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Rapid Recovery and Short-Term Culture of Gastric Circulating Tumor Cells Using Microcavity Array

Tomoko Yoshino[1] | Tomohiro Takabayashi[1] | Qian Bao[1] | Tsuyoshi Tanaka[1] | Ryo Negishi[1] | Tatsu Shimoyama[2] | Takeshi Sawada[2] | Yusuke Kanemasa[2] | Fumiaki Koizumi[3]

[1]Division of Biotechnology and Life Science, Institute of Engineering, Tokyo University of Agriculture and Technology, Koganei, Tokyo, Japan | [2]Department of Medical Oncology, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Tokyo, Japan | [3]Department of Laboratory Medicine, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Tokyo, Japan

Correspondence: Tomoko Yoshino (y-tomoko@cc.tuat.ac.jp)

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ABSTRACT

Circulating tumor cells (CTCs) hold significant promise for cancer diagnosis, prognosis, and treatment monitoring. We previously developed a technique for a single-cell filtering device known as the microcavity array (MCA), specifically designed for the efficient recovery of CTCs from whole blood samples. Efficient enrichment and release of cells from the MCA remains challenging because of cell adhesion that occurs on the MCA surface during the enrichment phase. This study investigated the effects of surface modification with 2-methacryloyloxyethyl phosphorylcholine (MPC) on the recovery efficiency of cancer cell lines from MCA. Scanning electron microscope (SEM) demonstrated reduced cell-substrate interactions, leading to improved recovery efficiency. Comparative analyses showed that the MCA method provided superior recovery efficiency and reduced processing time compared to traditional methods such as density gradient centrifugation (DGC), while maintaining cell viability and proliferative capacity. CTCs were successfully detected in patients with gastric cancer, and short-term cultures were achieved even when fewer than 20 CTCs per milliliter of blood were isolated. These findings emphasize the importance of surface modification for enhancing CTC isolation and the need for optimized culture conditions. The optimized MCA method offers a promising approach for rapid CTC recovery and potential integration with automated systems.

Practical application: The Microcavity array (MCA) is a device specifically designed for efficient recovery of CTCs from whole blood. However cell adhesion on the MCA surface can limit release efficiency. This study demonstrated that surface modification with MPC signigicantly reduces cell-substrate adhesion, improving recovery efficiency while maintaining cell viability and proliferative capacity. Compared to traditional density gradient centrifugation, the MPC-modified MCA offers shorter processing time and better performance. CTCs were successfully detected in gastric cancer, and short-term cultures were achieved even when fewer than 20 CTCs per mL of blood were isolated. The method supports downstearm applications such as cancer cell characterization and treatment monitoring. With potential for integration into automated system, the optimized MCA provides a practical, scalable solution for clinical liquid biopsy and personalized oncology.

Abbreviations: AGS, adenocarcinoma gastric stomach; CTC, circulating tumor cells; DGC, density gradient centrifugation; MCA, microcavity array; MPC, 2-methacryloyloxyethyl phosphorylcholine; NCI-H1975, National Cancer Institute-H1975 cell line; SEM, scanning electron microscopy; SNU-1, Seoul National University-1 cell line.

1 | Introduction

Circulating tumor cells (CTCs) are critical biomarkers for hematogenous metastasis and significantly contribute to cancer prognosis and treatment monitoring [1]. Despite their well-recognized clinical relevance, CTC detection and analysis remain challenging owing to their exceedingly low abundance, typically estimated at approximately 1 cell per 10 million white blood cells [2]. This extreme scarcity hinders reliable identification and severely limits the potential for detailed genetic analysis and drug sensitivity testing, which are essential for advancing personalized cancer therapies. Therefore, developing efficient strategies for CTC enrichment and culture is crucial for comprehensive molecular and functional studies.

Numerous studies have shown that CTC counts correlate strongly with clinical outcomes in a range of metastatic cancers such as breast, prostate, lung, pancreatic, and gastric cancers [3–8]. For instance, Li et al. [9] explored the potential of CTCs as prognostic markers for gastric cancer. They found that a higher number of CTCs was associated with shorter disease-free survival (DFS). Their findings suggest that CTC detection could be an independent predictor of prognosis in gastric cancer, particularly DFS. Khoo et al. [10] used patient-derived CTC cultures obtained from liquid biopsies to form CTC clusters using microwells and microfluidics for in situ drug screening. Their method provided rapid feedback on drug resistance within 2 weeks, showing potential for personalized cancer treatment. Similarly, Yu et al. [11] developed CTC cultures from patients with breast cancer to monitor drug susceptibility. They identified mutations in cancer-related genes, suggesting new therapeutic targets and highlighting the potential of CTC cultures for personalized cancer treatment. However, despite these advancements, existing techniques still struggle with low sensitivity and specificity, limiting their ability to reliably detect CTCs in early-stage cancers or in cases with minimal tumor burden. Consequently, refining the CTC enrichment and culture methods is essential to gain deeper insights into metastasis mechanisms and improve patient-specific treatment strategies.

In recent years, both positive and negative selection methods have been widely used to enrich and isolate CTCs from other blood components. Positive selection strategies generally employ cell surface markers, such as epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK), to directly capture and enrich CTCs. These methods are often combined with size-based filtration techniques [12] and microfluidic devices to enhance the efficiency of CTC separation and facilitate single-cell-level analysis [13]. However, the extreme rarity of CTCs in the bloodstream poses a considerable risk of contamination during enrichment. In contrast, negative selection, as exemplified by the RosetteSep technique, employs density gradient centrifugation (DGC) to remove non-target cells, thereby indirectly enriching CTCs while preserving their native state [14]. This method separates peripheral blood mononuclear cells (PBMCs) from peripheral blood using DGC, followed by leukocyte depletion using magnetic microparticles conjugated with anti-CD45 antibodies. Although this technique enables effective CTC enrichment without requiring labeling, multiple centrifugation steps lead to cell loss, ultimately diminishing the enrichment rate and increasing contamination from leukocytes. Furthermore, the entire process, from blood collection to culture setup, is time-consuming and contributes to a low overall success rate for CTC cultures.

To address the challenges associated with CTC enrichment and analysis, we developed a single-cell filtering device known as the microcavity array (MCA), specifically designed for the efficient enrichment of CTCs from whole blood samples [15–17]. The MCA has emerged as a transformative tool in CTC research, enabling automated high-throughput recovery that significantly enhances detection sensitivity and reproducibility [18–20]. Furthermore, we introduced a novel approach for manipulating individual CTCs based on hydrogel encapsulation, thereby augmenting the capability of genetic characterization suited for downstream analyses. Integration of MCAs with wide-field fluorescence imaging systems has accelerated the imaging process and significantly improved the throughput of CTCs. We also developed a multi-single-cell encapsulation system, which facilitates efficient single-cell genetic mutation analysis [21, 22]. This innovative multi-single-cell encapsulation system, combined with optimized illumination conditions, was demonstrated to be effective for the isolation of CTCs and other cellular targets from liquid biopsies [23]. Furthermore, single-cell transcriptomic analyses of CTCs derived from gastric cancer patients were successfully performed and characterized [24].

Building on these significant advancements, this study focuses on further enhancing the efficiency of MCA enrichment and release through innovative surface modifications and reflux technologies. This approach is designed to optimize CTC enrichment and to control their subsequent release, thereby addressing the limitations inherent in previous methodologies. We systematically optimized and compared our novel MCA-based technique with traditional DGC methods and assessed its application for CTC recovery from peripheral blood samples of patients with gastric cancer.

2 | Materials and Methods

2.1 | Cell Culture and Preparation

The human lung cancer cell line NCI-H1975 (ATCC CRL5908) and the human gastric cancer cell lines adenocarcinoma gastric stomach (AGS) (ATCC CRL-1739), and SNU-1 (ATCC CRL-5971) were used for cell release and culture experiments, based on their relevance in cancer research and drug testing. The cell lines were cultured in RPMI-1640 (Sigma-Aldrich, Irvine, UK) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO_2 atmosphere, ensuring optimal growth conditions for 3–4 days. Prior to experimentation, confluent cells were trypsinized and resuspended in PBS for detachment and maintenance of cell viability. Cells were then stained with 5 μM CellTracker Green CMFDA (Molecular Probes, Carlsbad, CA, USA) for 30 min, following the manufacturer's instructions, to ensure effective labeling. After two washes with PBS (Gibco, Grand Island, NY, USA), the cells were resuspended in PBS containing 2 mM EDTA and 0.5% BSA. For spike-in experiments, stained cells were aliquoted to achieve exact concentrations of 10–10^3 cells per tube using a BD FACS Aria II (BD, NJ, USA), which is suitable for precise cell sorting and quantification. Different

cell concentrations (10, 10^2, 10^3 cells) were spiked into either PBS (1 mL, 0.5% BSA, 2 mM EDTA) or human blood (1 mL). Human blood samples were collected from healthy donors at the Tokyo University of Agriculture and Technology after obtaining informed consent from all participants. All experimental protocols were approved by the Institutional Review Board (approval codes: No. 02–61), which ensured adherence to relevant ethical guidelines and regulations.

2.2 | Cell Enrichment and Release Using MCA

An MCA with a rectangular pore (8 μm × 100 μm, 6.7% porosity), was fabricated using electroplating methods and integrated into a microfluidic device as described previously [25]. For 2-methacryloyloxyethyl phosphorylcholine (MPC) modification, MCAs were dipped in a 0.5% MPC polymer ethanol solution for 6 min. After modification, the MCAs were washed with 99.5% ethanol and air-dried. Modified or unmodified MCAs were integrated into a CTC enrichment device, which included a sample inlet and reagent outlet. Samples were introduced into the CTC enrichment device at a flow rate of 200 μL/min using a peristaltic pump to enrich cancer cells on the MCA. To remove residual blood cells, 1 mL of PBS (0.5% BSA, 2 mM EDTA) and 1 mL of Dulbecco's modified Eagle medium (DMEM; Gibco) containing 20% FBS and 1% penicillin-streptomycin (PS) were introduced into the device at the same flow rate for 5 min. Following cell release, 1 mL DMEM (20% FBS, 1% PS) was introduced at reverse flow rates of 200, 400, 600, and 800 μL/min. The released cells were collected in 48-well plates. The entire process from blood introduction to CTC culture initiation was completed within 30 min.

Cell counts on the MCA were performed using a fluorescence microscope (BX53; Olympus Corporation, Tokyo, Japan) equipped with a 10× objective lens and a computer-operated motorized stage. Images were captured using a cooled digital camera (EXi Aqua; Q Imaging) and analyzed using Lumina Vision imaging software (Mitani, Tokyo, Japan). The released cells in 48-well plates were counted using an IN-Cell Analyzer 2000 (GE Healthcare Japan, Tokyo, Japan). Additionally, a wide-field fluorescence imaging system developed by our group was used to monitor the release of cancer cells from the MCA [22]. The Cell Enrichment Rate was defined as the ratio of enriched cells in the MCA to all introduced cells, whereas the Cell Release Rate was the ratio of released cells from the MCA to all enriched cells in the MCA. The Recovery Rate is defined as the ratio of cells released from the MCA to the total number of introduced cells, serving as an overall indicator of the combined efficiency of enrichment and release.

2.3 | Observation by SEM

After capturing cells on the CTC enrichment device with either unmodified and MPC polymer-modified MCA, PBS containing 2% paraformaldehyde and 2% glutaraldehyde was introduced to fix the cells at 4°C for 60 min. The MCA was then removed from the device and washed sequentially with PBS and ethanol solutions (25%, 50%, 75%, 90%, and 99.5%). Cells that adhered to the MCA were observed using a scanning electron microscope (SEM; VE-9800; KEYENCE, Osaka, Japan).

2.4 | Cell Collection by DGC

For DGC, 10, 10^2, or 10^3 cells spiked in blood were used as samples. The samples were diluted with 1 mL PBS and 100 μL of RosetteSep Human CD45 Depletion Cocktail (STEMCELL Technologies, Vancouver, BC, Canada) was added. After incubation at room temperature for 20 min, the samples were gently layered onto 3 mL of density gradient medium (Ficoll-Paque PLUS; GE Healthcare, MA, USA) and centrifuged at 1200 × *g* for 20 min. The enriched cell layer was pipetted into a new tube and suspended in 5 mL PBS. The cells were centrifuged at 300 × *g* for 10 min, and the pellet was resuspended in 5 mL PBS and centrifuged again at 300 × *g* for 10 min. The final pellet was resuspended in 1 mL DMEM (20% FBS, 1% PS) and seeded onto a 48-well plate.

2.5 | Patient Samples

The clinical study was conducted at the Tokyo Metropolitan Cancer and Infectious Diseases Center of Komagome Hospital. The study involving human subjects was approved by the Institutional Review Board of Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital (Approval Number: 1441). Blood samples (n = 8) were obtained from patients with gastric cancer. The detailed disease status and therapy are listed in Table S1. Written informed consent was obtained from all participants prior to blood collection. Blood samples were drawn by venipuncture into Venoject II 5 mL EDTA-2Na tubes (VP-NA050KN, Terumo, Japan). To avoid contamination with skin cells, the first few milliliters of blood were discarded. All samples were processed within 2 h of collection, which was crucial for maintaining sample integrity and ensuring accurate downstream analyses.

2.6 | Clinical Study

Blood samples (2–9 mL) from patients (Patient No: G1-G5) were divided into 1–3 groups for CTC counting and cultivation. The numbers in G4_1, G4_2, and G4_3 denote samples collected from the same patient on different dates. Similarly, G5_1 and G5_2 indicate samples obtained from the same patient at different dates. Patient bloods (1–3 mL) were introduced into the CTC recovery devices at a flow rate of 200 μL/min, as described for cancer cell-spiked samples. Subsequently, 1 mL PBS (0.5% BSA, 2 mM EDTA) was passed through the device at the same flow rate for 5 min. Captured cells were stained with 5 μM CellTracker Green, 1 μg/mL Hoechst 33342, and 0.2 μg/mL PE-labeled anti-CD45 antibody for 30 min. After staining, 1 mL PBS (0.5% BSA, 2 mM EDTA) and 1 mL DMEM (20% FBS, 1% PS) were introduced at a flow rate of 200 μL/min. Subsequently, 1 mL DMEM (20% FBS, 1% PS) was introduced at a reverse flow rate of 600 μL/min to collect the released cells in a 48-well plate for CTC counting.

For CTC cultivation, cells were collected without prior staining and subsequently cultured in 48-well plates for 7 days, with media changes every 3–4 days. On day 7, the cells were stained

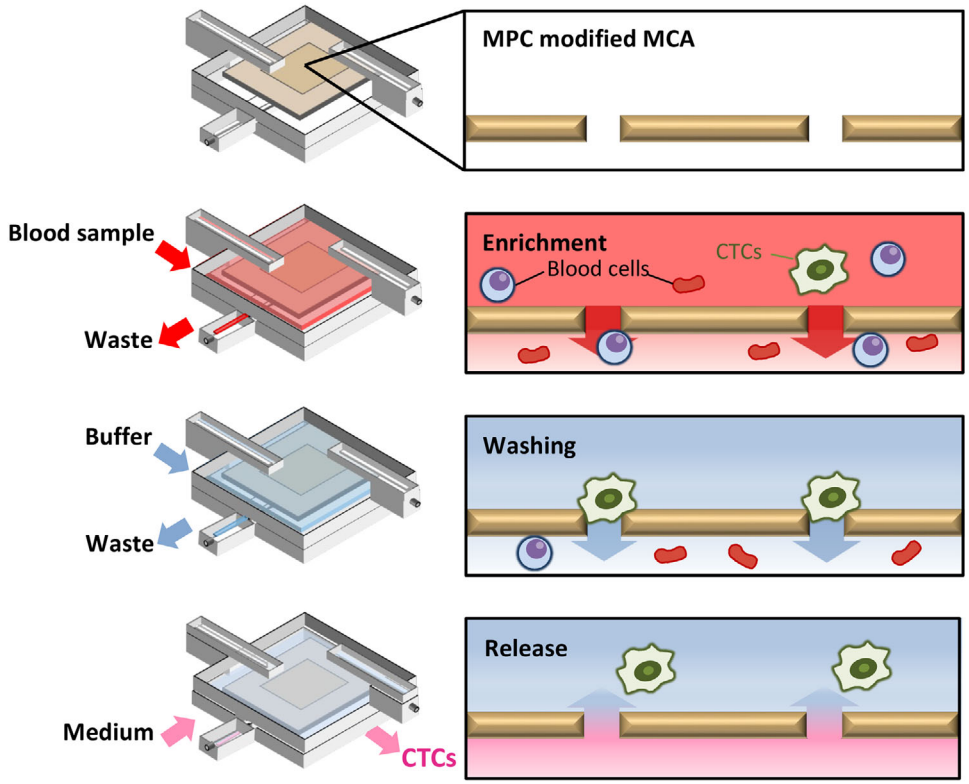

FIGURE 1 | The scheme of CTC recovery by MPC-modified MCA. The recovery process involves the application of MPC-modified MCA for sample filtration to capture target cells. Nontarget cells are then removed by buffer washing, and finally, a reverse flow releases the target cells for collection and culture. CTC, circulating tumor cell; MCA, microcavity array; MPC, 2-methacryloyloxyethyl phosphorylcholine.

with Calcein-AM, Hoechst 33342, and PE-conjugated anti-CD45 antibodies to facilitate quantification.

2.7 | Statistical Analysis

All experiments were calculated from three independent data sets. Additionally, statistical analyses were carried out using one-way ANOVA, with significance defined as $p < 0.05$ ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

3 | Results

3.1 | Effect of Surface Modification on Cell Recovery From MCA

The overall CTC recovery process is summarized in Figure 1. Initially, the enrichment step is performed to capture CTCs on the filtration pores of the MPC-modified MCA. Following this, a buffer wash is applied to remove non-target cells. Finally, backflow is utilized to release and recover the target CTCs. The recovered CTCs are applied for imaging, quantification analysis, and cultivation in subsequent steps. Figure 2A shows the enrichment and release rates of NCI-H1975 cells from unmodified MCA using blood samples spiked with 10^2 cells. While the enrichment efficiency reached 92 ± 3%, the release rate was limited to only 3 ± 2%, indicating strong cell adhesion to the MCA surface. SEM analysis (Figure 2B) confirmed this observation by demonstrating the tight adhesion of NCI-H1975 cells to the unmodified surface. In contrast, surface modification with MPC significantly enhanced the release efficiency to 71 ± 7% (Figures 1D and 2C), showing a marked reduction in cell adhesion. Wide-field imaging further corroborated these results, demonstrating the rapid release of NCI-H1975 cells from the MPC-modified MCA, with no detectable release from the unmodified version (Figure S1). Similar results were observed for the adherent gastric cancer cell line AGS (Figure 3A), in which recovery occurred only from the modified MCA. However, for the nonadherent SNU-1 cells, effective recovery was observed from both modified and unmodified MCAs, although MPC modification still resulted in a higher recovery rate (Figure 3B). All experiments were conducted at a flow rate of 400 μL/min, as tests at different flow rates showed no significant changes in performance above this threshold (Figure S2).

3.2 | Comparison Between MCA and DGC in CTC Recovery From Whole Blood

Figure 4A compares the recovery of NCI-H1975 cells from spiked healthy blood samples using the MPC-modified MCA and DGC methods. Across varying cell concentrations (10–10^3 cells), the MCA consistently achieved significantly higher CTC recovery rates than those with DGC. The MCA recovered over 80 ± 10% of cancer cells, regardless of the initial concentration, while DGC recovery peaked at 21.2 ± 2.4%. Furthermore, the MCA method achieved CTC recovery in less than 20 min, which was significantly faster than the 90-min processing time required for DGC. Importantly, cell viability after culturing in DMEM

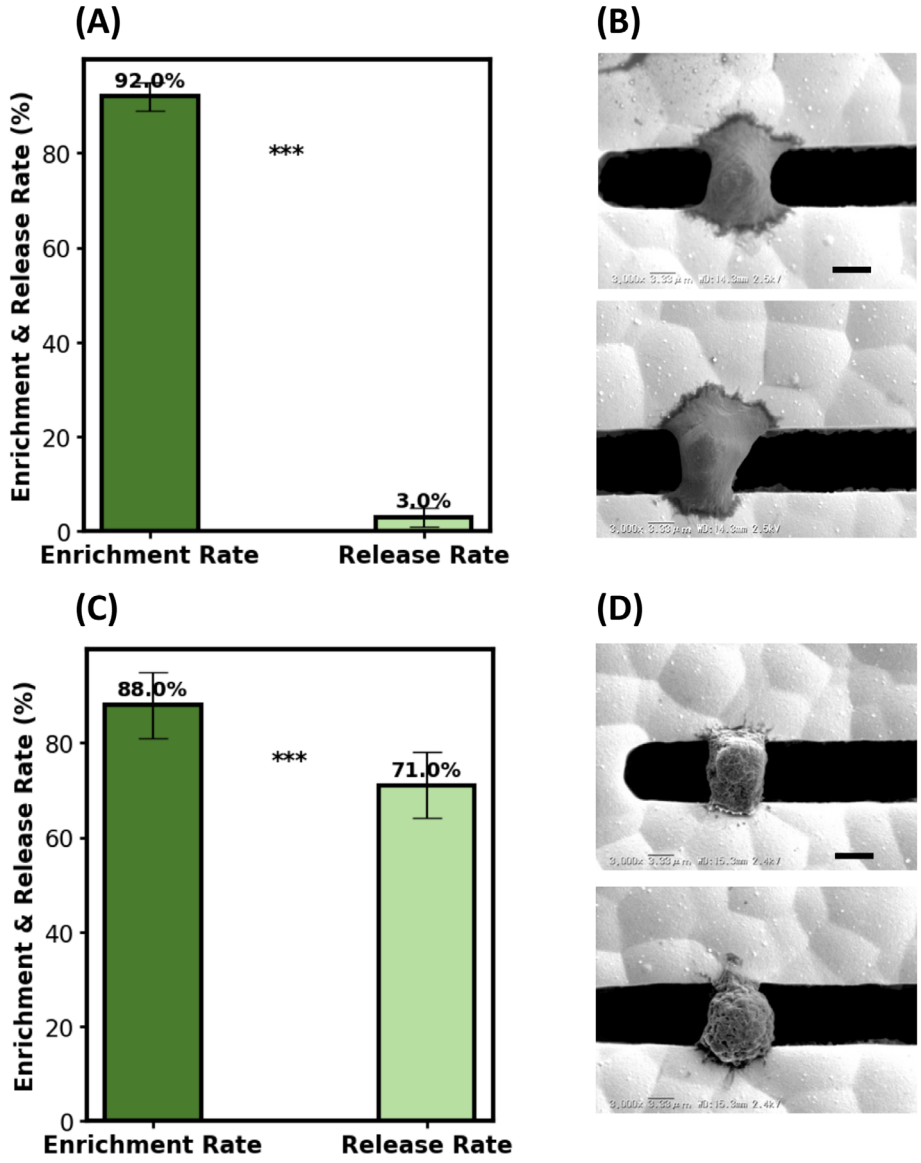

FIGURE 2 | Cell enrichment and release using unmodified MCA (A) and MPC-modified MCA (C) and SEM images of enriched NCI-H1975 cells both on unmodified MCA (B) and MPC-modified MCA (D). The significance level was determined based on the p value obtained from the t-test. Values are means ± SE of three independent experiments. ***:$p < 0.001$; Scale bar: 500 μm. MCA, microcavity array; MPC, 2-methacryloyloxyethyl phosphorylcholine; SEM, scanning electron microscopy.

was comparable between the MCA, DGC, and control methods, indicating that the MCA approach did not adversely affect cell viability (Figure 4B).

3.3 | Cultivation of CTCs From Patients With Metastatic Cancer Patients

This study aimed to recover and culture CTCs from the blood samples of patients with gastric cancer using the MCA method. Blood samples were processed as 1–3 mL aliquots using 2–3 CTC enrichment devices for CTC counting and cultivation. As shown in Table 1, CTC counts were measured in five patients with gastric cancer, with a total of eight blood samples collected. CTCs were detected in four of these patients, with counts ranging from 1 to 14 cells/mL of blood. Notably, CTC detection was unaffected by ongoing anticancer treatments (Table S1). While no cell proliferation was detected after 7 days in culture, cell adhesion and proliferation were successfully achieved for patient G4_3 (Table 1 and Figure 5). Overall, the method demonstrated notable efficiency, requiring less than 30 min to initiate a CTC culture from only 1 mL of blood. Overall, three of the eight CTC cultures (37.5%) remained viable after 7 days. Most cultures showed predominantly dead cells after 7 days, underscoring the significance of the successful culture observed for patient G4_3.

4 | Discussion

The low release efficiency of NCI-H1975 cells from the MCA is largely attributed to their strong adhesion to the unmodified surface. This observation aligns with those of previous studies

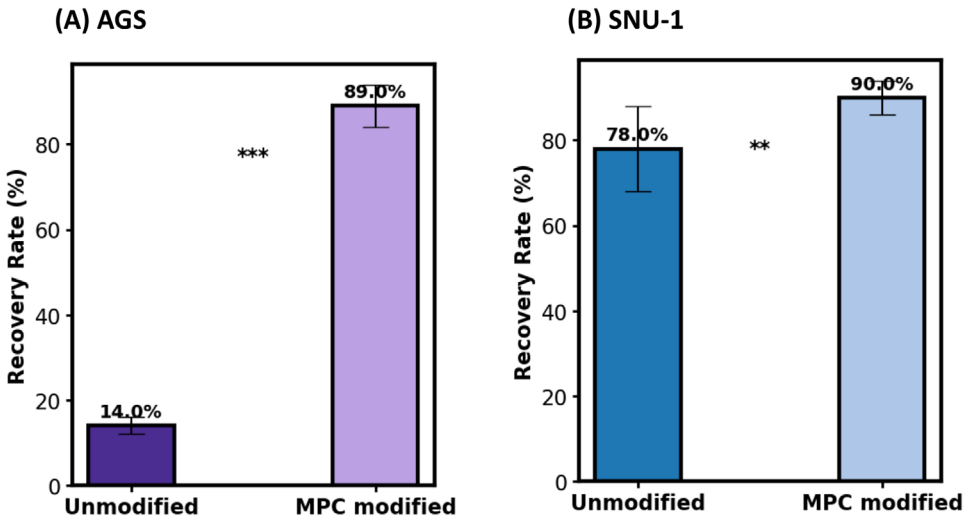

FIGURE 3 | Percentage of AGS (A), and SNU-1 (B) cells recovery using MPC-modified MCA. Cancer cell-spiked PBS (both A and B) samples were used. The significance level was determined based on the *p* value obtained from the *t*-test. If the *p* value was less than 0.001, the difference was considered highly significant and denoted by three asterisks ***. For *p* values between 0.001 and 0.01, the significance was marked by two asterisks **. AGS, adenocarcinoma gastric cell line; MCA, microcavity array; MPC, 2-methacryloyloxyethyl phosphorylcholine.

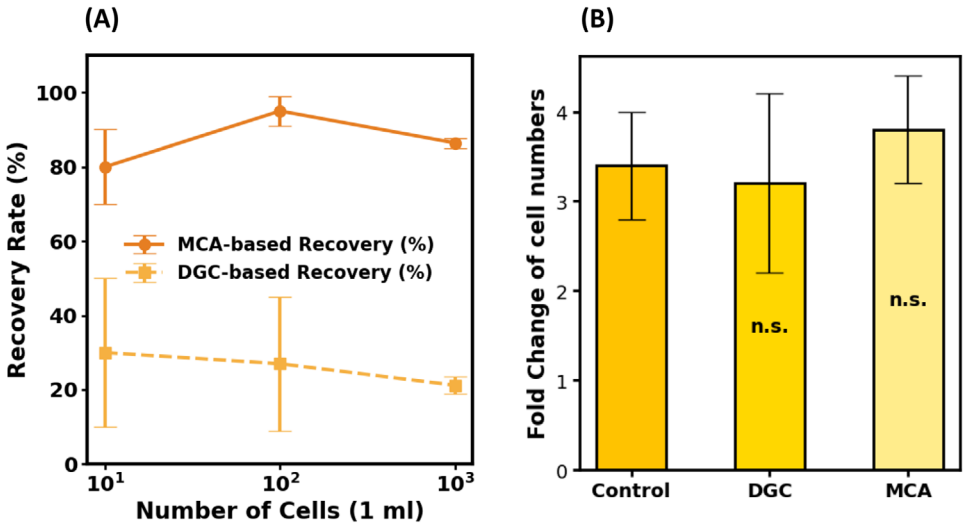

FIGURE 4 | Recovery rate with different fold-changes in cell number (A) and the fold-change in cell numbers (B). Cancer cell-spiked PBS (both A and B) samples were used. The significance level was determined based on the *p* value obtained from the *t*-test. A *p* value greater than 0.05 was considered ns. ns, not significant.

that emphasized the role of surface interactions in cell enrichment and release efficiency. SEM results further supported this, showing significant cell-substrate interactions within 30 min of confinement. Surface modification using MPC was explored to mitigate these adhesive forces. Studies have demonstrated that MPC can significantly reduce cell adhesion owing to its nonfouling properties [26]. Similarly, our results indicated that MPC modification notably improved the recovery efficiency of both NCI-H1975 and AGS cells. These findings underscore the critical importance of surface modification strategies in facilitating efficient CTC isolation and support prior research highlighting the utility of non-adhesive coatings in enhancing cell detachment.

The MCA method demonstrates high efficiency in CTC collection, primarily because of its streamlined operational procedure. In contrast to the DGC method, which involves multiple steps, the MCA approach is more direct and allows cell enrichment and release within 20 min. Our study confirms that the MCA not only enhances enrichment efficiency, but also effectively preserves the proliferative capacity of the enriched cells. This indicates that the MCA system may serve as a rapid and efficient tool for CTC recovery without compromising subsequent cell viability. Furthermore, we extended the high-throughput capabilities of MCA in other studies by achieving automated collection, thereby enhancing the efficiency and convenience of CTC collection [24].

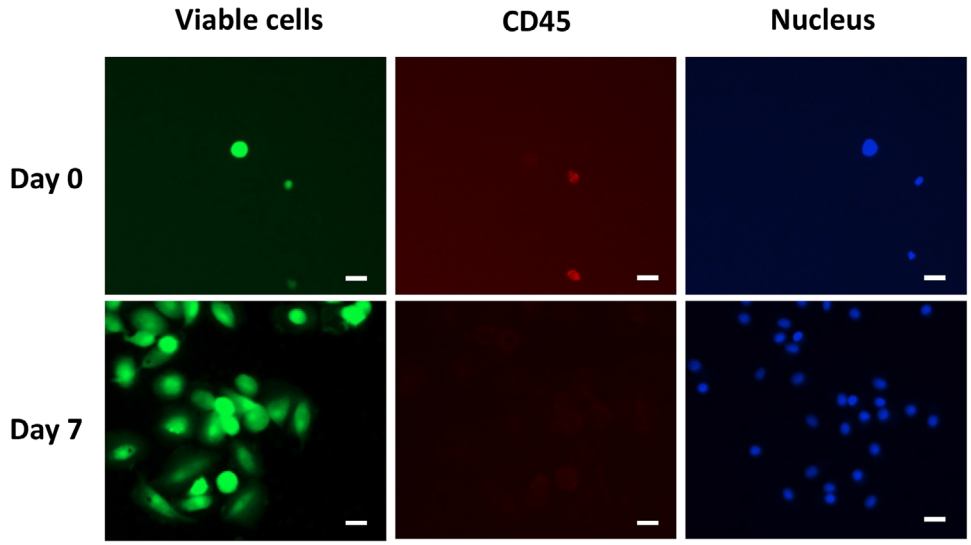

FIGURE 5 | Fluorescence images of released CTCs from a gastric cancer patient (patient no. G4) at day 0 and day 7. CTCs were identified as Calcein-AM$^+$, Hoechst 33342 stained$^+$, and CD45$^-$ cells$^-$. Scale bar: 20 μm. CTC, circulating tumor cell.

TABLE 1 | The number of released cells at day 0 and day 7.

Patient no.	Volume of blood (mL)	Number of viable CTCs[a] (at day 0)	Number of viable CTCs[a] (at day 7)
G1	2	2	0
G2	1	0	0
G3	3	16	0
G4_1	3	16	0
G4_2	1	12	0
G4_3	1	14	530
G5_1	3	7	2
G5_2	3	3	1

[a]Viable CTCs were identified as Calcein-$^-$AM+, Hoechst 33342 stained+, and CD45 negative cells−.

Despite the successful detection of CTCs in patients with gastric cancers, significant challenges remain in culturing these cells. The existing literature highlights a marked discrepancy between the number of enriched cells and their proliferative capacity, with larger initial cell counts (typically $> 10^2$ CTCs) being critical for a successful culture [27]. Short-term culture typically spans from 1 week to 3 months, with a 2-month duration being sufficient to evaluate the potential for continuous growth of CTCs. These findings provide valuable insights into the transition from short to long-term culture conditions [28, 29]. Future efforts to standardize the methods for detecting various CTCs and assessing their differences will provide valuable insights and hold significant potential for broad applications.

In conclusion, this study highlights the importance of surface modification with MPC in enhancing CTC release, which significantly reduces cell-substrate adhesion and improves recovery efficiency. The MPC-modified MCA method proved highly efficient for CTC collection, offering rapid enrichment and release without compromising cell viability. Our efforts emphasize the achievement of successful CTC culture, which is a critical step in personalized cancer therapy. These improvements underscore the broader significance of our research, highlighting its potential to advance single-cell-level analyses and significantly advance the field of CTC research. Although CTCs were successfully detected in patients with gastric cancer, their culture remained challenging, especially from small-volume samples. Intrinsic differences between cancer types may also affect CTC proliferation. Future studies are thus needed to optimize CTC detection and culture, increase throughput, and characterize CTCs at the single-cell level to standardize and advance CTC research.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information