Biotin-Triggered Decomposable Immunomagnetic Beads for Capture and Release of Circulating Tumor Cells

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S *Supporting Information*

ABSTRACT: Isolation of rare, pure, and viable circulating tumor cells (CTCs) provides a significant insight in early cancer diagnosis, and release of captured CTCs without damage for ex vivo culture may offer an opportunity for personalized cancer therapy. In this work, we described a biotin-triggered decomposable immunomagnetic system, in which peptide-tagged antibody designed by chemical conjugation was specifically immobilized on engineered protein-coated magnetic beads. The interaction between peptide and engineered protein can be reversibly destroyed by biotin treatment, making capture and release of CTCs possible. Furthermore, the peptide could mediate multiple antibodies' coimmobilization on engineered protein-coated magnetic beads, by which capture efficiency for CTCs was obviously improved. Quantitative results showed that 70% of captured cells could be released by biotin addition, and 85% of released cells remained viable. In addition, 79% of cancer cells spiked in human whole blood were captured and could also be successfully released for culture. Finally, immunomagnetic beads simultaneously loaded with anti-EpCAM, anti-HER2, and anti-EGFR were successfully applied to isolate and detect CTCs in 17 cancer patients' peripheral blood samples, and 2−215 CTCs were identified with high purity. These results suggest that our method is reliable and has great potential in CTC detection for CTC-based molecular profiling, diagnosis, and therapy.

KEYWORDS: *circulating tumor cells, peptide, capture, release, cell viability*
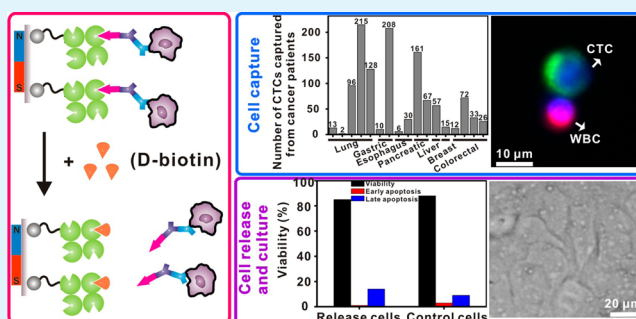
■ INTRODUCTION

Screening for circulating tumor cells (CTCs) in blood has evoked a lot of interest for early diagnosis of cancer.[1−4] CTCs are cancer cells disseminated from primary or secondary tumors that circulate in blood and may be responsible for metastasis.[5,6] However, the low occurrence of cancer cells in the bloodstream hampers the enrichment and characterization of CTCs.[7,8] Thus, high-efficiency isolation of CTCs is urgently needed.

Many techniques have been used to isolate CTCs, including filtration,[9,10] density-gradient centrifugation,[11,12] and affinity-based detection.[13−19] However, isolation on the basis of cell size and density is of low specificity. Immunoassay-based detection, including micro/nanostructured surfaces or immunomagnetic beads, is more commonly used and relies primarily on antigen−antibody affinity by recognizing tumor-specific markers especially epithelial cell adhesion molecules (EpCAM) to capture targeted cancer cells.[20−22] Micro/nanostructure-based approaches often use complicated structures, such as arrays of microposts,[23] nanopillars,[24,25] nanowires,[26] nanofibers,[27] and nanodots[28] to enhance the interactions with cells, improving the capture efficiency to target cells. Alternatively,

benefiting from easy manipulation, fast magnetic response, and high capture efficiency, immunomagnetic-based assays have been widely applied to CTC detection.[29−34]

To make significant strides in personalized cancer therapy, it is necessary to establish methods for ex vivo culture of viable CTCs that can be sequenced for genotyping and provide well-directed treatments of the primary tumor. Currently, isolation of viable CTCs is technically challenging because most methods captured few numbers of CTCs with low purity and damaged the CTCs viability during the cell purification process or in some cases CTCs were irreversibly immobilized on an adherent matrix.[35] Therefore, developing an isolating matrix that can capture and, especially, release viable CTCs with high purity for subsequent cell culture and analysis becomes increasingly urgent. The established methods used in release of captured tumor cells include electrochemical desorption,[36,37] photosensitive-induced cleavage,[38] thermodynamic release,[39]

enzyme degradation,[14] and chemical competitive-combination-triggered release.[33,40] However, these methods often need elaborate designs or are invasive, limiting the effectiveness of using these methods. Notably, although immunomagnetic-separation-based CTC capture has been fully developed, release of CTCs by means of immunomagnetic beads has rarely been reported.[34] In our previous study, we designed engineered decomposable nanobioprobes for capture and release of targeted cancer cells by EDTA-triggered disintegration of Ca^{2+}−alginate composite on the surface of fluorescent−magnetic nanospheres.[33] However, the viability of released cells was discounted by EDTA treatment to some extent. It is therefore desirable to design an immunomagnetic-bead-based strategy that can capture and release CTCs specifically and quickly and meanwhile highly maintain their viability.

Currently, Strep-tag II (a short peptide sequence) has been identified with binding affinity to Strep-Tactin (a mutated streptavidin molecule with the biotin-binding site). D-Biotin effectively competes with Strep-tag II for its higher affinity to Strep-Tactin, by which the Strep-tag system was established and widely used in purification and identification of protein complexes.[41−43] If immunomagnetic separation system for CTCs capture was constructed on the basis of the interaction of Strep-tag II with Strep-Tactin, then it should thus be possible to competitively destroy the immunomagnetic separation system in the presence of D-biotin, which makes cell-friendly release of CTCs possible. To realize our hypothesis, chemically synthesized Strep-tag II was oriented conjugated with antibody that were specifically interacted with Strep-Tactin-coated magnetic beads (STMBs), using Strep-tag II and Strep-Tactin as a bridge to obtain antibody-modified STMBs for capturing cancer cells. After addition of biotin, Strep-tag II derived antibody detached from STMBs, enabling release of cancer cells with highly maintained viability. As-prepared antibody-modified STMBs were used to isolate cancer cells from mimic clinical blood samples, and the captured cells could be released and kept their proliferation ability. Finally, multiple-antibody-functionalized STMBs were successfully applied to the isolation and detection of CTCs in cancer patients' peripheral blood samples, and rare CTCs were able to be detected and identified. These results support the idea that this system might provide a good opportunity for CTCs enrichment and enumeration.

■ MATERIALS AND METHODS

Materials and Instruments. Magnetic beads (MagStrep type2HC beads, binding capacity = 0.5−1 nmol/mg beads; bead size = 0.5−1.5 μm; ζ potential = −11.9 ± 1.1 mV) and washing buffer W were purchased from IBA Company. Goat antimouse IgG (Fc-specific) was bought from Jackson Company. FITC-labeled rabbit antigoat secondary antibody and nonfat dry milk were provided from Boster Company. Peptide sequences (WSHPQFEK) were supplied by Sangon Biotech Co., Ltd.. Millipore YM-30 was supplied by Merck Company. Bovine serum albumin (BSA), D-biotin, anti-EpCAM mouse monoclonal antibody, FITC-labeled goat antimouse secondary antibody, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. Anti-EGFR and anti-HER2 mouse monoclonal antibody, FITC-labeled mouse antihuman cytokeratin (FITC-CK), and PE-labeled mouse antihuman CD45 (PE-CD45) were obtained from Abcam Company. SK-BR-3 cells (a human breast cancer cell line), A431 cells (a human skin cancer cell line), and Hela cells (a human cervix cancer cell line) were purchased from China Type Culture Collection. Apoptosis detection kits (annexin V-FITC and PI) were provided by the Beyotime Institute of Biotechnology. Ultralow attachment plates were purchased from Corning. EGF, basic FGF, B27, and antibiotic−antimycotic were supplied by Life Technologies.

All blood samples were obtained from Renmin Hospital of Wuhan University. All the media used for cell culture was obtained from Gibco Corp. All other chemicals were supplied by Shanghai Chemical Reagent Company. ζ potential was obtained with ζ potential measurement (Zetasizer Nano, Malvern). Magnetic hysteresis loops were measured on a physical property measurement system (PPMS-9 T) with a vibrating sample magnetometer option (VSM, USA, Quantum Design). UV−vis absorption spectra were measured with a UV−vis spectrophotometer (UV-3600, Shimadzu Corporation). Fluorescence microscopic images were obtained using a Zeiss microscope (AxioObserver Z1, Zeiss, Germany).

Oxidation of Goat Antimouse IgG and Its Conjugation with Strep-Tag II. A 1 mg aliquot of goat antimouse IgG was dispersed in 1 mL of 0.1 M PBS (pH 6.4) and incubated 1:1 (v/v) with 20 mM sodium periodate (0.1 M PBS, pH 6.4) for 30 min in the dark. The excess sodium periodate was removed by filtration using a 30 K molecular-weight-cut-off centrifuge filter (Millipore YM-30). The retained IgG was resuspended in 0.1 M PBS (pH 7.2) to a concentration of 1 mg/mL. Subsequently, the oxidized IgG was incubated with Strep-tag II for 5 h at room temperature with constant shaking. The resultant solution was transferred to Millipore YM-30 in order to remove the excess Strep-tag II by filtration three times. The retained product was dispersed in 0.1 M PBS (pH 7.2) and stored at 4 °C before use.

Preparation and Characterization of Primary Antibody-Loaded IgG-STMBs. STMBs (0.5 mg) were first washed twice with buffer W and incubated on ice with Strep-tag II derived goat antimouse IgG (200 μL, 1 mg/mL) for 30 min to prepare IgG-STMBs. Subsequently, IgG-STMBs (0.5 mg) were dispersed in 200 μL of 0.1 M PBS (pH 7.2) to react with 10 μg of anti-EpCAM for 30 min with constant shaking. After removal of excess antibody, the reconstituted samples were blocked with 5% nonfat dry milk (dispersed in PBS containing 0.1% (v/v) Tween, biotin removed) for more than 2 h to reduce nonspecific adsorption. Then, the samples were washed three times and resuspended in 200 μL of 1× PBS, forming anti-EpCAM-IgG-STMBs.

To characterize anti-EpCAM-IgG-STMBs, anti-EpCAM-IgG-STMBs and the same amount of IgG-STMBs (already blocked) were incubated with 5 μL of FITC-labeled secondary antibody (100 μg/mL) for 30 min with shaking. Then, they were washed three times, followed by the acquiring of microscopic images. The FITC-labeled secondary-antibody-stained anti-EpCAM-IgG-STMBs were treated with biotin and observed with microscope.

The fabrication of anti-(EpCAM+EGFR)-IgG-STMBs and anti-(EpCAM+HER2)-IgG-STMBs was accomplished in a manner similar to the preparation of anti-EpCAM-IgG-STMBs, wherein 8 μg of anti-EpCAM and 2 μg of anti-EGFR or 8 μg of anti-EpCAM and 2 μg of anti-HER2 were used instead of 10 μg of anti-EpCAM, respectively.

Capture and Release of Cancer Cells Using Antibody-Modified IgG-STMBs. A 0.5 mg aliquot of anti-EpCAM-IgG-STMBs or anti-(EpCAM+EGFR)-IgG-STMBs was added to Eppendorf tubes containing 1×10^5 A431 cells in 1 mL of 1× PBS, incubated for 30 min at room temperature, and then separated with a magnetic scaffold. Similarly, the same concentrations of anti-EpCAM-IgG-STMBs or anti-(EpCAM+HER2)-IgG-STMBs were used to capture SK-BR-3 cells (1×10^5 cells/mL). The number of cells initially introduced and in the supernatant were all determined with a hemocytometer to calculate capture efficiencies. As controls, 0.5 mg of anti-EpCAM-IgG-STMBs was used to treat Hela cells (1×10^5 cells/mL) and 0.5 mg of IgG-STMBs was used to treat A431 cells and SK-BR-3 cells (1×10^5 cells/mL), respectively.

To release cells, the captured cells attracted by magnetic scaffold were further incubated with biotin for 5 min, which was repeated several times. The STMBs detached from cells were removed by magnetic scaffold, and the supernatants were recovered to obtain release efficiencies. Meanwhile, images were taken using a Zeiss microscope before and after capture and after release of DAPI-prestained SK-BR-3 cells.

Testing the Viability of Released Cells. Annexin V-FITC and PI were used according to the manufacturer's instructions for fluorescent

Scheme 1. Depiction of Capture and Release of Cancer Cells[a]
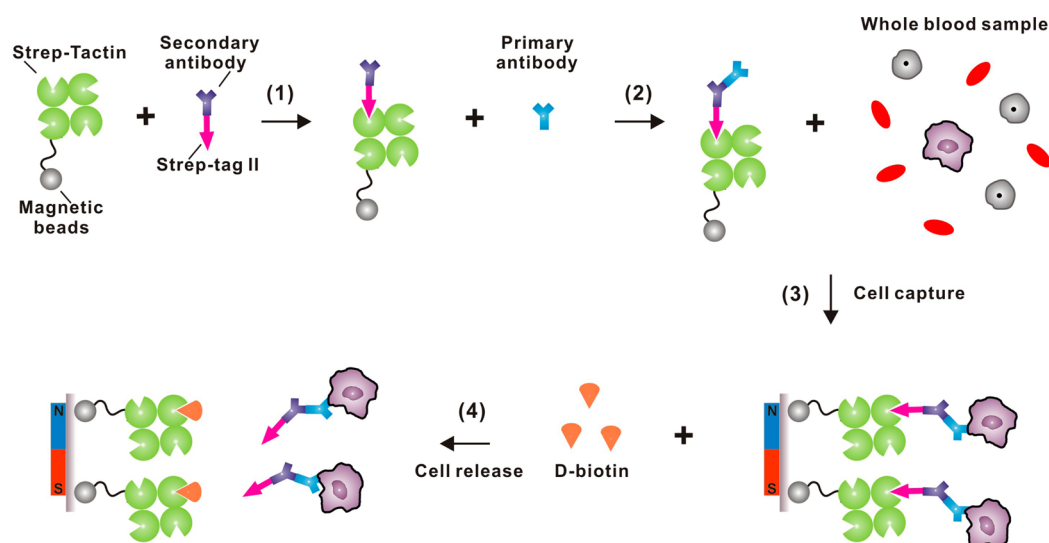
[a](1) Strep-tag II labeled IgG was reacted with STMBs, forming IgG-STMBs. (2) Anti-EpCAM, anti-EGFR, or anti-HER2 was interacted with IgG-STMBs for fabrication of antibody-modified IgG-STMBs. (3) Antibody-modified IgG-STMBs were used to capture cancer cells. (4) Captured cancer cells were released by addition of biotin.

staining of released A431 cells to evaluate their viability. The viability of A431 cells without capture-and-release treatment were also examined as control. For quantitative analysis, A431 cells that did not show fluorescence were counted as live cells.

Furthermore, the released A431 cells were cultured in an CO_2 incubator to detect their proliferation ability. The cells without capture-and-release treatment were also cultured under the same condition as control.

Capture, Release, and Culture of Spiked Cancer Cells from Whole Blood. IgG-STMBs (0.15 mg) were dispersed in 200 μL of 0.1 M PBS (pH 7.2) and reacted with 10 μg of anti-EpCAM for 30 min with constant shaking. After removal of excess antibody, the reconstituted samples were blocked with 5% nonfat dry milk. Then the samples were washed three times and resuspended in 100 μL of 1× PBS, forming anti-EpCAM-IgG-STMBs. A 100 μL aliquot of DAPI-prestained SK-BR-3 cell suspension (10^3 cells/mL) was added to Eppendorf tubes containing 1 mL of whole blood that were incubated with 100 μL of anti-EpCAM-IgG-STMBs for 30 min with gentle shaking. The same amount of cell suspension was distributed among three wells in a 96-well plate to calculate the mean of cells spiked into blood samples. Then, anti-EpCAM-IgG-STMBs captured cells were isolated, washed three times, resuspended in 1× PBS, and kept in a 96-well plate to be observed and counted using a Zeiss microscope to calculate the capture efficiency.

After magnetic separation, the captured cells were fixed with 4% paraformaldehyde (30 min), permeabilized with 0.2% Triton-X 100 (30 min), and stained with 10 μg/mL DAPI, FITC-labeled anti-CK19 monoclonal antibody, and PE-labeled anti-CD45 monoclonal antibody (according to kit instructions) for 2 h at 4 °C. After washing, the captured cells were put into a 96-well plate for fluorescence microscopy imaging. Captured cancer cells were classified as DAPI+/CK+/CD45−, and white blood cells were defined as DAPI+/CK−/CD45+. The purity was calculated by dividing the number of cells captured by the total number of nucleated cells (DAPI+) in the samples.

Furthermore, 100 μL of anti-EpCAM-IgG-STMBs (1.5 mg/mL) were added to 1 mL of whole blood containing SK-BR-3 cells or A431 cells and incubated for 30 min. After magnetic separation and three washes, the captured cells were treated with biotin (2 mM) and then collected for culture in a CO_2 incubator.

Isolation of CTCs from Patients with Cancer. IgG-STMBs (0.15 mg) were dispersed in 200 μL of 0.1 M PBS (pH 7.2) and reacted simultaneously with 8 μg of anti-EpCAM, 2 μg of anti-EGFR,

and 2 μg of anti-HER2 for 30 min with constant shaking. After washing, the reconstituted samples were blocked with 5% nonfat dry milk. Then, the samples were washed three times and resuspended in 100 μL of 1× PBS, forming anti-(EpCAM+EGFR+HER2)-IgG-STMBs.

Blood samples from 12 cancer patients and 7 healthy normal controls were prepared and incubated with 100 μL of anti-(EpCAM +EGFR+HER2)-IgG-STMBs prepared as described above. After magnetic separation, the captured cells were identified with three-color immunocytochemistry (ICC) and observed by a fluorescence microscope. Only cells that were DAPI+/CK+/CD45− with the appropriate size and morphology were counted as CTCs. In addition, CTC capture was further carried out by anti-(EpCAM+EGFR +HER2)-IgG-STMBs and CellSearch capture assay from blood samples of another five cancer patients for comparison.

Then, the captured CTCs were released as mentioned above. The released CTCs were cultured in ultralow-attachment plates containing RPMI-1640 medium, supplemented with EGF (20 ng/mL), basic FGF (20 ng/mL), B27, 1× antibiotic−antimycotic, and 5% CO_2.

■ RESULTS AND DISCUSSION

Strep-Tag System Integrated Immunomagnetic Separation for Capture and Release of Cancer Cells. Strep-tag II has the property of binding to Strep-Tactin, and D-biotin can competitively bind with Strep-Tactin to destroy the interaction of Strep-tag II and Strep-Tactin. On the basis of these features, a Strep-tag system was established for fast and easy purification of protein under very gentle conditions.[41] In addition, the Strep-tag system was also adopted for reversible T-cell staining, which maintained the specificity and sensitivity of cell staining while preserving the functional status of T lymphocytes.[44] These unique characteristics of the Strep-tag system make it an ideal tool for constructing a reversible attaching and detaching system for biological application. Therefore, we proposed to develop a versatile platform for capture and release of cancer cells under gentle conditions by combination of a Strep-tag system and an immunomagnetic separation system.

To realize the integration of these two systems, we first prepared Strep-tag II derived goat antimouse IgG that can be easily conjugated onto STMBs, followed by immobilization of

the primary antibody for recognizing its biotargets. Scheme 1 demonstrates the processes of fabrication of primary antibody-functionalized magnetic beads for the capture and release of cancer cells. First, STMBs (whose magnetic hysteresis loop and capture efficiencies at different attraction times with a commercial magnetic scaffold were demonstrated in Figure S1) were used as supporter to load Strep-tag II derived goat antimouse IgG, forming IgG-STMBs. After that, anti-EpCAM or multiple antibodies were immobilized onto IgG-STMBs to obtain anti-EpCAM-IgG-STMBs (or anti-(EpCAM+EGFR)-IgG-STMBs, anti-(EpCAM+HER2)-IgG-STMBs, and anti-(EpCAM+EGFR+HER2)-IgG-STMBs) by the specific recognition between secondary antibody and primary antibody. Subsequently, Strep-tag II mediated primary-antibody-functionalized STMBs were employed for capturing and isolating cancer cells. Because biotin binds to Strep-Tactin by several orders of magnitude more tightly than the Strep-tag II peptide,[41] biotin was used to interact with Strep-Tactin in competition with Strep-tag II, thus allowing the disintegration of anti-EpCAM-IgG-STMBs and release of the captured cancer cells.

Preparation of Strep-Tag II Derived Secondary Antibody. Generally, the enrichment of CTCs on the basis of a single cancer cell marker, most commonly EpCAM, often encounters major challenges because of tumor heterogeneity and epithelial to mesenchymal transition (EMT).[13,18,45] Consequently, new approaches that can target multiple clinically relevant cancer biomarkers are urgently needed for improving capture efficiency of CTCs. Therefore, we conjugated Strep-tag II labeled secondary antibody onto STMBs, where multiple primary antibodies could be simultaneously immobilized for recognizing various cancer biomarkers.

Strep-tag II comprises the amino acid sequence of Trp-Ser-His-Pro-Gln-Phe-Glu-Lys,[43] where the carboxyl group of Glu residue plays an important role in binding with Strep-Tactin.[46] Therefore, to prepare a Strep-tag II—secondary antibody biological composite while preserving the carboxyl group of Strep-tag II, the amino group of Strep-tag II was employed to conjugate with the aldehyde group of IgG produced by oxidation of the carbohydrate moiety on the IgG's Fc region. By this strategy, the binding affinity of Strep-tag II to Strep-Tactin was conserved. Furthermore, the reaction between the aldehyde group and the amino group is also helpful to obtain high labeling efficiency of Strep-tag II to secondary antibody.[47] Meanwhile, Strep-tag II was controllably linked to the Fc region of IgG, allowing the active Fab zone to remain fully exposed for connecting primary antibody. We have to mention that chemical conjugation was used to build Strep-tag II—secondary antibody biological composite, which was easier and more general than molecular-biology-based protein labeling,[48−51] improving the universality of this method.

To evaluate whether our design is applicable, oxidized IgG (containing aldehyde group) was incubated with amino-terminated nanospheres (representing Strep-tag II), followed by incubation with FITC-labeled rabbit antigoat secondary antibody as a reporter. As demonstrated in Figure S2, strong fluorescence was observed on the IgG-labeled nanospheres (Figure S2b), whereas the fluorescence of control samples (nanospheres without incubation with oxidized IgG) was rarely observed (Figure S2d). Hence, we knew that IgG was successfully immobilized onto the surface of the nanospheres through the aldehyde group of IgG and the amino group of the nanospheres, meaning that the aldehyde group was successfully

generated by $NaIO_4$ oxidation. Accordingly, Strep-tag II derived goat antimouse IgG was successfully constructed.

Integration and Disintegration of Strep-Tag II Mediated Antibody-Functionalized STMBs. Originating from the specific interaction between Strep-tag II and Strep-tactin, Strep-tag II derived IgG (Fc-specific) could be immobilized onto STMBs for fabricating IgG-STMBs (whose integration and disintegration ability was successfully evaluated by FITC-labeled rabbit antigoat antibody, see Figure S3). Then, primary antibody was oriented immobilized on IgG-STMBs with fully exposed Fab fragments for recognizing cell makers effectively. Here, anti-EpCAM was immobilized onto IgG-STMBs to construct anti-EpCAM-IgG-STMBs, and FITC-labeled secondary antibody was employed to report this construction. Compared with faint fluorescence of IgG-STMBs (Figure 1d), anti-EpCAM-IgG-STMBs had strong
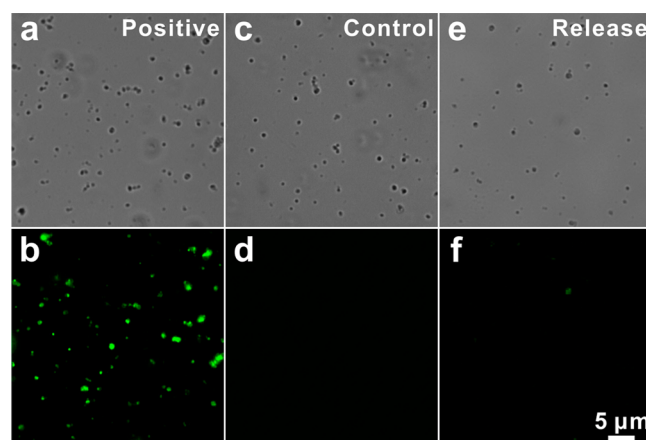

Figure 1. Construction of anti-EpCAM-IgG-STMBs. (a and b) Incubation of FITC-labeled secondary antibody with anti-EpCAM-IgG-STMBs. (c and d) Incubation of FITC-labeled secondary antibody with IgG-STMBs as control. (e and f) Release of anti-EpCAM from the surface of STMBs by biotin addition. Bright field, a, c, and e; fluorescence, b, d, and f.

fluorescence (Figure 1b) when IgG-STMBs and anti-EpCAM-IgG-STMBs were incubated with FITC-labeled secondary antibody. Furthermore, if anti-EpCAM-IgG-STMBs stained by FITC-labeled secondary antibody were further incubated with biotin, the fluorescence from anti-EpCAM-IgG-STMBs was rarely observed (Figure 1f). These results illustrated the successful conjugation and detachment of anti-EpCAM from the surface of STMBs. Therefore, we concluded that anti-EpCAM can be reversibly attached to and detached from the surfaces of STMBs, enabling capture and release of cancer cells by using Strep-tag II mediated antibody labeling on STMBs.

Capture and Release of Cancer Cells Using Biotargeting IgG-STMBs. Strep-tag II derived goat antimouse secondary IgG was immobilized on STMBs that can specifically interact with any primary IgG from a mouse source. Therefore, multiple primary-antibody-modified IgG-STMBs can be fabricated by combination of different antibodies against various biomarkers on cancer cells surface. The specificity of antibody-modified IgG-STMBs was evaluated by three cell lines: (1) A431, a model of skin cancer that is positive for EpCAM and epidermal growth factor receptor-1 (EGFR) and negative for epidermal growth factor receptor-2 (HER2), namely, EpCAM+/EGFR+/HER2−; (2) SK-BR-3, a model of breast cancer which is EpCAM+/EGFR−/HER2+; (3) Hela, a model of cervix cancer
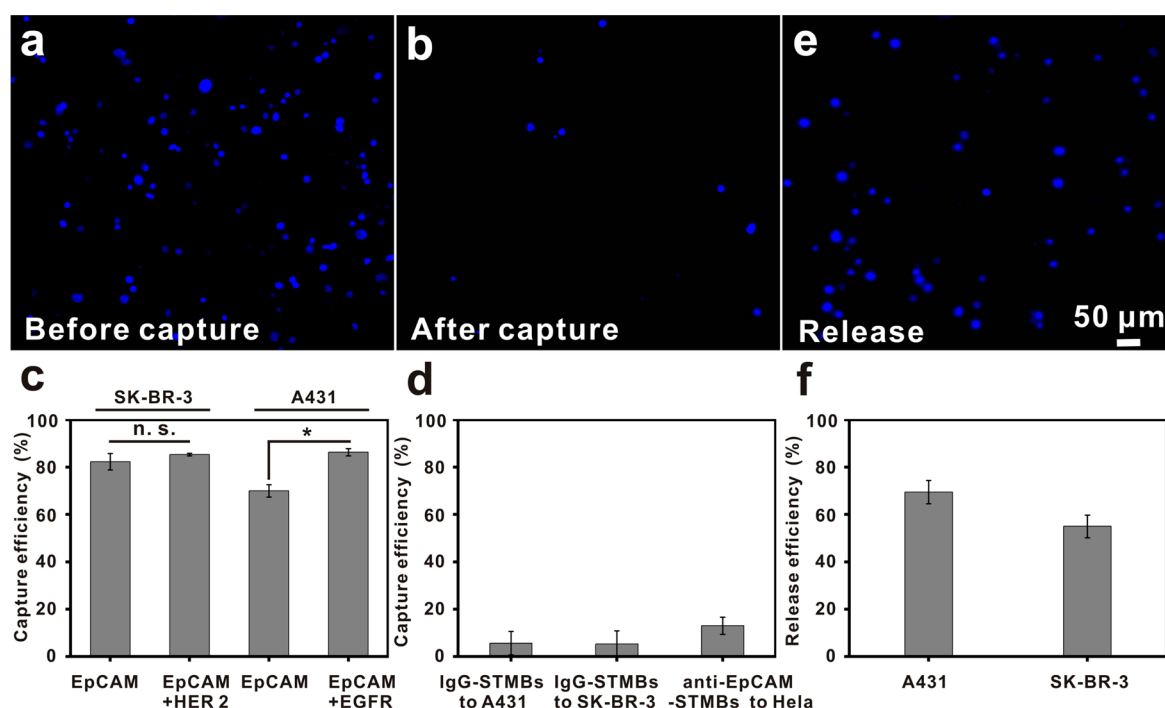
Figure 2. Capture and release of cancer cells. Fluorescent microscopy images of SK-BR-3 cells (a) before and (b) after incubation with anti-EpCAM-IgG-STMBs. (c) Capture efficiencies of antibody modified IgG-STMBs to A431 and SK-BR-3 cells. (d) Performance of IgG-STMBs mixed with A431 and SK-BR-3 cells, as well as anti-EpCAM-IgG-STMBs mixed with Hela cells. (e) Fluorescence microscopy images of released SK-BR-3 cells. (f) Release efficiencies of anti-EpCAM-IgG-STMBs captured A431 and SK-BR-3 cells by biotin addition; error bars, standard error (n = 3). Independent samples t test; n.s., not significant; *, $p < 0.05$.

that is EpCAM−.[18,27] As demonstrated in Figure 2a,b, SK-BR-3 cells stained by DAPI were imaged before (Figure 2a) and after (Figure 2b) incubation with anti-EpCAM-IgG-STMBs, indicating that SK-BR-3 cells can be recognized by anti-EpCAM-IgG-STMBs. Quantitative results are shown in Figure 2c; when 0.5 mg/mL of anti-(EpCAM+HER2)-IgG-STMBs were applied to 1×10^5 SK-BR-3 cells for cell isolation, capture efficiency was increased (85 ± 1%) when compared to anti-EpCAM-IgG-STMBs-based SK-BR-3 cell capture (82 ± 4%). Meanwhile, compared with anti-EpCAM-IgG-STMBs, STMBs simultaneously loaded with anti-EpCAM and anti-EGFR (namely, anti-(EpCAM+EGFR)-IgG-STMBs) increased the capture efficiency of A431 cells from 70 ± 3 to 86 ± 2% ($p < 0.05$). Thus far, it is clear that STMBs simultaneously loaded with multiple antibodies could improve capture efficiency, meeting the urgent demand on obtaining high capture efficiency for heterogeneous cancer cells. As controls (Figure 2d), IgG-STMBs (without anti-EpCAM) rarely interacted with A431 and SK-BR-3 cells, and anti-EpCAM-IgG-STMBs only isolated a few Hela cells (low EpCAM expression level). Hence, it was concluded that antibody-modified IgG-STMBs could specifically recognize and isolate targeting cancer cells.

Strep-tag II mediated antibody immobilization on STMBs could be destroyed by biotin treatment, facilitating release of captured cells. Figure 2e shows the captured SK-BR-3 cells that were imaged after release by biotin addition. Quantitative results showed that 70 ± 5% of captured A431 cells and 55 ± 5% of captured SK-BR-3 cells could be released. These results suggested that antibody-modified IgG-STMBs can be used for capture and release of cancer cells. Currently, a number of methods have been established for reversible capture and release of cancer cells,[14,33,36−40] among which an aptamer-based approach is a brilliant star because it gives high efficiency for

both cell capture and release. Generally, more than 90% of cancer cells can be captured by aptamer-based devices, and the captured cells were reversibly released by enzyme treatment or antisense DNA with efficiency higher than 90%.[26,52,53] Although an aptamer-based method is promising for CTC detection, our strategy gives an alternative choice because of its universal, mild, and cell-friendly features for CTC capture and release.

Viability of Released Cells. Annexin V-FITC and PI were used to evaluate the viability of released cells.[33] Cells with good bioactivity could not be stained by annexin V-FITC and PI, whereas cells in early stages of apoptosis were labeled by annexin V-FITC and cells in late apoptotic stage were stained by PI. From Figure 3a−d, it can be seen that majority of released A431 cells did not have fluorescence, exhibiting results similar to cells without capture-and-release treatment (Figure 3e−h). Quantitative results (Figure 3i) showed that 85% of released cells had good viability, 1% of cells underwent early apoptotic process, and 15% of cells almost lost their viability. The percentages of viability, early apoptosis, and late apoptosis of control cells corresponded to 88, 3, and 9%, respectively. Obviously, there were no significant differences between the released cells and cells without capture-and-release treatment. The main reason is that the release process was conducted under physiological conditions by biotin treatment that was mild and cell friendly.

To detect proliferation ability, the released A431 cells were further cultured in a CO_2 incubator and routinely cultured A431 cells were used as control. The results showed that the released A431 cells adhered well and proliferated on the tissue culture plates, exhibiting growth behavior similar to that of routinely cultured cells. As displayed in Figure 3j−m, the released A431 cells successfully underwent multiple (>5)
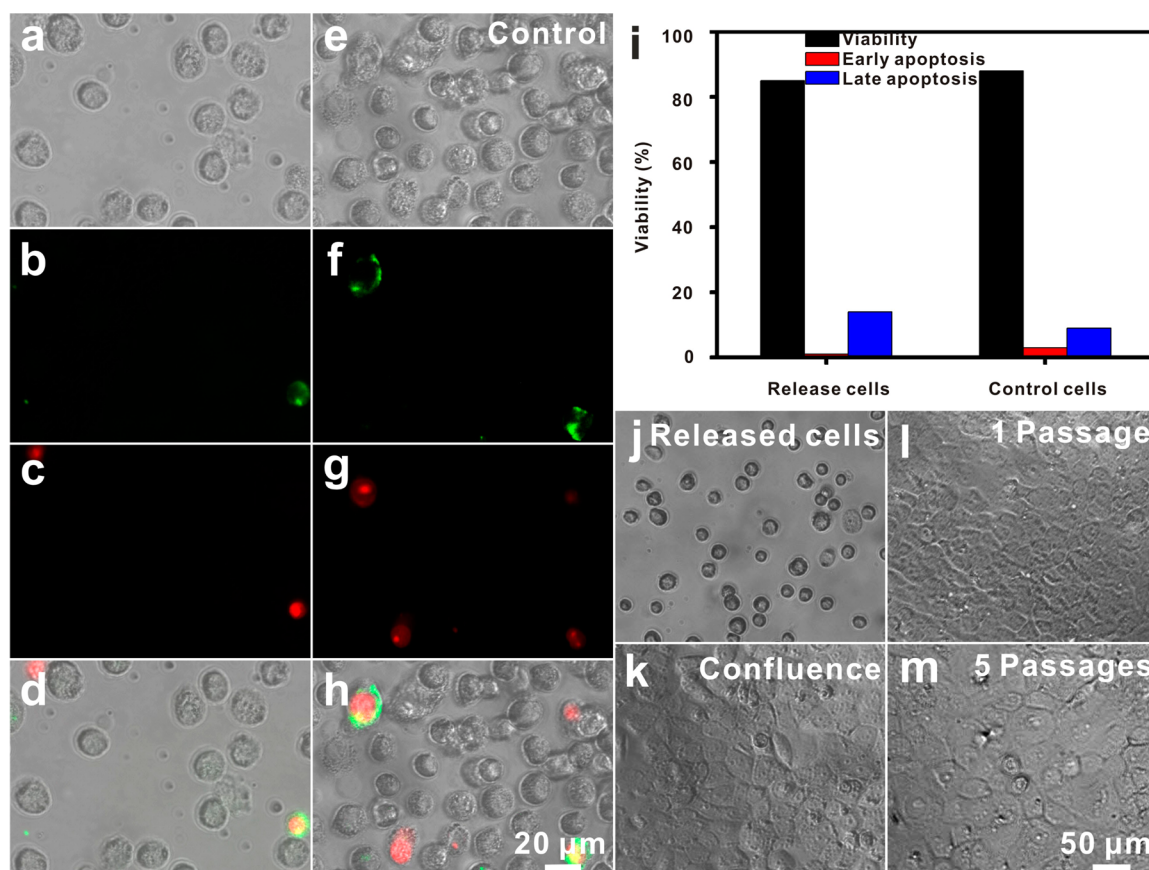
Figure 3. Viability of the released cells. (a–d) Released A431 cells stained by annexin V-FITC and PI; (e–h) A431 cells without capture-and-release treatment stained by annexin V-FITC and PI. (i) Quantitative results of viability of A431 cells with and without capture-and-release treatment. Microscopic images of released A431 cells (j) just seeded onto culture plate, (k) at confluence, and cells that have reached confluence (l) after one passage and (m) after five passages. Bright field, a, e, and j–m; fluorescence, b, c, f, and g; merged images, d and h.
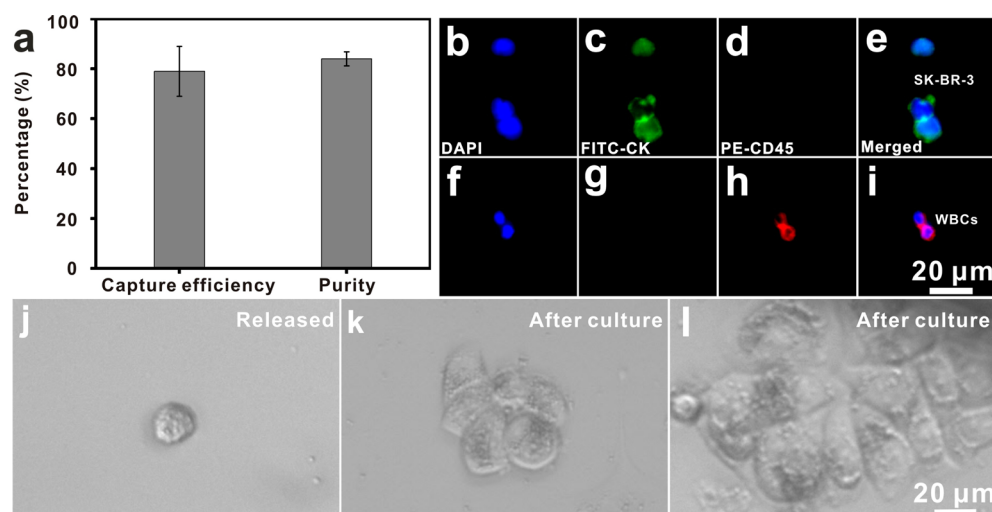

Figure 4. Capture, release, and culture of cancer cells from mimic clinical blood samples. (a) Capture efficiency and purity of cancer cells captured from whole blood; (b–d, f–h) Microscopic images of cells captured from blood samples, identified with three-color ICC; b–d, SK-BR-3 cells, and f–h, white blood cells. (e and i) Merged images of nucleus (DAPI), CK (FITC) and CD45 (PE). Microscopic images of (j) released SK-BR-3 cell and (k and l) cell growth after culture.

passages without detectable changes in behavior. These results clearly demonstrated that cancer cells after capture-and-release procedures retained both their viability and proliferation ability. Taken together, we could conclude that the cancer cells with

capture-and-release treatment were suitable for subsequent cell culture, which was vital for further research.

Capture, Release, and Culture of Spiked Cancer Cells from Whole Blood. To further explore the potential clinical application of anti-EpCAM-IgG-STMBs, capture, release, and
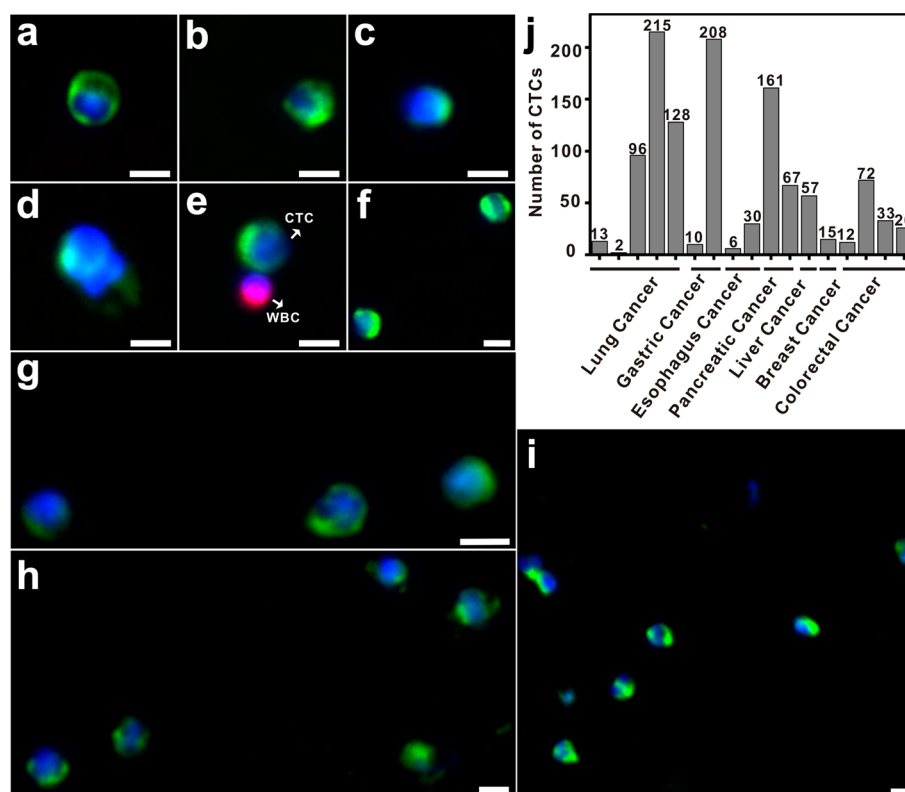
Figure 5. Capture of CTCs in whole blood samples from cancer patients. (a−i) Fluorescent microscope images of CTCs captured from patient blood. (j) Quantification of CTCs from cancer patients' blood samples. Scale bar = 10 μm.

culture of cancer cells were carried out in mimic clinical blood samples. First, we spiked human whole blood samples with DAPI-stained SK-BR-3 cells at a concentration of approximately 10^2 cells/mL. Figure 4a shows the recovery rate calculated by dividing number of captured cells by number of spiked cells, and a recovery rate of 79 ± 10% was obtained.

To determine the purity of SK-BR-3 cells separated by anti-EpCAM-IgG-STMBs, three-color ICC was conducted by treatment with DAPI nuclear stain, FITC-labeled anti-CK19 (a marker for epithelial cells) monoclonal antibody, and PE-labeled anti-CD45 (a marker for white blood cells (WBCs)) monoclonal antibody. As shown in Figure 4b−i, SK-BR-3 cells were DAPI+/CK+/CD45−, and WBCs were DAPI+/CK−/CD45+. From the observed results, we calculated that the purity of separated SK-BR-3 cells was 84 ± 3% (Figure 4a).

Furthermore, anti-EpCAM-IgG-STMBs were applied to capture and release SK-BR-3 cells spiked in human whole blood. As shown in Figure 4j, the SK-BR-3 cells captured from blood were successfully released by biotin treatment, showing good morphology. After culture, the released cells spread and divided normally, showing the tendency to grow to a single clone from single cell (Figure 4k,l). When we carried out this experiment by using A431 cells, the behavior (Figure S4) was similar to the growth behavior of A431 cells proceeded in PBS media (Figure 3j−m). These results exhibited that the capture-and-release treatment by anti-EpCAM-IgG-STMBs had little influence on cells' proliferation ability, suggesting the potential clinical application of this method in isolation of undamaged CTCs for ex vivo culture.

Isolation of CTCs from Cancer Patients. Although EpCAM-based enrichment methods have been frequently used by many groups, several studies have revealed that the presence of EpCAM on tumor cells varies with tumor type.[54,55] To overcome this obstacle, we simultaneously immobilized anti-EpCAM, anti-EGFR, and anti-HER2 onto IgG-STMBs (namely, anti-(EpCAM+EGFR+HER2)-IgG-STMBs) for high-efficiency and high-purity capture of CTCs from peripheral whole blood samples of cancer patients. Here, 12 cancer patients (including lung, gastric, esophagus, pancreatic, and liver cancer patients) and 7 healthy donors were investigated by anti-(EpCAM+EGFR+HER2)-IgG-STMBs, and the captured CTCs were successfully identified with the three-color ICC. Some representative fluorescent microscope images of CTCs are shown in Figure 5a−i: CTCs were DAPI+/CK+/CD45− with the appropriate size and morphology, whereas WBCs were DAPI+/CK−/CD45+. The number of isolated CTCs ranged from 2 to 215 CTCs per 1 mL of blood from 12 cancer patients (Table S1), and high purities were achieved in all samples (as shown in Figure 5g−i, rare WBCs were found in larger observed areas). No identifiable CTCs were detected from the blood samples of 7 normal healthy individuals, indicating that our anti-(EpCAM+EGFR+HER2)-IgG-STMBs could be successfully applied to real patient blood samples. Furthermore, we compared our method with the gold standard in clinical CTCs detection: the FDA-cleared CellSearch assay. Because the CellSearch system is efficient for detecting CTCs in cancer patients with metastatic breast, prostate, or colorectal cancer,[6] we simultaneously carried out CTCs enumeration by using anti-(EpCAM+EGFR+HER2)-IgG-STMBs and the CellSearch system for 5 cancer patients (4 colorectal cancer patients and 1 breast cancer patient). As displayed in Table S2, anti-(EpCAM +EGFR+HER2)-IgG-STMBs recovered 12−72 CTCs from 1 mL of blood, whereas the CellSearch system detected 0−23 CTCs from 7.5 mL of blood. These results demonstrated that

our method outperformed the CellSearch system for CTC detection because multiple antibodies were simultaneously immobilized on STMBs with fully exposed antigen recognition sites that could conspicuously improve capture efficiency, indicating that anti-(EpCAM+EGFR+HER2)-IgG-STMBs might be a powerful tool for early cancer diagnosis and relapse prediction.

To test whether CTCs captured from cancer patients were viable and had proliferation ability, we released the specifically captured CTCs and cultured them in vitro. Initially, we could observe single CTC or CTC spheres in ultralow-attachment plates under bright field, but the low density of CTCs may lose their proliferation ability in current culture conditions and were washed out during the media change process. We suspect that a large volume of blood samples would be beneficial to acquire a greater number of CTCs for ex vivo culture; meanwhile, finding suitable culture conditions for specific kinds of CTCs is also a critical factor.[35] Hence, more exploration of culturing CTCs in vitro is needed to promote personalized treatment of cancer.

CONCLUSIONS

We successfully developed a novel strategy for reversibly capturing and releasing CTCs by biotin-triggered decomposable immunomagnetic beads. The active Strep-tag II derived IgG fabricated by chemical conjugation was reversibly loaded onto and discharged from STMBs, owing to the competitive binding between Strep-tag II and D-biotin toward Strep-Tactin, which made capture and release of CTCs possible. As-prepared IgG-STMBs could immobilize multiple primary antibodies to achieve higher capture efficiency for CTC model cells, and biotin treatment could release 70% of captured cells with highly preserved viability because of the mild and cell-friendly release conditions. It is worth noting that the IgG-STMBs simultaneously loaded with anti-EpCAM, anti-HER2, and anti-EGFR were successfully applied to isolate and detect CTCs in cancer patients' peripheral blood samples with high purity, and rare CTCs in the whole blood samples were detected and identified by routine immunostaining. We foresee that the presented strategy may provide promising guidance for enumeration and release of viable CTCs for ex vivo culture, facilitating CTC-based molecular profiling, diagnosis, and therapy.

ASSOCIATED CONTENT

ⓢ Supporting Information
Figures SI-1–SI-4, Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Balic, M.; Williams, A.; Lin, H.; Datar, R.; Cote, R. J. Circulating Tumor Cells: From Bench to Bedside. *Annu. Rev. Med.* **2013**, *64*, 31–44.

(2) Lianidou, E. S.; Markou, A.; Strati, A. Molecular Characterization of Circulating Tumor Cells in Breast Cancer: Challenges and Promises for Individualized Cancer Treatment. *Cancer Metastasis Rev.* **2012**, *31*, 663–671.

(3) Joosse, S. A.; Pantel, K. Biologic Challenges in the Detection of Circulating Tumor Cells. *Cancer Res.* **2013**, *73*, 8–11.

(4) Budd, G. T. Let Me Do More Than Count the Ways: What Circulating Tumor Cells Can Tell Us about the Biology of Cancer. *Mol. Pharmaceutics* **2009**, *6*, 1307–1310.

(5) Steeg, P. S. Tumor Metastasis: Mechanistic Insights and Clinical Challenges. *Nat. Med. (N.Y., NY, U. S.)* **2006**, *12*, 895–904.

(6) Arya, S. K.; Lim, B.; Rahman, A. R. A. Enrichment, Detection and Clinical Significance of Circulating Tumor Cells. *Lab Chip* **2013**, *13*, 1995–2027.

(7) Paterlini-Brechot, P.; Benali, N. L. Circulating Tumor Cells (CTC) Detection: Clinical Impact and Future Directions. *Cancer Lett.* **2007**, *253*, 180–204.

(8) Riethdorf, S.; Pantel, K. Advancing Personalized Cancer Therapy by Detection and Characterization of Circulating Carcinoma Cells. *Ann. N.Y. Acad. Sci.* **2010**, *1210*, 66–77.

(9) Hosokawa, M.; Hayata, T.; Fukuda, Y.; Arakaki, A.; Yoshino, T.; Tanaka, T.; Matsunaga, T. Size-Selective Microcavity Array for Rapid and Efficient Detection of Circulating Tumor Cells. *Anal. Chem.* **2010**, *82*, 6629–6635.

(10) Hofman, V. J.; Ilie, M. I.; Bonnetaud, C.; Selva, E.; Long, E.; Molina, T.; Vignaud, J. M.; Fléjou, J. F.; Lantuejoul, S.; Piaton, E.; Butori, C.; Mourad, N.; Poudenx, M.; Bahadoran, P.; Sibon, S.; Guevara, N.; Santini, J.; Vénissac, N.; Mouroux, J.; Vielh, P.; Hofman, P. M. Cytopathologic Detection of Circulating Tumor Cells Using the Isolation by Size of Epithelial Tumor Cell Method: Promises and Pitfalls. *Am. J. Clin. Pathol.* **2011**, *135*, 146–156.

(11) Huh, D.; Bahng, J. H.; Ling, Y.; Wei, H. H.; Kripfgans, O. D.; Fowlkes, J. B.; Grotberg, J. B.; Takayama, S. Gravity-Driven Microfluidic Particle Sorting Device with Hydrodynamic Separation Amplification. *Anal. Chem.* **2007**, *79*, 1369–1376.

(12) Park, J. M.; Lee, J. Y.; Lee, J. G.; Jeong, H.; Oh, J. M.; Kim, Y. J.; Park, D.; Kim, M. S.; Lee, H. J.; Oh, J. H.; Lee, S. S.; Lee, W. Y.; Huh, N. Highly Efficient Assay of Circulating Tumor Cells by Selective Sedimentation with a Density Gradient Medium and Microfiltration from Whole Blood. *Anal. Chem.* **2012**, *84*, 7400–7407.

(13) Myung, J. H.; Gajjar, K. A.; Chen, J.; Molokie, R. E.; Hong, S. Differential Detection of Tumor Cells Using a Combination of Cell Rolling, Multivalent Binding, and Multiple Antibodies. *Anal. Chem.* **2014**, *86*, 6088–6094.

(14) Adams, A. A.; Okagbare, P. I.; Feng, J.; Hupert, M. L.; Patterson, D.; Göttert, J.; McCarley, R. L.; Nikitopoulos, D.; Murphy, M. C.; Soper, S. A. Highly Efficient Circulating Tumor Cell Isolation from Whole Blood and Label-Free Enumeration Using Polymer-Based Microfluidics with an Integrated Conductivity Sensor. *J. Am. Chem. Soc.* **2008**, *130*, 8633–8641.

(15) Dharmasiri, U.; Njoroge, S. K.; Witek, M. A.; Adebiyi, M. G.; Kamande, J. W.; Hupert, M. L.; Barany, F.; Soper, S. A. High-Throughput Selection, Enumeration, Electrokinetic Manipulation, and Molecular Profiling of Low-Abundance Circulating Tumor Cells Using a Microfluidic System. *Anal. Chem.* **2011**, *83*, 2301–2309.

(16) Stott, S. L.; Hsu, C. H.; Tsukrov, D. I.; Yu, M.; Miyamoto, D. T.; Waltman, B. A.; Rothenberg, S. M.; Shah, A. M.; Smas, M. E.; Korir, G. K.; Floyd, F. P., Jr.; Gilman, A. J.; Lord, J. B.; Winokur, D.; Springer, S.; Irimia, D.; Nagrath, S.; Sequist, L. V.; Lee, R. J.; Isselbacher, K. J.; Maheswaran, S.; Haber, D. A.; Toner, M. Isolation of Circulating Tumor Cells Using a Microvortex-Generating Herringbone-Chip. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 18392–18397.

(17) Xu, H.; Aguilar, Z. P.; Yang, L.; Kuang, M.; Duan, H.; Xiong, Y.; Wei, H.; Wang, A. Antibody Conjugated Magnetic Iron Oxide Nanoparticles for Cancer Cell Separation in Fresh Whole Blood. *Biomaterials* 2011, 32, 9758−9765.

(18) Wu, C. H.; Huang, Y. Y.; Chen, P.; Hoshino, K.; Liu, H.; Frenkel, E. P.; Zhang, J. X. J.; Sokolov, K. V. Versatile Immunomagnetic Nanocarrier Platform for Capturing Cancer Cells. *ACS Nano* 2013, 7, 8816−8823.

(19) Wen, C. Y.; Wu, L. L.; Zhang, Z. L.; Liu, Y. L.; Wei, S. Z.; Hu, J.; Tang, M.; Sun, E. Z.; Gong, Y. P.; Yu, J.; Pang, D. W. Quick-Response Magnetic Nanospheres for Rapid, Efficient Capture and Sensitive Detection of Circulating Tumor Cells. *ACS Nano* 2014, 8, 941−949.

(20) Patriarca, C.; Macchi, R. M.; Marschner, A. K.; Mellstedt, H. Epithelial Cell Adhesion Molecule Expression (CD326) in Cancer: a Short Review. *Cancer Treat. Rev.* 2012, 38, 68−75.

(21) Zhang, P.; Zhang, R.; Gao, M.; Zhang, X. Novel Nitrocellulose Membrane Substrate for Efficient Analysis of Circulating Tumor Cells Coupled with Surface-Enhanced Raman Scattering Imaging. *ACS Appl. Mater. Interfaces* 2014, 6, 370−376.

(22) Wang, H.; Yue, G.; Dong, C.; Wu, F.; Wei, J.; Yang, Y.; Zou, Z.; Wang, L.; Qian, X.; Zhang, T.; Liu, B. Carboxybetaine Methacrylate-Modified Nylon Surface for Circulating Tumor Cell Capture. *ACS Appl. Mater. Interfaces* 2014, 6, 4550−4559.

(23) Nagrath, S.; Sequist, L. V.; Maheswaran, S.; Bell, D. W.; Irimia, D.; Ulkus, L.; Smith, M. R.; Kwak, E. L.; Digumarthy, S.; Muzikansky, A.; Ryan, P.; Balis, U. J.; Tompkins, R. G.; Haber, D. A.; Toner, M. Isolation of Rare Circulating Tumour Cells in Cancer Patients by Microchip Technology. *Nature* 2007, 450, 1235−1239.

(24) Wang, S.; Wang, H.; Jiao, J.; Chen, K. J.; Owens, G. E.; Kamei, K. i.; Sun, J.; Sherman, D. J.; Behrenbruch, C. P.; Wu, H.; Tseng, H. R. Three-Dimensional Nanostructured Substrates toward Efficient Capture of Circulating Tumor Cells. *Angew. Chem., Int. Ed.* 2009, 48, 8970−8973.

(25) Wang, S.; Liu, K.; Liu, J.; Yu, Z. T. F.; Xu, X.; Zhao, L.; Lee, T.; Lee, E. K.; Reiss, J.; Lee, Y. K.; Chung, L. W. K.; Huang, J.; Rettig, M.; Seligson, D.; Duraiswamy, K. N.; Shen, C. K. F.; Tseng, H. R. Highly Efficient Capture of Circulating Tumor Cells by Using Nanostructured Silicon Substrates with Integrated Chaotic Micromixers. *Angew. Chem., Int. Ed.* 2011, 50, 3084−3088.

(26) Shen, Q.; Xu, L.; Zhao, L.; Wu, D.; Fan, Y.; Zhou, Y.; OuYang, W. H.; Xu, X.; Zhang, Z.; Song, M.; Lee, T.; Garcia, M. A.; Xiong, B.; Hou, S.; Tseng, H. R.; Fang, X. Specific Capture and Release of Circulating Tumor Cells Using Aptamer-Modified Nanosubstrates. *Adv. Mater.* 2013, 25, 2368−2373.

(27) Zhang, N.; Deng, Y.; Tai, Q.; Cheng, B.; Zhao, L.; Shen, Q.; He, R.; Hong, L.; Liu, W.; Guo, S.; Liu, K.; Tseng, H. R.; Xiong, B.; Zhao, X. Z. Electrospun TiO$_2$ Nanofiber-Based Cell Capture Assay for Detecting Circulating Tumor Cells from Colorectal and Gastric Cancer Patients. *Adv. Mater.* 2012, 24, 2756−2760.

(28) Sekine, J.; Luo, S. C.; Wang, S.; Zhu, B.; Tseng, H. R.; Yu, H. h. Functionalized Conducting Polymer Nanodots for Enhanced Cell Capturing: the Synergistic Effect of Capture Agents and Nanostructures. *Adv. Mater.* 2011, 23, 4788−4792.

(29) Riethdorf, S.; Fritsche, H.; Müller, V.; Rau, T.; Schindlbeck, C.; Rack, B.; Janni, W.; Coith, C.; Beck, K.; Jänicke, F.; Jackson, S.; Gornet, T.; Cristofanilli, M.; Pantel, K. Detection of Circulating Tumor Cells in Peripheral Blood of Patients with Metastatic Breast Cancer: A Validation Study of the CellSearch System. *Clin. Cancer Res.* 2007, 13, 920−928.

(30) Hoshino, K.; Huang, Y. Y.; Lane, N.; Huebschman, M.; Uhr, J. W.; Frenkel, E. P.; Zhang, X. Microchip-Based Immunomagnetic Detection of Circulating Tumor Cells. *Lab Chip* 2011, 11, 3449−3457.

(31) Kang, J. H.; Krause, S.; Tobin, H.; Mammoto, A.; Kanapathipillai, M.; Ingber, D. E. A Combined Micromagnetic-Microfluidic Device for Rapid Capture and Culture of Rare Circulating Tumor Cells. *Lab Chip* 2012, 12, 2175−2181.

(32) Kim, S.; Han, S. I.; Park, M. J.; Jeon, C. W.; Joo, Y. D.; Choi, I. H.; Han, K. H. Circulating Tumor Cell Microseparator Based on Lateral Magnetophoresis and Immunomagnetic Nanobeads. *Anal. Chem.* 2013, 85, 2779−2786.

(33) Xie, M.; Lu, N. N.; Cheng, S. B.; Wang, X. Y.; Wang, M.; Guo, S.; Wen, C. Y.; Hu, J.; Pang, D. W.; Huang, W. H. Engineered Decomposable Multifunctional Nanobioprobes for Capture and Release of Rare Cancer Cells. *Anal. Chem.* 2014, 86, 4618−4626.

(34) Huang, C.; Yang, G.; Ha, Q.; Meng, J.; Wang, S. Multifunctional "Smart" Particles Engineered from Live Immunocytes: Toward Capture and Release of Cancer Cells. *Adv. Mater.* 2015, 27, 310−313.

(35) Yu, M.; Bardia, A.; Aceto, N.; Bersani, F.; Madden, M. W.; Donaldson, M. C.; Desai, R.; Zhu, H.; Comaills, V.; Zheng, Z.; Wittner, B. S.; Stojanov, P.; Brachtel, E.; Sgroi, D.; Kapur, R.; Shioda, T.; Ting, D. T.; Ramaswamy, S.; Getz, G.; Iafrate, A. J.; Benes, C.; Toner, M.; Maheswaran, S.; Haber, D. A. Ex Vivo Culture of Circulating Breast Tumor Cells for Individualized Testing of Drug Susceptibility. *Science* 2014, 345, 216−220.

(36) Zhang, P.; Chen, L.; Xu, T.; Liu, H.; Liu, X.; Meng, J.; Yang, G.; Jiang, L.; Wang, S. Programmable Fractal Nanostructured Interfaces for Specific Recognition and Electrochemical Release of Cancer Cells. *Adv. Mater.* 2013, 25, 3566−3570.

(37) Jeon, S.; Moon, J. M.; Lee, E. S.; Kim, Y. H.; Cho, Y. An Electroactive Biotin-Doped Polypyrrole Substrate That Immobilizes and Releases EpCAM-Positive Cancer Cells. *Angew. Chem., Int. Ed.* 2014, 126, 4685−4690.

(38) Lee, H. J.; Oh, J. H.; Oh, J. M.; Park, J. M.; Lee, J. G.; Kim, M. S.; Kim, Y. J.; Kang, H. J.; Jeong, J.; Kim, S. I.; Lee, S. S.; Choi, J. W.; Huh, N. Efficient Isolation and Accurate In Situ Analysis of Circulating Tumor Cells Using Detachable Beads and a High-Pore-Density Filter. *Angew. Chem., Int. Ed.* 2013, 125, 8495−8498.

(39) Ke, Z.; Lin, M.; Chen, J. F.; Choi, J.-s.; Zhang, Y.; Fong, A.; Liang, A. J.; Chen, S.-F.; Li, Q.; Fang, W.; Zhang, P.; Garcia, M. A.; Lee, T.; Song, M.; Lin, H.-A.; Zhao, H.; Luo, S.-C.; Hou, S.; Yu, H.-h.; Tseng, H.-R. Programming Thermoresponsiveness of NanoVelcro Substrates Enables Effective Purification of Circulating Tumor Cells in Lung Cancer Patients. *ACS Nano* 2015, 9, 62−70.

(40) Liu, H.; Li, Y.; Sun, K.; Fan, J.; Zhang, P.; Meng, J.; Wang, S.; Jiang, L. Dual-Responsive Surfaces Modified with Phenylboronic Acid-Containing Polymer Brush To Reversibly Capture and Release Cancer Cells. *J. Am. Chem. Soc.* 2013, 135, 7603−7609.

(41) Schmidt, T. G.; Skerra, A. The Strep-tag System for One-Step Purification and High-Affinity Detection or Capturing of Proteins. *Nat. Protoc.* 2007, 2, 1528−1535.

(42) Vasilescu, J.; Figeys, D. Mapping Protein-Protein Interactions by Mass Spectrometry. *Curr. Opin. Biotechnol.* 2006, 17, 394−399.

(43) Voss, S.; Skerra, A. Mutagenesis of a Flexible Loop in Streptavidin Leads to Higher Affinity for the Strep-tag II Peptide and Improved Performance in Recombinant Protein Purification. *Protein Eng.* 1997, 10, 975−982.

(44) Knabel, M.; Franz, T. J.; Schiemann, M.; Wulf, A.; Villmow, B.; Schmidt, B.; Bernhard, H.; Wagner, H.; Busch, D. H. Reversible MHC Multimer Staining for Functional Isolation of T-cell Populations and Effective Adoptive Transfer. *Nat. Med. (N.Y., NY, U. S.)* 2002, 8, 631−637.

(45) Wan, Y.; Winter, M.; Delalat, B.; Hardingham, J. E.; Grover, P. K.; Wrin, J.; Voelcker, N. H.; Price, T. J.; Thierry, B. Nanostructured Polystyrene Well Plates Allow Unbiased High-Throughput Characterization of Circulating Tumor Cells. *ACS Appl. Mater. Interfaces* 2014, 6, 20828−20836.

(46) Schmidt, T. G. M.; Koepke, J.; Frank, R.; Skerra, A. Molecular Interaction Between the Strep-tag Affinity Peptide and its Cognate Target, Streptavidin. *J. Mol. Biol.* 1996, 255, 753−766.

(47) Wang, G. P.; Song, E. Q.; Xie, H. Y.; Zhang, Z. L.; Tian, Z. Q.; Zuo, C.; Pang, D. W.; Wu, D. C.; Shi, Y. B. Biofunctionalization of Fluorescent-Magnetic-Bifunctional Nanospheres and Their Applications. *Chem. Commun.* 2005, 4276−4278.

(48) Kleymann, G.; Ostermeier, C.; Ludwig, B.; Skerra, A.; Michel, H. Engineered Fv Fragments as a Tool for the One-Step Purification of Integral Multisubunit Membrane Protein Complexes. *Nat. Biotechnol.* 1995, 13, 155−160.

(49) Sturm, A.; Schierhorn, A.; Lindenstrauss, U.; Lilie, H.; Brüser, T. YcdB from *Escherichia coli* Reveals a Novel Class of Tat-dependently Translocated Hemoproteins. *J. Biol. Chem.* **2006**, *281*, 13972−13978.

(50) Niebisch, A.; Kabus, A.; Schultz, C.; Weil, B.; Bott, M. Corynebacterial Protein Kinase G Controls 2-Oxoglutarate Dehydrogenase Activity via the Phosphorylation Status of the OdhI Protein. *J. Biol. Chem.* **2006**, *281*, 12300−12307.

(51) Stemberger, C.; Dreher, S.; Tschulik, C.; Piossek, C.; Bet, J.; Yamamoto, T. N.; Schiemann, M.; Neuenhahn, M.; Martin, K.; Schlapschy, M.; Skerra, A.; Schmidt, T.; Edinger, M.; Riddell, S. R.; Germeroth, L.; Busch, D. H. Novel Serial Positive Enrichment Technology Enables Clinical Multiparameter Cell Sorting. *PLoS One* **2012**, *7*, e35798.

(52) Sheng, W.; Chen, T.; Tan, W.; Fan, Z. H. Multivalent DNA Nanospheres for Enhanced Capture of Cancer Cells in Microfluidic Devices. *ACS Nano* **2013**, *7*, 7067−7076.

(53) Wan, Y.; Liu, Y.; Allen, P. B.; Asghar, W.; Mahmood, M. A. I.; Tan, J.; Duhon, H.; Kim, Y. t.; Ellington, A. D.; Iqbal, S. M. Capture, Isolation and Release of Cancer Cells with Aptamer-Functionalized Glass Bead Array. *Lab Chip* **2012**, *12*, 4693−4701.

(54) Chen, W.; Weng, S.; Zhang, F.; Allen, S.; Li, X.; Bao, L.; Lam, R. H. W.; Macoska, J. A.; Merajver, S. D.; Fu, J. Nanoroughened Surfaces for Efficient Capture of Circulating Tumor Cells without Using Capture Antibodies. *ACS Nano* **2013**, *7*, 566−575.

(55) Wang, L.; Asghar, W.; Demirci, U.; Wan, Y. Nanostructured Substrates for Isolation of Circulating Tumor Cells. *Nano Today* **2013**, *8*, 374−387.