyses. The electrically induced switch is mainly composed of Prussian Blue (PB), which is a well-known, nontoxic, FDA-approved inorganic iron hexacyanoferrate compound with many excellent properties, such as electrochromism, electrochemical, and magnetic properties. A potential of 1.25 V transforms this compound from the PB (negative) to the Prussian Brown (PX, neutral) state, thereby switching between "closed" and "open" states. This may provide a strategy for integrating bioelectronics devices with this material platform and its binary control scheme.

EXPERIMENTAL SECTION

Materials. Cell culture media (Dulbecco's modified eagle medium, RPMI-1640 growth medium) were obtained from Invitrogen (Life Technologies, CA, U.S.). Fetal bovine serum (FBS) was obtained from Sigma-Aldrich (St. Louis, MO, U.S.). Indium tin oxide (ITO)-coated glass substrates were obtained from Delta Technologies (Chicago, IL, U.S.). Biotinylated antihuman EpCAM antibody was purchased from R&D systems (Minneapolis, MN, U.S.). 1-(3-(Dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC) was obtained from TCI (Tokyo, Japan). All other chemicals were purchased from Wako (Tokyo, Japan).

Cell Line Experiments. Tumor cell lines of different cancer types were all obtained from ATCC. They were maintained and grown to confluence in the appropriate medium supplemented with 10% FBS at 37 °C with 5% CO_2. The cells were digested by trypsin and suspended. The cell number was counted with a hemocytometer. The concentrations of suspended cells ranging from 10^2 to 10^5 per mL were prepared by serial dilutions of starting cell suspensions in PBS, whole blood, or lysed blood.

Preparation of Layer-by-Layer Solutions. The preparation of Prussian Blue (PB) nanoparticle solution was accomplished as described previously.[27] The concentration of chitosan (CHI, low molecular weight, Aldrich) was prepared as 1.5 mg/mL in 0.1 M acetic acid. Poly(acrylic sodium) (PAA) and poly(diallyldimethylammonium chloride) (PDADMAC) were dissolved in 0.5 M NaCl at a concentration of 5 g/L. Deionized water was employed for preparation of all the solutions.

Construction of the Electroactive Films by Electropolymerization. ITO glass substrates were cleaned via ultrasonication in a series of dichloromethane, acetone, methanol, and deionized water for 15 min separately. To ensure clean surfaces with abundant hydroxyl groups, the oxygen plasma etching was carried out with Harrick PCD 32G (Ithaca, NY, U.S.) for 5 min. Subsequently, freshly prepared glass substrates were immersed in CHI solution for 1 h, and constructed with $CHI(PB/CHI)_{27}$ as follows. Each individual layer was assembled by separately immersing in the polyelectrolyte CHI or PB solution for 10 min, followed by a successive rinsing cycle in pH 4.0 water baths three times to remove weakly bound polyelectrolytes. The films were dried under nitrogen finally.

Characterization of the Electroactive Films. Absorbance of the films was measured by UV spectrophotometry. The thickness of the films was determined in the range of 190−1700 nm by a Spectroscopic

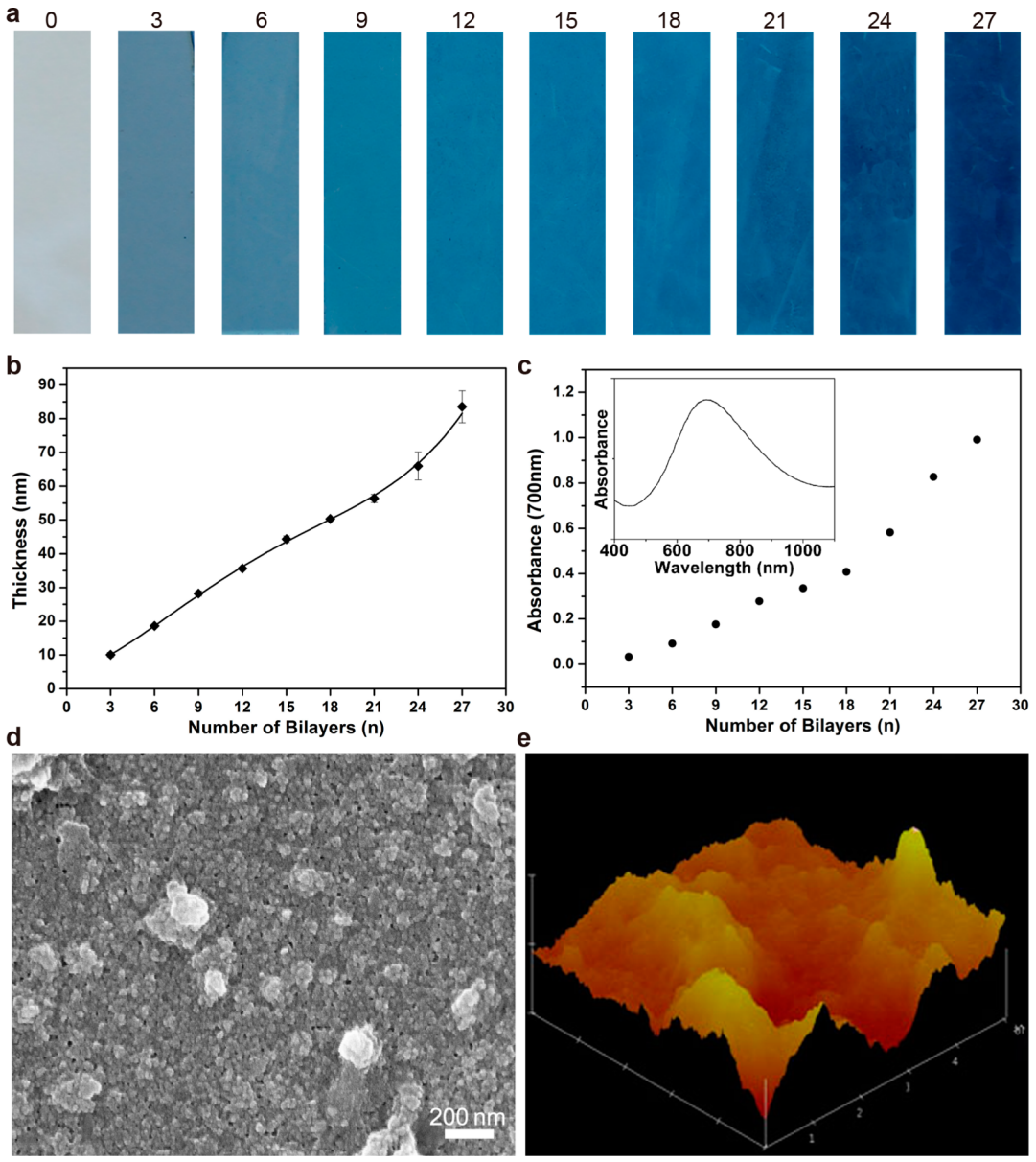

Figure 2. Characterization of the electroactive thin films. (a) Photographs of the CHI(PB/CHI)$_n$ films when n = 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27. (b) Thickness and (c) absorbance values at 700 nm vs the number of deposited bilayers. (d) SEM and (e) AFM images of the film of 27 bilayers. The scale bar is μm.

Ellipsometer (M-2000 Ellipsometer, J.A. Woollam, NE, U.S.). Surface morphology and roughness were characterized via scanning electron microscopy (SEM) (JSM-35CF, JEOL, Japan) and atomic force microscopy (AFM) (Veeco, Santa Barbara, CA, U.S.).

Deconstruction of the Electroactive Film. Electrochemical deconstruction studies were conducted with a computer-controlled EG&G PACR 263 potentiostat/galvanostat (Princeton Applied Research, Oak Ridge, TN, U.S.). The setup of electrode and electrolyte was adopted from a previous report.[26] Spectroscopic characterization and cell release were performed and recorded during the process.

Bioconjugation on the Electroactive Film. Carboxylic functionalized films were fabricated with PAA and PDADMAC on a layer-by-layer basis. Then, the film surface was modified with EDC (0.2 M) and *N*-hydroxysuccinimide (0.05 M) in water. The substrate was treated with avidin (10 μg/mL) for 90 min. Subsequently, biotinylated antihuman EpCAM antibody (25 μL, 10 μg/mL in 1× PBS containing 0.1% BSA) was placed on the substrate and incubated for 60 min at 37 °C.

Cell Capture Experiment. The glass substrates were placed in a 12-well plate, and 1 mL of cell suspensions (10^5 cells/mL) were loaded. After incubation for 45 min at 37 °C with 5% CO_2, the substrates were washed three times with PBS. The captured cells on the substrates were fixed with 4% paraformaldehyde PBS for 10 min.

Cell Viability. To determine cell viability, we used the LIVE/DEAD viability assay kit. The captured cells on the chip were processed with the molecular probes and according to protocols provided by the manufactory. Afterward, the chip was washed with PBS and observed by way of fluorescence microscope.

Microfluidic-Chip Fabrication. To fabricate the microfluidic devices, we employed a single inlet and outlet linked by one microchannel (100 μm × 0.5 cm × 1.5 cm) using the micromolding method with poly(dimethylsiloxane) (PDMS). A syringe pump of Harvard Apparatus PHD 22/2000 (Holliston, MA, U.S.) was used to drive the fluid flow.

Blood Specimen Collection and Processing. With informed consent, blood specimens were drawn from healthy donors or cancer patients. Anticoagulant ethylenediaminetetraacetic acid was added to

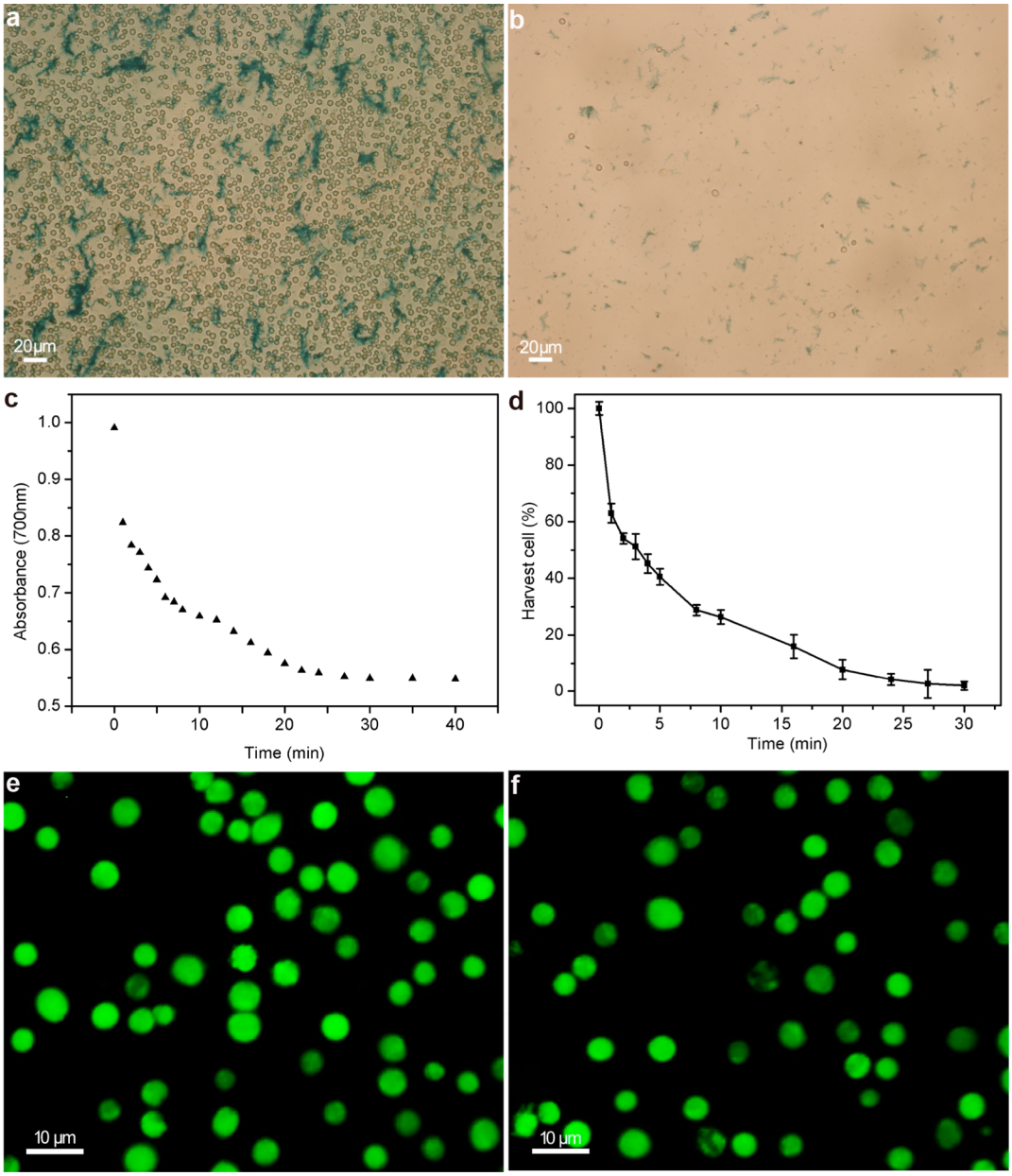

Figure 3. Cell capture and electronic mediated harvest on the CHI(PB/CHI)$_n$ film (n = 27). (a) Microscopy image of the HepG2 cells trapped on the film. (b) Substrate free of cells after the release triggered by electronic reactions. (c) The absorbance change of the film with the triggering time. (d) The number of cells vs the time of electrochemically induced deconstruction of the film at 1.25 V. (e) Viability analysis of captured and released cells (f). The cells were treated with a mixture of calcein AM (green: live) and ethidium homodimer-1 (red: dead) using a live/dead cell staining kit.

all the specimens, and whole blood specimens were stored at 4 °C. To obtain lysed blood, whole blood was added to NH_4Cl in a 10:1 ratio (v/v) and mixed for 15−20 min at room temperature.

CTCs Identification and Enumeration by Confocal Laser Scanning Microscopy. Immunofluorescence staining with cytokeratin 18 as well as morphological characteristics, such as cell shape and nuclear size, were adopted as criteria to positively identify CTCs.

RESULTS AND DISCUSSION

Figure 1 shows the process of creating the smart nanomaterial platform. Layer-by-layer (LbL) assembly was utilized as the approach to create the smart nanomaterial platform. First, the ITO-coated glass substrate was subjected to oxygen plasma etching. Empirically, 5 min after the etching, we obtained clean surfaces that were abundantly full of hydroxyl groups. Then, the substrates were constructed with positively charged CHI and negatively charged PB through LbL assembly. Next, the substrates were coated with polyacrylic sodium (PAA)/poly dimethyl diallyl ammonium chloride (PDADMAC) to bind carboxylic acid groups. Finally, the carboxylic acid groups were activated with 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) and then conjugated with avidin. The avidin-grafted substrates were then chemically functionalized with antiepithelial cell adhesion molecule (EpCAM) antibodies. Given that most solid tumors are epithelial cell-derived, that diverse carcinomas of head and neck, breast, colorectal,[28] stomach, prostate, lung,[29] ovarian,[30] bladder,[31] and hepatic origins overexpress EpCAM, and that EpCAM is absent in hematologic cells,[32,33] it is commonly accepted that anti-EpCAM antibodies can provide the specificity for CTC capture.

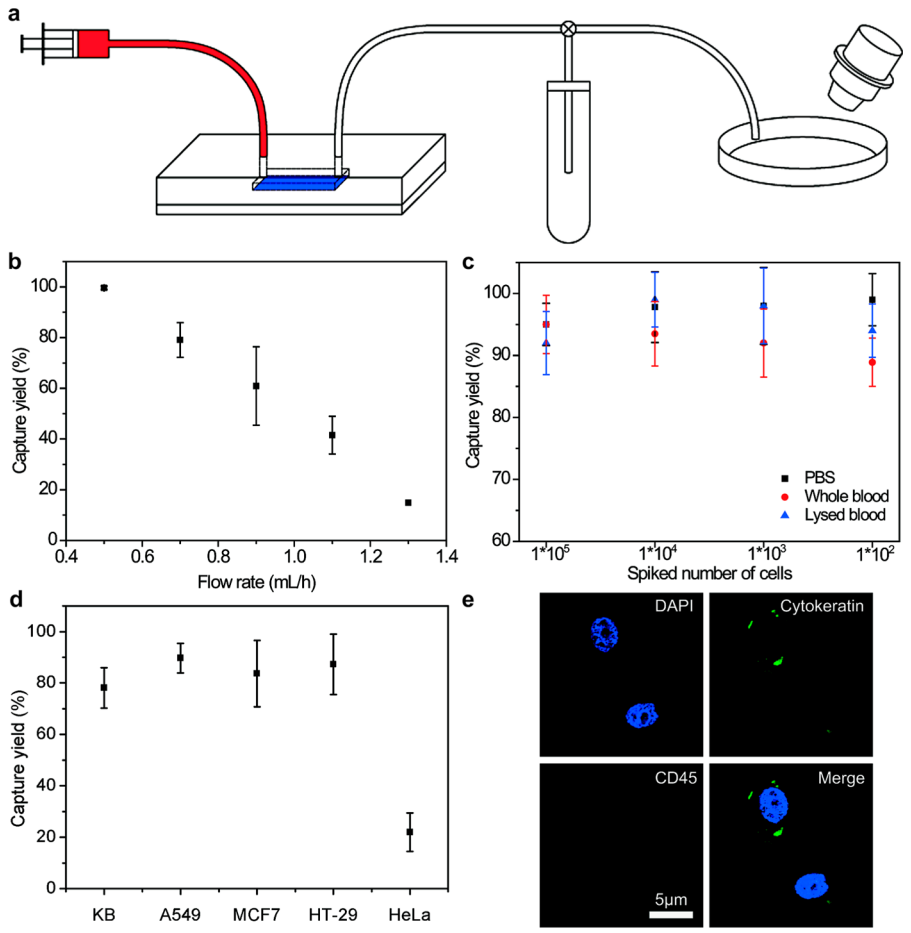

Figure 4. Microfluidic device based on the electroactive thin films for cell sorting applications. (a) Sketch of the microfluidic device. The cell collection can be switched from the undesired cells (red blood cells or white blood cells) to the desired ones (CTCs) based on the electronic on−off or capture and release pattern. (b) The cell capture yield functions at different cell flow rates and (c) different cell concentrations and solution environments by using HepG2 cell line. (d) Capture yields from buffers spiked with 100 cells per milliliter of five different cell lines: oral squamous cell carcinoma (KB), lung cancer cells (A549), human breast cancer cell (MCF7), human colorectal adenocarcinoma cell (HT-29), and human cervical cancer cell (HeLa). (e) The fluorescent images from confocal laser scanning microscopy of HepG2 cells sorted from whole blood samples by the microfluidic device integrated with the electroactive thin films.

As shown in Figure 2a, with increased numbers of layers, the color of the films deepened gradually, which resulted from the deposition of PB nanoparticles. Meanwhile, profilometry was used to measure the thickness of the CHI(PB/CHI)$_n$ films (Figure 2b). Given that PB shows a maximal absorbance at ~700 nm as revealed by the UV−visual spectroscopy (Figure 2c inset), the fact that the absorbance of the PB-deposited film at 700 nm increased gradually suggests that films grow linearly in thickness when numbers of layers are increased. The surface morphology of the CHI(PB/CHI)$_{27}$ films was investigated with scanning electron microscopy (SEM) and also atomic force microscopy (AFM), as shown in Figure 2d,e. The substrate was covered with spherical nanoparticles with an average size of approximately 10−20 nm.

To test specific cell affinity of the electroactive film, we incubated the film in a suspension of EpCAM-positive human hepatoma carcinoma cells (HepG2). Many cells were observed on the surface of the film (Figure 3a) after 30 min of incubation, even after washing three times with the PBS solution, indicating the cells could bind to the electroactive film successfully. Then, whether or not cell binding could be converted into cell release under a potential of 1.25 V was investigated. When the voltage was applied for 20 min, the density of binding cells decreased to ~15 cells/mm^2 (Figure 3b). The efficiency for cell release was approximately 95%. In contrast, the same film without electric treatment or the glass with the EpCAM modification but without electroactive payload layer did not induce any cell release under electric treatment (Supporting Information, Figure S1). The above results, taken together, suggest that bound cells can be successfully released via applying a small voltage. Figure 3c shows the deconstruction of CHI(PB/CHI)$_{27}$ films under an applied voltage held constant at 1.25 V. The absorbance at 700 nm was decreased over time in this case. In the first 10 min, we observed a rapid decline of the absorbance, which reached a value equal to 65% as compared with that of the film at the start. The disintegration of the film is most likely due to a loss of electroneutrality and the repulsion of adjacent layers that take place during the PB to PX transition, which results in the repulsion of adjacent layers. Microscopy images of the captured HepG2 cells versus the time of electrochemically induced deconstruction of the film are also exhibited. Quantitatively, the kinetics analysis suggests that more than 50% of the cells were released in 5 min, and nearly 95% cells were released at t = 30 min (Figure 3d, Supporting Information, Figure S2). To monitor the viability of the released cells, a live/dead cell assay

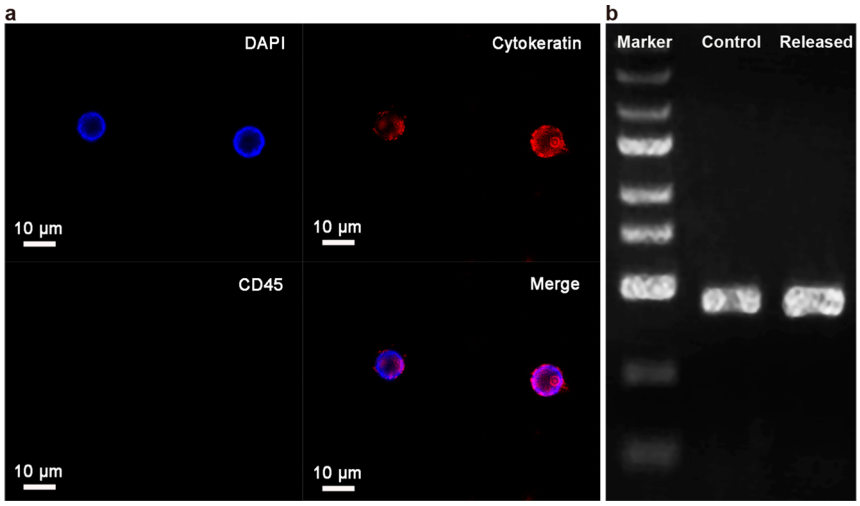

Figure 5. Material-based cell sorting devices are ready for clinical practice and also for molecular analysis. (a) The fluorescent images from confocal laser scanning microscopy of CTCs obtained from the peripheral blood of pancreatic cancer patients by the microfluidic device integrated with the electroactive thin films. (b) The extraction of mRNA that encodes the translation of the G6PD enzyme from the controlled and captured groups.

was employed to evaluate the viability of the released cells. It clearly shows that there is essentially no difference between the captured (Figure 3e) and harvested cells (Figure 3f), and the percentage of viable in the released is ~95%. These results, hence, suggest that, by way of an electrically induced switch, CTCs can be effectively captured and released.

After evaluating the ability of the film equipped with the electrically induced switch to capture and release cells, we designed a microfluidic device on the basis of the electroactive film (Figure 4a). The chip consists of a glass substrate patterned with an electroactive film and an overlaid polydimethylsiloxane (PDMS) chip with a rectangular channel (10 mm length × 5 mm width × 0.1 mm height). To find the optimal flow rates of the device, cell suspensions containing 100 cells mL^{-1} HepG2 cells in PBS buffer were poured into the microdevices at flow rates of 0.5, 0.7, 0.9, 1.1, and 1.3 $mL{\cdot}h^{-1}$. Subsequent to the detachment of the PDMS component from the substrate, captured cells were rinsed, fixed, nuclear stained with 4,6-diamidino-2-phenylindole (DAPI), and analyzed by means of fluorescence microscope. As shown in Figure 4b, superb efficiency of cell capture (>95%) was achieved at flow rates of 0.5 $mL{\cdot}h^{-1}$. Therefore, an optimal flow rate of 0.5 $mL{\cdot}h^{-1}$ for cell-capture was determined (Supporting Information, Movie S1). The capture efficiency for the optimal capture at the corresponding fluid-flow rate was further validated under the circumstances in which peripheral blood was simulated to contain cancer cells. To this end, a series of mimetic samples with densities at approximately 10^2, 10^3, 10^4, and 10^5 cells mL^{-1} were prepared by spiking DAPI-stained HepG2 cells to bloods drawn from healthy donors. As can be seen (Figure 4c), more than 95% HepG2 cells can be captured from the "artificial" CTC samples regardless of whether in the whole blood or the sample, in which the red blood cells were previously lysed, was used. In the meanwhile, HepG2 cells suspended in PBS at similar cell densities were also examined thoroughly, and high-efficacy capture was achieved. To test the commonality of the optimal condition for cell recovery, four additional EpCAM-positive tumor cell lines including human oral squamous cell carcinoma KB cells, the human lung cancer cell line A549, the human breast cancer cell line MCF7, and the human colorectal adenocarcinoma cell line HT-29, and also EpCAM-negative human cervical cancer cell line HeLa were tested in the devices. Various cell lines (total of five) suspended at a density of 10^2 cells mL^{-1} in whole blood were analyzed, and virtually all EpCAM-positive cells, except for the KB cell line (78.15 ± 7.92%), yielded greater than 80% harvest rate; as a comparison, the harvest rate for the HeLa cell line was 22.05 ± 7.50% (Figure 4d). The HeLa cell line was characterized to be EpCAM-negative, but the capture, albeit at a much lower rate, presumably suggests that EpCAM may also be weakly expressed in HeLa cells. We believe that the efficiency of tumor cell capture is affected by the varied cellular EpCAM expression among different cancer cell lines. Furthermore, the purity of captured cells is illustrated by the immunofluorescence assays of biomarkers on the cancer cell surface (Figure 4e). We suggest that the high efficiency for tumor cell capture resulted from the inherently rough morphology of the electroactive thin film, which is composed of PB nanoparticles (Supporting Information, Figure S3). It was also recently reported by other investigators that nanoparticle-based cell capture systems could achieve ultrahigh efficiency of cell capture.[34] These results could be owing to augmented cell–substrate interactions, which is inherent to the cell-capturing system involving nanoparticles.

The optimized conditions of cell capture were further utilized to investigate CTCs in the patients of pancreatic cancer (Supporting Information, Table S1). Peripheral bloods were drawn from pathologically examined/defined pancreatic cancer patients with informed consent. Blood (1.0 mL) was injected into the integrated device in each study. To verify the viabilities of the cells in the peripheral blood samples from cancer patients, a live/dead cell assay was applied. As shown in Supporting Information, Figure S4, the captured cells exhibited superior viability. Meanwhile, cell type was identified through an immunocytochemistry method. The microchip was rinsed with PBS, followed by administration of fixation reagents and incubation at 4 °C for 10 min. Then FITC-labeled anti-CD45 and APC-labeled anti-Cytokeratin (CK), as well as 4,6-diamidino-2-phenylindole (DAPI) were used to stain captured cells. CD45 is a marker for WBCs, and CK is a marker for epithelial cells, whereas DAPI stains nuclei.[35] The DAPI intensity and the expression levels of CK and CD45 in captured

cells were quantified through fluorescence microscopy. As seen in Figure 5a, the trapped cells exhibit strong CK expression, negligible CD45 signals, and intact nuclei validated by DAPI staining.

Furthermore, whether the materials platform is suitable or not for subsequent molecular analyses was determined. The extraction of mRNA that encodes the G6PD enzyme from the control and captured groups was tested. We confirmed specific expression of this mRNA, as evidenced by direct lysis of CTCs harvested by the responsive materials-based microdevices followed by amplification of the reverse-transcribed product of the transcript with PCR (RT-PCR) (Figure 5b and Supporting Information, Figure S5). These results confirm that the materials platform is friendly and could provide powerful opportunities for CTC-based molecular analyses.

CONCLUSIONS

Demonstrably, programmable electroactive multilayered materials can be manufactured to realize cell-type specific capture on the basis of cell−antibody and ligand−receptor interactions. In addition, with controlled thickness, the coating can survive a wide variety of buffers and physiological fluids for extended periods. Furthermore, factors destructive to captured cells are not involved in the entire procedure of achieving regulatory cell-interface interactions. Finally, and most importantly, applying a tiny voltage to multilayered films leads to their disassembly due to changes of PB oxidation state from negative to zerovalent (PX), hence making a gentle and effective release of captured cells possible and paving the way for further functional analyses. The inherent rough characteristics of the nanoparticle-composed surface achieve high capture efficiency and purification by integration with a simple microfluidic device. This materials system represents a strategy for the integration of bioelectronics devices with a materials platform and makes subsequent molecular and cell biological analyses achievable. Therefore, the system has broad implications for advancing fundamental research of cancer biology, clinical disease management, and treatments based on blood purification,[36] and the system may also be applicable to microbe capture and enrichment.[37]

ASSOCIATED CONTENT

Supporting Information

The details of the RNA extraction test, some other control experiments, images obtained by optical microscopy showing cell capture and release, and results of clinical trials. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Y.X. and B.W. designed the project. Y.X., H.Z., N.X., M.C., Y.R., and B.W. conducted the experiments. Y.X., Y.L., B.W., and R.T. analyzed the data. The manuscript was written with contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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