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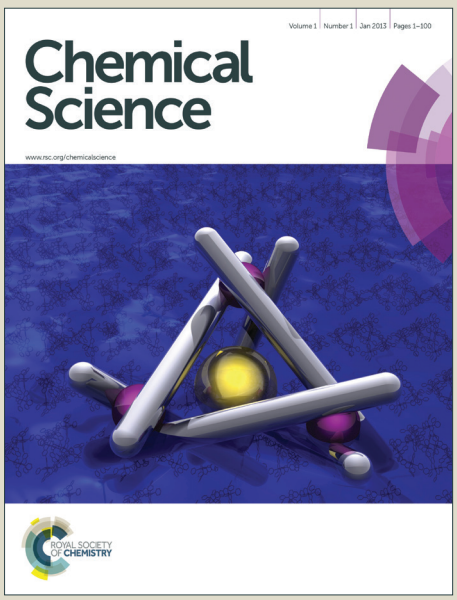

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EDGE ARTICLE

Photoresponsive Immunomagnetic Nanocarrier for Capture and Release of Rare Circulating Tumor Cells

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Song-Wei Lv,[a †] Jing Wang,[b †] Min Xie,[a] Ning-Ning Lu,[a] Zhen Li,[a] Xue-Wei Yan,[a] Si-Liang Cai,[a] Ping-An Zhang,[c] Wei-Guo Dong[b] * and Wei-Hua Huang[a] *

Isolation, release and culture of rare circulating tumor cells (CTCs) are an important capability that may promote the progress of individualized anti-tumor therapies. To realize release of CTCs without disruption of their viability for further culture and analysis, we designed an effective photocontrolled CTCs capture/release system by combination of photochemistry and immunomagnetic separation. 7-Amino coumarin was synthesized as the phototrigger to bridge the connection of anti-EpCAM antibody and magnetic beads. The coumarin moieties produced cleavage of a C-O under both ultraviolet (UV) and near-infrared (NIR) light illumination, breaking the bridge and releasing CTCs from the immunomagnetic beads. Compared with conventional immunomagnetic separation system, the negative influence of absorbed immunomagnetic beads on further CTCs culture and analysis was effectively eliminated. The system can specifically recognize 10^2 MCF-7 cells in 1 mL of human whole blood samples with 90 % efficiency and 85 % purity. Under the irradiation of UV and NIR light, 73 ± 4 % and 52 ± 6 % of captured cells were released with the viability of 90 % and 97 %, respectively. Furthermore, this technique has been used to detect CTCs from whole blood of cancer patients with high purity. This study demonstrates that the photochemical-based immunomagnetic separation method for isolating, releasing and culturing CTCs from clinic patients may provide new opportunities for cancer diagnosis and personalized therapy.

Introduction

Metastasis is the cause of most cancer deaths in patients with solid tumors.[1, 2] Circulating tumor cells (CTCs) are cells released from the primary tumor into the bloodstream that are considered the main promoters of metastasis.[3, 4] Compared to biopsy (the gold standard of current cancer diagnosis), CTCs detection offers convenient and non-invasive access to tumor cells before fatal metastasis occurs.[5, 6] To exploit CTCs as a "liquid biopsy" for disease progression and guide implementation of therapy, over the past decade, many techniques have been developed for CTCs isolation and enrichment, for instance, flow cytometry,[7, 8] microfluidic chips,[9-11] immunomagnetic separation,[12-15] and CTCs filters.[16, 17] Among them, magnetic separation is a promising tool for CTCs enrichment, because of its easy modification, fast magnetic response and high capture efficiency. The current FDA cleared CellSearch Assay[18, 19] is also based on immunomagnetic separation of CTCs and shows a good stability and reproducibility for CTCs detection.

The present-day CTCs detections, not only focus on capture of CTCs from patient, but also subsequent culture and analysis, since further independent study in the CTCs isolated from patient sample can provide additional information that lead to progress in individualized anti-tumor therapies. However, CTCs are usually captured and tightly adhered on the substrates of capture platforms, and must be released from these substrates for further culture and analysis. Although magnetic beads (MBs)-based techniques can isolate individual CTCs from whole blood, the adsorption of numerous magnetic nanoparticles on cells leads to severely negative influences for further analysis such as inhibition on cell re-culture and distortion effect on accurate image analysis.[14, 20-23] Therefore, releasing the captured CTCs from carrier surface becomes a very important and challenging step. Methods like thermodynamic release,[24-26] chemical competitive combination triggered release,[14, 27, 28] electrochemical desorption[29-31] and proteolytic enzyme degradation[10, 20] have been used to release captured tumor cells. However, the majority of these methods are invasive, with the potential to harm the completeness of cell structure and disturb the cell microenvironment.

[a] *Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China. E-mail: whhuang@whu.edu.cn; Fax: +86-27-68754067; Tel: +86-27-68752149.*
[b] *Department of gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, China. E-mail: dwg@whu.edu.cn.*
[c] *Department of Clinical Laboratory, Renmin Hospital of Wuhan University, Wuhan 430060, China.*
† These authors contributed equally.
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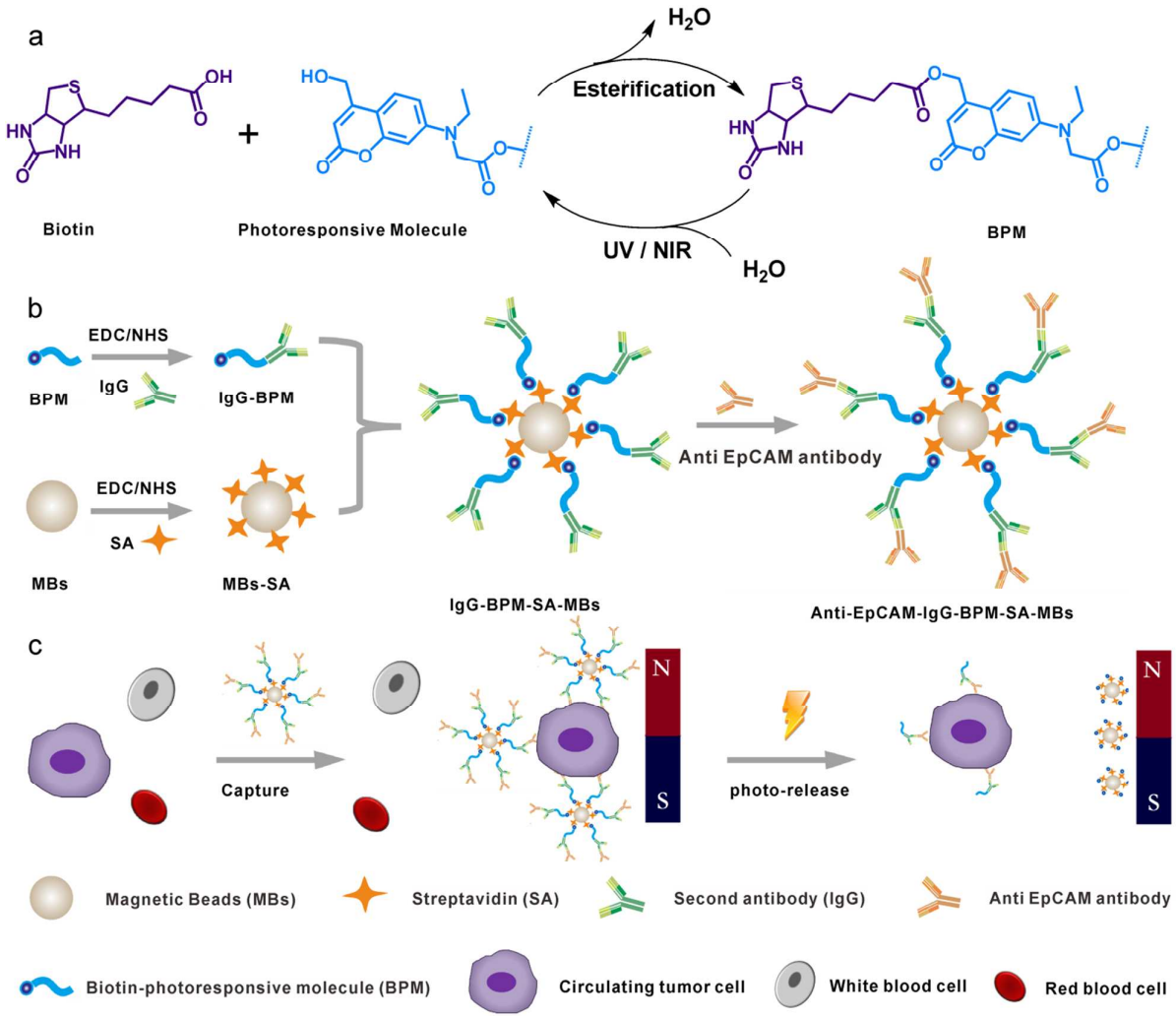
Recently, photocontrolled release systems based on light-induced bond cleavage or structural changes have attracted much attention for their applications in the area of drug/gene delivery[32-38] and photoswitched cell adhesion.[39-41] Photocontrolled release systems have non-invasiveness to the biological system and possess the possibility of remote spatiotemporal control. Cell release can be controlled precisely by external manipulation, through changing the irradiation parameters such as wavelength, intensity and time, providing the possibility for the site-specific cell release.[42] However, applying photocontrolled system to CTCs release has hitherto rarely been reported.[43, 44]



Scheme 1 Schematic diagram showing the photoresponsive immunomagnetic system for capture and release of CTCs. (a) Synthesis and photo-induced cleavage of biotin-photoresponsive molecule (BPM); (b) Construction of photoresponsive immunomagnetic beads; (c) Capture and photo-induced release of CTCs.

Herein, we constructed a novel CTCs capture and release system by combination of photochemistry and immunomagnetic separation. 7-Amino coumarin was synthesized, and reacted with biotin to form a photoresponsive linker (Scheme 1a). This photoresponsive linker was then used to bridge the capture antibody and Strepavidin (SA) modified MBs (magnetic hysteresis loop and time-dependent magnetic separation efficiency are shown in Figure S1) (Scheme 1b). Thus the whole system constructed as antibody - photoresponsive linker - magnetic beads falls into three functions: specific capture, magnetic-separation and photo-release. After CTCs capture, upon the application of a non-invasive UV or NIR light irradiation, the coumarinylmethy moieties produced cleavage of a C-O[45, 46](Scheme 1a), which realized the release of the immunomagnetic immobilized CTCs (Scheme 1c). 73 ± 4 % and 52 ± 6 % of captured cells were released under the UV and NIR light irradiation with the viability of 90 % and 97 %, respectively. This strategy effectively eliminates the optical distortion effect of beads and ensuring accurate image analysis for CTCs, more importantly CTCs were relieved from the side-effect created by the presence of adsorbed beads, promoting further cell re-culture.

Furthermore, this system has been used to detect CTCs from whole blood of cancer patients with high purity, indicating the photochemical-based immunomagnetic separation method may provide new opportunities for cancer diagnosis and personalized therapy.

RESULTS AND DISCUSSION

Synthesis of Photoresponsive Linker. In this work, a small molecule, 7-Amino coumarin compound was picked and synthesized as the core part to construct photoresponsive linker, due to its excellent property in photo-response. The coumarin photo-cleavable groups have high molar extinction coefficient, rapid photolysis rate, NIR excitation and low toxicity of photolysis side-product for biological systems.[47-49] The coumarin moieties produce cleavage of a C-O under illumination, leading to a separation of the two ends. Furthermore, as a synthetic product, 7-Amino coumarin can be designed into various functional groups to fit different application system. Here, a 7-Amino coumarin compound introduced a hydroxyl group and a carboxyl group was reacted with biotin via the hydroxyl group to construct biotin-7-Amino coumarin compound (biotin-photoresponsive molecule, BPM) as the photoresponsive linker, and the carboxyl group was subsequently used to conjugate with antibodies (Figure S2). Fluorescence spectrum was conducted by using single- and two-photo excitation light source to test the two-photon absorption properties of the photoresponsive linker. The results showed the linker processed both single- and two-photon fluorescence properties (Figure 1a).

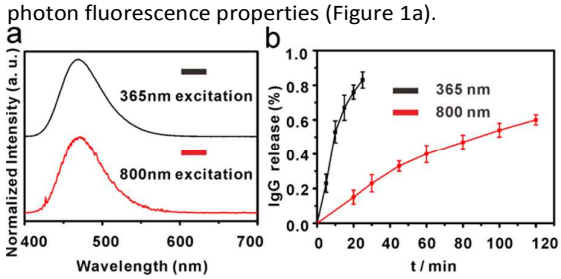


Figure 1. (a) Emission spectra of compound 7 (upper: single photon excitation at 365 nm, lower: two photon excitation at 800 nm); (b) Time course of photolysis controlled IgG release from IgG-BPM-SA-MBs (left: 365 nm, 10 mW/cm^2; right: 800 nm, 10 mW/cm^2). (Error bars represent standard deviations, n = 3)

Fabrication of IgG-BPM-SA-MBs. Scheme 1 illustrates the entire construction process of photoresponsive immunomagnetic beads and the photo-induced release of cells from MBs. To construct photoresponsive system, SA-immobilized MBs (SA-MBs) (Figure S3) and BPM caged IgG (IgG-BPM) were prepared, and IgG-BPM-SA-MBs were then obtained by the specific interaction between SA and biotin. The successful preparation of IgG-BPM-SA-MBs was evaluated by fluorescent labelling (Figure S4) as well as particle size analysis (Figure S5). Here, SA-biotin was employed to mediate the connection of phototrigger caged antibody and MBs which avoided the direct interaction between antibody and MBs, guaranteeing the high photo-induced release efficiency. To determine the optical exposure time for bond cleavage, the time courses of the IgG release under photolysis at both 365 nm light and 800 nm NIR light (Figure 1b) were monitored by UV-visible spectra. Both photolytic processes progressed effectively and the release controlled by 365 nm (10 mW/cm^2) irradiation reached 75 % after 15 min while 800 nm (10 mW/cm^2) irradiation reached 60 % after 2 h.

Fabrication and Characterization of Anti-EpCAM-IgG-BPM-SA-MBs. Anti-EpCAM was immobilized onto IgG-BPM-SA-MBs through specific recognition between secondary antibody and primary antibody to construct Anti-EpCAM-IgG-BPM-SA-MBs. Subsequently, FITC-labelled secondary antibody (Figure 2) was used to report the attachment and light-induced detachment of anti-EpCAM onto/from the surface of IgG-BPM-SA-MBs. Compared to faint fluorescence of IgG-BPM-SA-MBs (Figure 2e), anti-EpCAM-IgG-BPM-SA-MBs showed strong FITC fluorescence (Figure 2c). Upon light irradiation treatment, the 7-Amino coumarin in BPM produced a cleavage of the ester bond, leading to the release of Anti-EpCAM-IgG-BPM from MBs surface, while the remained MBs part could not be labelled by FITC-labelled secondary antibody (Figure 2g).

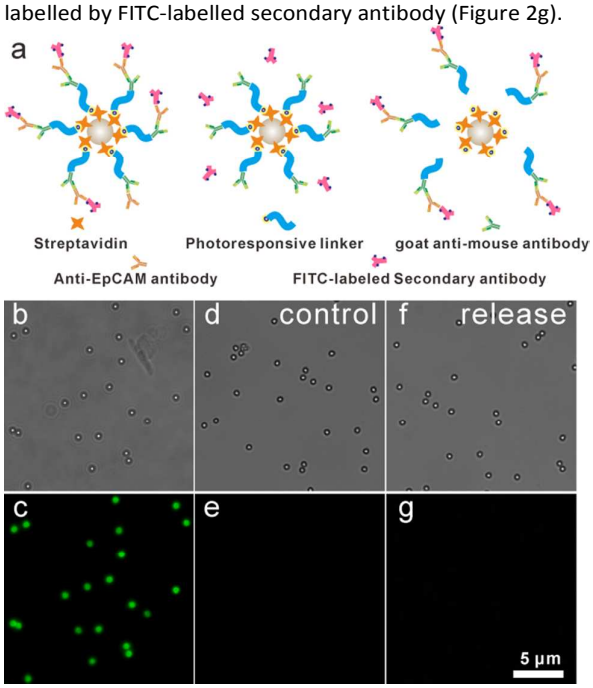


Figure 2. 7-Amino coumarin enabled conjugation and release of anti-EpCAM on IgG-BPM-SA-MBs. (a) schematic graph showing the principle of confirming conjugation of anti-EpCAM on IgG-BPM-SA-MBs; (b, c) incubation of FITC-labelled secondary antibody with anti-EpCAM-IgG-BPM-SA-MBs; (d, e) incubation of FITC-labelled secondary antibody with IgG-BPM-SA-MBs; (f, g) after light irradiation (365 nm, 10 mW/cm^2, 15 min) to anti-EpCAM-IgG-BPM-SA-MBs, the remaining MBs part reported by FITC-labelled secondary antibody (Panels b, d, and f show bright-field images; Panels c, e, and g show fluorescence images).

Capture and Release of Cancer Cells Using Anti-EpCAM-IgG-BPM-SA-MBs. To explore the specific cell recognition performance of anti-EpCAM-IgG-BPM-SA-MBs (Figure 3a), two EpCAM-positive cancer cell lines (MCF-7, SK-BR-3) were chosen as the target cell lines[50], and Hela (EpCAM-negative cancer cell lines)[51] was selected as control. EpCAM is frequently over-expressed by many kinds of solid-cancer cells and is absent from hematologic cells.[52] Compared with the EpCAM-negative cells (Hela), the anti-EpCAM positive cells (MCF-7 and SK-BR-3 cells) display high specific cell capture efficiency through anti-EpCAM-IgG-BPM-SA-MBs (MCF-7, 91 ± 5 %; SK-BR-3, 87 ± 4 %). Meanwhile, the IgG-BPM-SA-MBs could hardly capture MCF-7 cells (Figure 3a), indicating that the binding between anti-EpCAM-IgG-BPM-SA-MBs and MCF-7 cells was effective and specific. The capability of anti-EpCAM-IgG-BPM-SA-MBs to capture rare tumor cells in synthetic CTCs samples was investigated. DAPI-stained MCF-7 cells were spiked into whole blood with a concentration of 10^2, 10^3, 10^4, 10^5 cells mL^{-1}, for comparison, capture efficiencies were also examined in PBS buffer spiked with similar concentrations of MCF-7 cells. As shown in Figure 3b, regression analysis of captured cell number versus spiked cell number obtained y= 0.90 x (R^2 = 0.9991), y= 0.86 x (R^2 = 0.9994) respectively in PBS and whole blood. It can be seen that anti-EpCAM-IgG-BPM-SA-MBs could specifically and efficiently capture target cells.

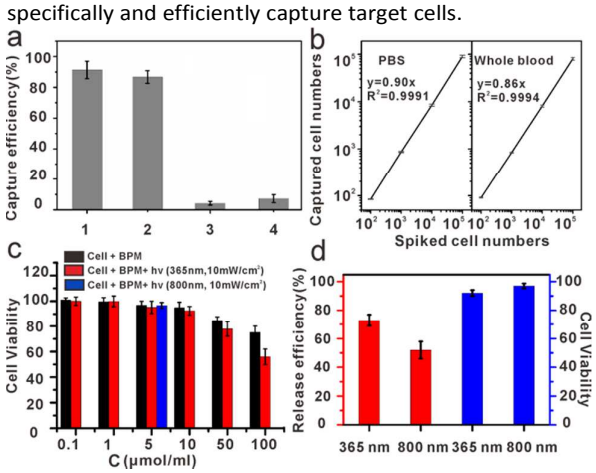


Figure 3. Capture and release of cancer cell. (a) Capture efficiencies of cancer cells. 1: anti-EpCAM-IgG-BPM-SA-MBs to MCF-7 cells; 2: anti-EpCAM-IgG-BPM-SA-MBs to SK-BR-3 cells; 3: anti-EpCAM-IgG-BPM-SA-MBs to Hela cells; 4: IgG-BPM-SA-MBs to MCF-7 cells; (b) Regression analysis of the number of the MCF-7 cells captured by the anti-EpCAM-IgG-BPM-SA-MBs versus the number of the cells spiked in two different types of samples: left PBS, right Whole blood (Error bars represent standard deviations, n = 3); (c) Cytotoxicity Assay: Effect of photosensitive molecule with different concentrations on the cell viability of MCF-7 cell line, the light exposure time is 15 min (λ = 365 nm, 10 mW/cm^2) and 2 h (λ =800 nm, 10 mW/cm^2); (d) Release efficiencies of captured MCF-7 cells by light irradiation (left: 365 nm, 10 mW/cm^2; right: 800 nm, 10 mW/cm^2), and cell viability after release by different wavelength of light (left: 365 nm, right: 800 nm).

Toxicity of Materials and the Viability of Released Cells. To study the effect of photosensitive molecules and optical radiation on cell viability, cytotoxicity of materials was determined by MTT method in breast cancer cell line MCF-7. As demonstrated in Figure 3c, in a photoresponsive molecule concentration of 0.1 to 10 μmol/ml, the MCF-7 cells did not lose the viability or have detectable changes in behavior after 24 hours of incubation, which demonstrated the synthesized material was of good biocompatibility.

According to the results of photo-release of IgG-BPM from SA-MBs (Figure 1) and previously optimized conditions for photo-cleavage of coumarin[53, 54], cell release was carried out under two different wavelengths of radiation. Before cell capture, there have large population of MCF-7 cells on the 96-well cell-culture plate (Figure S6a). After incubation with anti-EpCAM-IgG-BPM-SA-MBs for 30 min and magnetic scaffold separation (in the dark condition), there were few cells remained on the 96-well cell-culture plate (Figure S6b). When the light irradiation (365 nm, 10 mW/cm^2) was applied for 15 min, an average of 73 ± 4 % of the captured MCF-7 cells were released from the anti-EpCAM-IgG-BPM-SA-MBs (Figure 3d and Figure S6c), and an average of 52 ± 6 % of the captured cells were released under condition of NIR light (800 nm, 10 mW/cm^2, 2 h) (Figure 3d and Figure S6d). Meanwhile, the released cancer cells can be directly cultured and propagated in vitro (Figure S7).

Calcein AM and propidium iodide (PI) were used to stain the released tumor cells to analyze their viability. Calcein AM can penetrate the live cell membrane and react with the intracellular esterase to form calcein with green fluorescence, while PI is a membrane-impermeable nuclear stain that can stain only dead cells, resulting in red fluorescence.[55, 56] From Figure 3d and Figure S8, it can be seen that the majority of the released cells showed green fluorescence, and the viability rate was calculated to be 90 % (365 nm) and 97 % (800 nm). The results showed that released cells kept good viability under irradiation under both 365 nm and 800 nm, while 365 nm irradiation induced a faster and more efficient cell release performance. Cell viability under different UV-irradiation time was further tested. The results showed that cell viability was obviously sacrificed with elongated irradiation time (Table S1), though increasing irradiation time could improve the release efficiency (Figure 1b). Therefore, we finally selected the UV-irradiation time of 15 min in our following photo-induced releasing experiments, which can keep a good balance between release efficiency and cell viability. With this approach, the anti-EpCAM-IgG-BPM-SA-MBs on the cancer cells surface could be rapidly and efficiently released without damaging the cells, which was crucial for CTCs cellular analysis.

Capture and ICC Identification of Spiked Cancer Cells from Mimic Clinical Blood Samples. To demonstrate the isolation of cancer cells from human whole blood, DAPI-stained MCF-7 cells were spiked into healthy human whole blood with a concentration of approximately 10^2 cells mL^{-1}. As shown in Figures 4, as few as 10^2 MCF-7 cells were effectively isolated and detected from 1 mL mimic patient blood with 90 % ± 5 % capture efficiency (n = 3), demonstrating that anti-

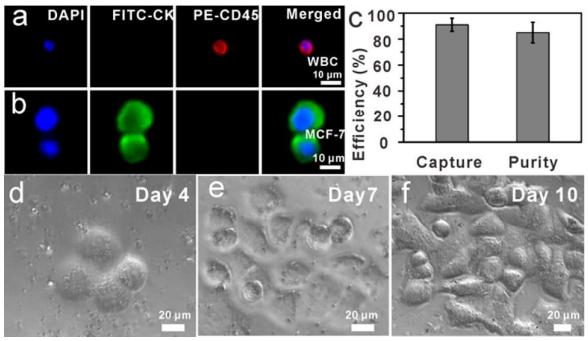


Figure 4. Capture of MCF-7 cells from whole blood. (a, b) microscopic images of cells captured from mimic clinical blood samples and identified with the three-color ICC. Merged: merge of nucleus (DAPI), CK (FITC), and CD45 (PE); (c) Capture efficiency of MCF-7 in the whole blood and purity; (d-f) Re-culture of released cancer cells from mimic clinical blood samples (5×10^2 MCF-7 cells were spiked into 1 mL whole blood) (d) Day 4. (e) Day 7. (f) Day 10.

EpCAM-IgG-BPM-SA-MBs were applicable in the isolation and detection of rare CTCs. Further, the captured cancer cells could be used for common three-color immunocytochemistry (ICC) identification by FITC-labelled anti-CK19 (a marker for epithelial cells) monoclonal antibody, PE-labelled anti-CD45 (a marker for WBCs), and DAPI nuclear staining. As shown in Figures 4a − 4b, the MCF-7 cell was DAPI+/CK+/CD45− and WBCs were DAPI+/CK−/CD45+. Meanwhile, we counted that the purity of separated MCF-7 cells was 85 % ± 8 % (Figure 4c). These results showed that cancer cells can be isolated by anti-EpCAM-IgG-BPM-SA-MBs from whole blood.

To determine whether the released tumor cells can be cultured, 5×10^2 MCF-7 cells were spiked into 1 mL whole blood and subjected to the capture and release process as discussed above. The released cells were then seeded into cell culture dishes for propagation in culture (Figure 4 (d-f)). Compared with the control group, re-culture of the released cells showed the same viability, which might have great potential for the subsequent molecular and functional analysis.

Isolation of CTCs from Cancer Patients Blood Samples. Further, we applied anti-EpCAM-IgG-BPM-SA-MBs to the detection of CTCs in the whole blood samples from 13 cancer patient's samples (including colon, liver, lung, and breast cancer patients). We also processed blood from healthy individuals as controls (n=8). The isolated cells were also identified with the three-color ICC as described above, and CTCs were DAPI+/CK+/CD45-, and WBCs were DAPI+/CK-/CD45+. The images of a part of CTCs captured with our photoresponsive immunomagnetic beads from 1.5 mL of blood of patient #4 are shown in Figure 5(a-c) and the results are summarized in Figure 5d and Table S2-S3 (Supporting Information). CTCs in the blood of the 13 cancer patients could be captured and detected, while no CTC was found in any healthy samples.

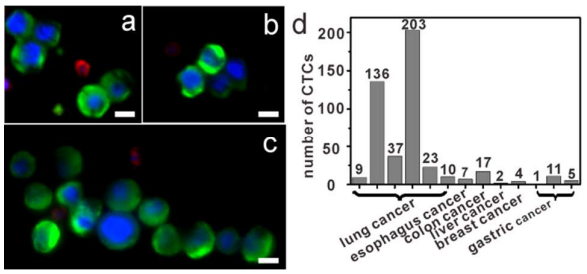


Figure 5. Capture of CTCs from whole blood of cancer patients. (a-c) Micrographs of CTCs (a) and CTCs clusters (b, c) isolated from a metastatic lung cancer patient based on anti-EpCAM-IgG-BPM-SA-MBs, immunofluorescence staining (DNA (blue), CK (green), and CD45 (red)); (d) Quantification of CTCs of blood samples from patients. All scale bars represent 10 μm.

An important finding of this study is the successful isolation of CTCs clusters from the blood of a patient with metastatic lung cancer. Although there is evidence to show that compared with single CTCs, CTC clusters have 23- to 50-fold increased metastatic potential, the presence and biological significance of such CTCs clusters in blood are still not well understood.[57, 58] The successful capture of CTCs clusters in the blood of patients with cancer may provide insight into the process of metastasis in human cancer. Further research for these clusters will provide more possibilities to clarify the mechanism of tumor metastasis.

Conclusions

In conclusion, we developed a strategy for isolating and releasing CTCs using biotin-7-Amino coumarin as phototrigger to cage anti-EpCAM antibody to constitute photoresponsive immunomagnetic system. This system can not only isolates CTCs with high specificity, but also releases CTCs without disruption of its viability and biological functions. The anti-EpCAM-IgG-BPM-SA-MBs could specifically recognize 102 MCF-7 cells in 1 mL of human whole blood samples with 90 % efficiency and 85 % purity. Under the irradiation of UV and NIR light, 73 ± 4 % and 52 ± 6 % of captured cells were released from MBs with the viability of 90 % and 97 %, respectively. The released cells well kept the ability to proliferation, which is a critical requirement for personalized medicine. Furthermore, the photoresponsive immunomagnetic beads were applied to the clinic CTCs detection, including isolation of individual CTC or CTCs cluster from metastatic cancer patients and characterization based on three-color ICC method. Therefore, our CTCs capture and release system shows great potential for efficient CTCs enrichment, isolation and culture. Our future efforts will include releasing and culturing the captured CTCs from cancer patients, as well as cellular and genetic analysis of the isolated CTCs.

Acknowledgements

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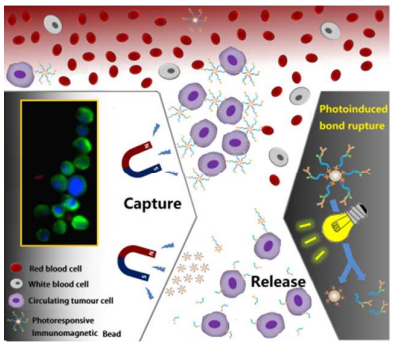
TOC



7-aminocoumarin compound was synthesized and used as phototrigger to cage EpCAM-antibody to construct a photocontrolled CTCs capture and release system.