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Highly dense, optically inactive silica microbeads for the isolation and identification of circulating tumor cells

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ABSTRACT

Efficient isolation of circulating tumor cells (CTCs) from whole blood is a major challenge for the clinical application of CTCs. Here, we report an efficient method to isolate CTCs from whole blood using highly dense and transparent silica microbeads. The surfaces of silica microbeads were fully covered with an antibody to capture CTCs, and blocked by zwitterionic moieties to prevent the non-specific adsorption of blood cells. Owing to the high density of the silica microbeads, the complexation of CTCs with silica microbeads resulted in the efficient sedimentation of CTC-microbead complexes, which enabled their discrimination from other blood cells in density gradient media. Model CTCs (MCF-7, HCC827, and SHP-77) with various levels of epithelial cell adhesion molecule (EpCAM) were isolated efficiently, especially those with low EpCAM expression (SHP-77). Moreover, the transparency of silica microbeads enabled CTCs to be clearly identified without interference caused by microbeads. The improved sensitivity resulted in increased CTC recovery from patient samples compared with the FDA-approved CellSearch system (14/15 using our method; 5/15 using the CellSearch system). These results indicate that the isolation method described in this report constitutes a powerful tool for the isolation of CTCs from whole blood, which has important applications in clinical practice.

1. Introduction

Circulating tumor cells (CTCs) are rare tumor cells that are disseminated from primary tumors or metastatic sites, and enter the bloodstream; they are believed to play a critical role in the spread of disease throughout the body [1]. CTCs can be a noninvasive and repeatedly accessible source of tumor material; thus, they may provide clinically feasible diagnostic and prognostic markers of cancer and of an increased likelihood of metastasis, and their analysis is more readily accomplished than conventional biopsy approaches [2–5]. Therefore, CTCs could be an attractive source of tumor cells for contemporary and repeatable tumor biopsies [6].

There has been considerable interest in analyzing CTCs as a potential source of clinical information related to patient diseases [7]. For example, the enumeration of CTCs using the CellSearch system (Veridex, Raritan, NJ, USA) is FDA-approved and has clinical utility as a prognostic marker in patients with metastatic breast cancer, colorectal cancer, and prostate cancer [5,8,9]. Genomic analysis of CTCs by sequencing and fluorescence in-situ hybridization also has potential clinical utility for the targeted treatment of lung cancer [10,11].

A major challenge for the accurate enumeration and molecular characterization of CTCs is the isolation of CTCs from whole blood with high purity despite the extremely low number of CTCs, which has been estimated to be as low as 1–10 cells per 10^9 blood cells [12]. A number of techniques have recently been developed to efficiently isolate rare CTCs from peripheral blood [12]. These techniques are based on the properties of CTCs that differ from those of other blood cells, particularly (a) physical properties, such

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as size, density, electrical properties, or (b) biological properties, such as the expression of protein markers, cancer-specific antigen–antibody interactions, and a combination of these two characteristics [13–19].

Immunomagnetic separation, which targets a specific antigen with an antibody that is coupled to magnetic beads and subsequently separates the antigen–antibody complex via exposure to a magnetic field, is the most widely used technique for isolation of CTCs from whole blood. The FDA-approved CellSearch system is based on this technique; it uses ferrofluids, which are coupled with antibodies against epithelial cell adhesion molecule (EpCAM) on the cell surface. To increase the cell recovery rate and minimize contamination by other blood cells, various microfluidic devices for immunomagnetic separation using magnetic microbeads have been developed [7,17,18]. Using these devices, contaminant blood cells are removed by inertial focusing in a microfluidic device, and CTC-magnetic microbead complexes are separated in a magnetic field [17] or washed out by continuous flow while CTC-magnetic microbead complexes are held to a magnet in the device [7,18].

As an alternative to immunomagnetic separation, CTC-magnetic microbead complexes can be separated using their physical properties. The high density and large size of CTC-magnetic microbead complexes are sufficient to discriminate them from other blood cells [20]. We previously reported an isolation technique based on this change in the physical properties of CTC-magnetic microbead complexes [20,21]. In this technique, CTCs in whole blood are bound by EpCAM-conjugated magnetic microbeads. Due to large size and high density, CTC-magnetic microbead complexes could be selectively separated from other blood cells, such as erythrocytes and leukocytes, in density gradient media via centrifugal force (i.e., selective sedimentation) and a microfilter. Greater than 90% of spiked CTCs were recovered and the purity of these recovered CTCs (considering the contamination of CTCs with white blood cells [WBCs]) was very high, enabling their molecular characterization. Moreover, this selective sedimentation technique could be easily adapted to the disc platform because the driving force for separation is centrifugal force (i.e., selective sedimentation on a disc) [21].

Although this selective sedimentation technique has been conceptually proven to isolate CTCs effectively and with high purity from whole blood, some issues require consideration when applying this technique to clinical samples. First, this technique has only been applied to cell lines with relatively high levels of EpCAM expression (DMS-79, MCF-7, and HCC827). In real CTCs, the expression level of EpCAM varies widely among cells, ranging from 9900 to 246,000 per cell [22]. Therefore, this technique may be not effective for the isolation of CTCs with low EpCAM expression due to the minimal increase in the size and density of CTC-magnetic microbead complexes relative to CTCs with high EpCAM expression [23]. Second, magnetic microbeads bound to CTCs may affect light scattering, quenching, or eclipse [24]. These interferences may affect the precise detection and analysis of CTCs via fluorescence, especially in the case of opaque microbeads, such as magnetic microbeads.

Although the magnetic microbeads used in our previous study are sufficiently large (2–5 μm in diameter) and dense (1.3–1.8 g/mL) to effectively increase the size and density of CTC-microbead complexes, several shortcomings remain. First, magnetic beads are not dense enough to discriminate between CTC-magnetic microbead complexes and other blood cells in the case of CTCs with low EpCAM expression in density gradient medium (DGM). Although this problem may be overcome by increasing bead size, beads that exceed 6 μm in diameter are not suitable for capturing CTCs [25]. Second, with respect to their opaqueness, precise cellular and subcellular image analyses of isolated CTCs are not easy, owing to the eclipse of fluorescence by the magnetic beads bound to CTCs.

To overcome the shortcomings mentioned above, we used silica microbeads instead of magnetic microbeads to increase the size and density of CTC-microbead complexes (Fig. 1A). Silica microbeads have favorable properties to overcome the shortcomings of magnetic microbeads. First, their high density (2.0 g/mL) enables the effective separation of CTC-microbead complexes, even when EpCAM expression is low, from erythrocytes and leukocytes via selective sedimentation on a disc platform (Fig. 1B). Second, their optical transparency enables precise cellular and subcellular imaging of isolated CTCs without interference from microbeads (Fig. 1C). The surfaces of silica microbeads were modified to bind CTCs effectively and these microbeads were used to isolate CTCs on a disc platform (i.e., selective sedimentation on a disc using silica microbeads, SSDS). To confirm the isolation efficiency of silica microbeads, we examined the recovery rate of several cells lines with various levels of EpCAM expression and performed an image analysis of recovered CTCs. We compared the number of CTCs isolated from patient samples using this method with the number isolated using the CellSearch System.

2. Materials and methods

2.1. Reagents

All materials were used as received, unless otherwise noted. Silica microbeads were purchased from Bangs Laboratories, Inc. (Fishers, IN, USA) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and buffer reagents were purchased from Sigma (St. Louis, MO, USA). Anti-mouse IgG was also purchased from Sigma. Human EpCAM antibody was purchased from Novus Biologicals, LLC (Littleton, CO, USA).

2.2. Blood sample processing

Healthy human blood samples were obtained from individuals at the Yonsei University College of Medicine (Seoul, Korea) and lung cancer patient blood samples were obtained from the Samsung Medical Center (Seoul, Korea). In all cases, informed written consent was obtained from all participants, and this study was approved by the Institutional Review Board (IRB) at Yonsei University and at Samsung Medical Center. Whole blood was drawn into a CellSave Preservative Tube (Veridex, Raritan, NJ, USA). Samples were maintained at room temperature (RT) and processed within 72 h after collection.

2.3. Bead preparation

The overall synthetic scheme is presented in the supporting information (Fig. S1). Carboxylated silica microbeads (30 mg; 5.0 μm diameter) were suspended in 1 mL of 25 mM MES buffer (pH 6.0) and washed twice with the same buffer. After transferring 300 μL of bead solution to an Eppendorf tube, 100 μL each of EDC and NHS solution (50 mg/mL in 25 mM MES buffer) were added to the bead solution as activating agents. The beads were then reacted in a rotator at RT for 30 min. After the activation step, the beads were washed and re-suspended with 300 μL of 25 mM MES buffer (pH 6.0) and reacted with 100 μL of anti-mouse IgG solution in a rotator at RT for 1 h. After the reaction, 100 μL of sulfobetaine [26] solution (50 mg/mL in deionized water) was added to the solution. The beads were then reacted in a rotator at RT for 2 h. After the reaction, the beads were washed twice with 1 × phosphate-buffered saline (PBS). After the removal of the buffer by centrifugation, 3 mL of anti-EpCAM solution was added to the beads and the mixture was incubated in a rotator at 4 °C for 4 h. The prepared