Spatially gradated segregation and recovery of circulating tumor cells from peripheral blood of cancer patients

Peitao Lv,¹ Zhewen Tang,¹ Xingjie Liang,² Mingzhou Guo,³ and Ray P. S. Han^{1,a)}

¹*Department of Materials Science and Engineering, Peking University, Beijing 100871, China*

²*Laboratory for Nanobiomedicine and Nanosafety, National Center for Nanoscience and Technology, Beijing 100190, China*

³*Department of Gastroenterology and Hepatology, Chinese PLA General Hospital, Beijing 100853, China*

(Received 12 April 2013; accepted 21 May 2013; published online 6 June 2013)

For cancer patients, the enumeration of rare circulating tumor cells (CTCs) in peripheral blood is a strong prognostic indicator of the severity of the cancer; for the general population, the capture of CTCs is needed for use as a clinical tool for cancer screening, early detection, and treatment assessment. Here, we present a fast, high-purity (~90%) and high-efficiency (>90%) method for the segregation and undamaged recovery of CTCs using a spatially gradated microfluidic chip. Further, by lysing the red blood cells we achieved not only a significant reduction in the overall processing time but also mitigated the blood clogging problem commonly encountered in microfluidic-based CTC isolation systems. To clinically validate the chip, we employed it to detect and capture CTCs from 10 liver cancer patients. Positive CTC enumeration was observed in all the blood samples, and the readings ranged from a low of 1–2 CTCs (1 patient) to a high of >20 CTCs (2 patients) with the balance having 3–20 CTCs per 3-ml blood sample. The work here indicates that our system can be developed for use in cancer screening, metastatic assessment, and chemotherapeutic response and for pharmacological and genetic evaluation of single CTCs. © 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4808456>]

INTRODUCTION

Molecular studies suggest that circulating tumor cells (CTCs) are responsible for the hematogenous spread of cancer from the primary tumor to other parts of the human body.¹ Approximately 90% of all deaths in cancer patients are caused by CTCs spread, which is far more lethal than the primary tumor.^{2–4} The presence of CTCs in blood of cancer patients is linked to tumor cell density and, thus, to tumor growth,⁵ and the number of CTCs in blood correlates with overall survival in cancer patients.⁶ For example, breast cancer patients with more than 5 CTCs per 7.5 ml of whole blood had a shorter median progression-free survival compared with patients with less than 5 CTCs per 7.5 ml.⁷ Therefore, the segregation, capture, and recovery of CTCs in human peripheral blood are of clinical importance in cancer screening, diagnosis, and treatment.

Today, the 2 major challenges of CTC capture are the need for a high-flow rate, high-purity capture of the rare CTCs and the lack of a comprehensive suite of biomarkers for the positive identification of the captured CTCs. Current CTC capture flow rates that also yield high capture purity are still too slow for hospital use. The capture purity has improved considerably to over 90%, but most devices still occasionally entrap white blood cells (WBCs). As for the slow speeds, current methods are still not able to efficiently handle the numerically immense orders of magnitude difference in the main constituents (RBCs (red blood cells) vs

^{a)} Author to whom correspondence should be addressed. Electronic mail: ray-han@pku.edu.cn. Tel.: +8610 6276-7394.

WBCs vs CTCs) of a cancer patient's blood. The other 2 issues of blood clogging and accurate distinction of the rare CTCs from WBCs and other non-tumor epithelial cells also need to be addressed before the technology can be adopted. Hence, despite the rapid improvements in CTC capture technology, it has not attained the stage where the method can be clinically used for cancer screening, metastatic assessment, and chemotherapeutic monitoring.

Traditional methods for detecting CTCs in human blood samples include methods based on flow cytometry, immunocytochemistry, immunofluorescence, and reverse transcription-polymerase chain reaction (RT-PCR).^{8–12} Compared to conventional biochemical methods, a microfluidic system constitutes an attractive alternative for the detection and recovery of CTCs from human blood samples. One of the earliest microfluidic platforms is based on the interaction of CTCs with epithelial-cell-adhesion-molecule or EpCAM antibody coated micro-posts under controlled flow conditions and has been demonstrated to successfully separate and recover viable CTCs from whole blood samples.¹³ However, the EpCAM coated chip is complicated not only to be fabricated but also to be employed to isolate CTCs expressing a specific antigen, and the antigen may be lost when cells undergo the epithelial to mesenchymal transition.¹⁴ Hence, CTC capture technologies that are antibody-independent (or label-free) and based solely on the physical properties of cancer cells to detect a wide-range of CTCs are being increasingly developed. Table I summarizes some of the widely used antibody-independent technologies by the CTC community. It is organized in accordance to the source of the cancer cells: cell-line or peripheral blood of cancer patients. Further, methods based on the same physical property for cell capture are grouped together in the table. The carrier medium could be spiked phosphate buffered saline (PBS), spiked blood, or peripheral blood of cancer patients. We have also listed 3 metrics pertaining to the device characterization: capture efficiency (CE), capture purity (CP), and flow rate.

Size-based devices that capture cancer cells based solely on their physical size are able to isolate and enumerate the rare CTCs in one step,^{15,16} but if not properly designed they can suffer from blood clogging issue and can take comparatively longer time for cell retrieval and recovery. Microfluidic devices based on hydrodynamic forces to isolate CTCs have generally performed well in terms of their high flow rates and high capture efficiency.^{17–23,29} Since the captured cells are all lumped together at the outlet, it is difficult to retrieve single CTCs for molecular research. They also require additional effort for cell enumeration. Dielectrophoresis (DEP) employs dielectric properties of a cancer cell to manipulate its flow trajectory in non-uniform electric field.^{24–28} However, it is known that the dielectric properties of blood of cancer patients are different from that of healthy people, and therefore the method may not be robust enough to handle a wide spectrum of human cancers. DEP-based devices also suffer from the same cell handling difficulties as the hydrodynamics-based chips.

In our paper, we present a microfluidic chip system to segregate, enumerate, and recover CTCs from cancer patient blood, and the method is based on the physical differences of CTCs with the main constituents of the human peripheral blood. To significantly improve the flow rates and reduce the blood clogging we lysed the RBCs prior to passage through the chip. Our chip not only performed with high capture efficiency and purity but also was able to easily recover undamaged single cancer cells. We have also clinically assessed our chip performance by using it to detect and capture CTCs in 3 ml blood samples drawn from 10 liver cancer patients.

MATERIALS AND METHODS

Microfluidic chip design and fabrication

Fig. 1 shows the design of the microfluidic chip. It contains two main regions: the pre-filter cone and the cell capture chamber (Fig. 1(a)). The pre-filter cone consisting of micro-pillars with a gap spacing of 50 μm (Fig. 1(b)) is designed to minimize the blood clogging problem. In the cell capture chamber, specially shaped micropillars that are spatially graded over a 10-layer spread (Fig. 1(c)) are used to segregate and capture the CTCs while allowing the main blood constituents pass through. The gap spacings of the micropillars are gradiently distributed from 12 μm down to 4 μm . Most cancer cells have sizes that range from a large of 20 μm down to a small of 6 μm and, thus, are expected to be trapped between the micropillars as they flow

TABLE I. Antibody-independent technology for CTC segregation, capture, and recovery.

Author	CTC capture principle	Carrier medium	Cancer cell source	Capture efficiency	Capture purity	Flow rate
Tan <i>et al.</i> ¹⁵	Size	Diluted whole blood	Cell-line: MCF-7, HT-29 MDA-MB-231	>80%	>80%	~0.7 ml/h
Hosokawa <i>et al.</i> ¹⁶	Size	Whole blood	Cell-line: NCI-H358	~97%	...	~60 ml/h
Chen <i>et al.</i> ¹⁷	Size	Diluted whole blood	Cell-line: SPC-A-1	~99%	...	...
Zheng <i>et al.</i> ¹⁸	Size	Diluted whole blood (10×)	Cell-line: LNCaP, MCF-7	~86.5%	...	120 ml/h
Liu <i>et al.</i> ¹⁹	Size	Diluted whole blood (10×)	Cell-line: MCF-7 MDAMB231	~99% (MCF-7) ~80% (MDAMB231)	...	120 ml/h
Hur <i>et al.</i> ²⁰	Size	RBC-lysed blood	Cell-line: HeLa, MCF-7	~43% (HeLa) ~20% (MCF-7)	...	240 ml/h
Bhagat <i>et al.</i> ²¹	Hydrodynamics	Diluted whole blood	Cell-line: MCF-7	~90%	...	~4 ml/h
Moon <i>et al.</i> ²²	Hydrodynamics	Diluted whole blood (100×)	Cell-line: MCF-7	98.9%	...	~8 ml/h
Sun <i>et al.</i> ²³	Hydrodynamics	Diluted whole blood (20×)	Cell-line: HeLa	~90%	...	60 ml/h
Alshareef <i>et al.</i> ²⁴	DEP	Diluted PBS	Cell-line: MCF-7 HCT-116	93%	...	~0.006 ml/h
Yang <i>et al.</i> ²⁵	DEP	PBS	Cell-line: CCHCT-116	~98%	~95%	~0.006 ml/h
Gascoyne <i>et al.</i> ²⁶	DEP	Peripheral blood mononuclear cells (PBMCs)	Cell-line: MDA-MB-435 DA-MB-468 MDA-MB-231	~90% ~75% (SKOV3) ~70% (MDA-MB-231)	...	...
Gupta <i>et al.</i> ²⁷	DEP	PBMCs	Cell-line: SKOV3 MDA-MB-231	~88%	...	~1.5 ml/h
Shim <i>et al.</i> ²⁸	DEP	PBMCs	CTC: colon cancer patients	~85%	...	~1.2 ml/h
Hou <i>et al.</i> ²⁹	Hydrodynamics	Diluted peripheral blood	CTC: lung cancer patients	~95%	...	3 ml/h
Present paper	Size	RBC-lysed peripheral blood	CTC: liver cancer patients	~90%	~90%	0.5-2 ml/h

FIG. 1. Microfluidic device for CTC detection, capture, and recovery. (a) Design of the chip. (b) Pre-filter with a gap space of $50\ \mu\text{m}$ to minimize blood clogging. (c) Enlarged view of the cells capture region. The size distribution of the micro-pillars varies from $12\ \mu\text{m}$ down to $4\ \mu\text{m}$. (d) Details of the connection setup with a syringe pump to drive the cells through the microfluidic chip.

through the cell capture region of the chip. Like most size-based microfluidic chips, our chip can also occasionally trap the larger and stiffer WBCs (neutrophils and lymphocytes, which accounted for $>75\%$ of the leukocytes have an average size of $8\ \mu\text{m}$). RBCs on the other hand are not only smaller (average size $6\ \mu\text{m}$) but also highly malleable; therefore, they can easily pass through the micropillars unimpeded.

The inner structure of the microfluidic chip is created using lithography technology. The masks are made by spin coating SU8 on 3-in. silicon wafer. The depth of the micro channel is $20\ \mu\text{m}$ and cast from polydimethylsiloxane (PDMS). Pre-cured PDMS is degassed in a desiccator and cured in an oven preset at $60\ ^\circ\text{C}$ for 4 h. After peeling the PDMS off the master, fluidic ports are punched using a flat tip needle. Irreversible bonding of the PDMS to a glass slide is achieved using an oxygen plasma treatment.

The microfluidic system setup

The microfluidic system consists of the chip itself, tubing, fluid connectors, syringes, and syringe pumps. Tubes and fluid connectors are used to connect the syringes, and fluidic ports punched into the microfluidic chip (Fig. 1(d)). Five syringe pumps are used to inject or extract fluid samples from the chip at a specified flow rate. The microfluidic chip is placed in an inverted microscope for monitoring cell movements. The tubes and fluid connectors are thoroughly sterilized with 70% ethanol before use, and the micropillars inside the chip are pre-washed with PBS and ethylenediaminetetraacetic acid (EDTA) prior to starting the experiment.

Sources of CTCs

Spiked PBS and spiked blood experiments are employed for evaluating 2 important chip characteristics; i.e., capture efficiency $CE = [(\text{captured CTCs})/(\text{captured} + \text{escaped CTCs})] \times 100\%$ and capture purity $CP = [(\text{captured CTCs})/(\text{captured CTCs} + \text{captured WBCs})] \times 100\%$. In our work, we use the cell-line Hela to represent CTCs. The cancer cells are cultured in a 3-in.

cell culture flask and maintained with Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G/streptomycin/amphotericin B. For clinical test experiments, we used blood samples taken from 10 late-stage liver cancer patients to harvest the hepatocellular carcinoma cells (HCCs). The 3-ml blood samples are drawn from the arms of cancer patients at Beijing 301 Hospital and preserved in anticoagulation tubes prior to testing.

Detection and recovery of CTCs

In a typical experiment, the blood sample is injected into the chip at the inlet and extracted at the waste outlet. As the blood sample flows through the cell capture chamber, CTCs are being continuously captured due to their larger size and poorer deformability compared to other blood constituents.^{30–32} Hence, the much smaller-sized and malleable RBCs pass through the micropillars virtually unhindered. Most WBCs, which are of comparable dimension but more deformable than CTCs, also pass through the micropillars with relative ease. However, it is common to have a small amount of WBCs trapped in the chamber, together with CTCs. To retrieve the CTCs and WBCs trapped in the micropillars, PBS is injected into the chip at the retrieval inlet in the cross-flow direction, and the fluidic sample is extracted at the retrieval outlet.

Lysis of the RBCs

Red blood cell lysis buffer or Ammonium-Chloride-Potassium (ACK) lysis buffer (*Beyotime, Nantong, Jiangsu, China*) is used to remove RBCs from the whole blood. It is an improved and milder product that works on the anuclear red blood cells while having little effects on lymphocytes and other nuclear cells.

Distinguishing CTCs from WBCs

The HeLa cells used in our spiked blood experiments inherently express a red fluorescence protein (RFP), which emits red light in a fluorescence microscope. The WBCs are marked with

FIG. 2. (a) Cell capture chamber depicting trapped HeLa. (b) The *DE* ranges from a high of 95% to a low of 74% for the 4 flow rates (0.5, 1.0, 1.5, 2.0 ml/h) being studied. (c) A comparison of the 5-day cell proliferation process between captured cells (at the max flow rate of 2 ml/h) and normal cultured cells shows no perceptible difference between them. Thus, it appears that the viability of the cancer cells is unaffected by the 4 flow rates used for their capture. Further, our micropillars are designed with an unusual shape to minimize damage to a cell caught in-between 2 micropillars. Scale bar is 100 μm.

FIG. 3. A comparison of the severity of blood clots in the capture region of the chip when working with (a) unlysed blood versus (b) lysed blood.

CD45 antibody bind with fluorescein isothiocyanate (FITC). For experiments with patient blood, all the trapped human cells are marked with 4',6-diamidino-2-phenylindole (DAPI), the HCCs are marked with anti-EpCAM and CK18 antibodies bind with FITC, and the WBCs are marked with CD45 antibody bind with a red fluorescent dye. The DAPI and the antibodies are sourced from *Cytointelligen, Inc.*, Jiangsu, China.

RESULTS AND DISCUSSION

Experiments with spiked PBS: Capture efficiency of chip

Capture efficiency rates a chip's performance by assessing its ability to capture CTCs versus the number that escaped, that is, it measures the efficiency of a chip in capturing CTCs. For clinical use, a *CE* of about 90% is required. To test the *CE* of the chip, cancer cells spiked in PBS are injected into the microfluidic system. The concentration of cancer cells is diluted out of the cell culture suspension to less than 100 cells/ml. Fig. 2(a) shows the trapped cancer cells in the cell capture chamber. The *CE* is evaluated for varying flow rates controlled by a syringe

FIG. 4. (a) Bright field and fluorescence and merged images in the microfluidic chip. Hela and WBCs are marked with red and green fluorescence, respectively. Scale bar is 10 μm . (b) The *CP* of the chip seems to be unaffected by the varying flow rates.

pump. As expected, this ratio is highly dependent on the flow rates with the lower flows being more efficient in the cell capture (Fig. 2(b)), but they require a longer processing time. The captured cancer cells are then recovered and reseeded onto a 96 well plate (cell culture flask). Fig. 2(c) shows that the 5-day proliferation record of the recovered cancer cells is comparable to the normal cultured cancer cells, indicating that the quality of these 2 cell types is similar.

Experiments with spiked human blood: Capture purity of chip

Capture purity rates a chip's performance by assessing the amount of captured CTCs versus the inadvertently captured WBCs, that is, it gives a measure of the capture purity. Usually, a *CP* of at least 85% is required for acceptable performance.

Most microfluidic devices for CTC segregation and capture suffer from the blood clogging problem that can significantly affect their performance. The clogging issue can be largely mitigated by the removal of anuclear cells from blood and the easiest way of doing so is by lysing the RBCs. Fig. 3 gives a comparison of the clogging problem for unlysed and lysed blood situations. Further, RBC lysing overwhelmingly reduces the fluidic content by some 96% leading to a greatly decreased processing time. We work with 3 ml blood samples, and the RBC lysing reduces the total fluid volume down to 0.3–0.5 ml, which, in turn, reduces the processing time to below an hour at our lowest flow rate (0.5 ml/h). This advantage persists even when a much larger sample size (10–0 ml) is used as the lysed blood sample is still <1 ml. Hence, RBC lysing yields another advantage—the processing time is largely unaffected by the collected sample size.

To evaluate the *CP*, spiked blood obtained by injecting cancer cells (Hela express RFP) into healthy human blood samples donated by volunteers drawn at Peking University Hospital is lysed and stained with CD45, so the relative quantities of captured cancer cells versus captured WBCs can be determined. Fig. 4(a) depicts the fluorescence result of the captured cells in

FIG. 5. Positive CTC enumeration was detected in all blood samples taken from patients with liver cancer. (a) CTC count per 3-ml blood sample and it ranged from a low of 1–2 (1 patient) to a high of >20 (2 patients). (b) Captured cells from blood of liver cancer patients. Scale bar is 10 μ m. (c) Immunofluorescence staining is performed on recovered CTCs. To distinguish the inadvertently captured WBCs from the captured CTCs, the former are stained with blue and red fluorescence, while the latter are stained with blue and green fluorescence. The merged slide clearly depicts the distinction between the 2 cell types. Scale bar is 10 μ m.

the microfluidic chip. The *CP* of the chip not only averages close to 90% but also seems to be independent of the flow rates tested (Fig. 4(b)).

Clinical validation with blood drawn from liver cancer patients

Blood samples donated by liver cancer patients from Beijing 301 Hospital are used for a clinical assessment of the microfluidic system. Ten liver cancer patients are selected as testees. CTCs are found in the 3 ml blood samples of all 10 testees (Fig. 5(a)) with 9 of them having CTCs >2 cells/3 ml blood (Fig. 5(b)). The captured HCC-CTCs shown in Fig. 5(c) depict the fluorescence labeling results of the recovered CTCs. Since the HCCs are DAPI+/CK18+ and EpCAM+ and the WBCs are DAPI+/CK18– and EpCAM–, the result is that the HCCs are stained with blue and green fluorescence while the WBCs are stained with blue and red fluorescence. Hence, using appropriate bio-markers we were able to clearly distinguish the captured CTCs from the inadvertently trapped WBCs. We have shown that our microfluidic chip is capable of segregating and capturing CTCs not only in spiked blood but also in cancer patient whole blood.

CONCLUSION

Experimental results show that our microfluidic system is a fast, accurate, and efficient system for the detection, capture, and recovery of CTCs. By lysing the RBCs, we not only achieved a significant reduction in the overall processing time but also mitigated the blood clogging problem encountered in most microfluidic-based CTC filtering systems. We were also able to successfully capture CTCs in the peripheral blood of ten liver cancer patients with our chip. Our rapid CTC detection, segregation, and recovery system appears promising for use in cancer screening using blood samples (or *liquid biopsy*), for monitoring cancer development and assessing chemotherapeutic response, and for evaluating pharmacological and genetic analysis of single CTCs.

ACKNOWLEDGMENTS

This work was financially supported in part by grants from the Key Project of National Natural Science Foundation (No. 91123034), National Science and Technology support program (No. 2012BAF13B05), Chinese Natural Science Foundation project (Nos. 30970784 and 81171455), National Key Basic Research Program of China (2009CB930200), and the Chinese Academy of Sciences “Hundred Talents Program” (07165111ZX).

¹P. Paterlini-Brechot and N. L. Benali, *Cancer Lett.* **253**, 180–204 (2007).

²E. C. Kohn and L. A. Liotta, *Cancer Res.* **55**, 1856–1862 (1995).

³C. Wittekind and M. Neid, *Oncology* **69**, 14–16 (2005).

⁴J. S. Michealson *et al.*, *Br. J. Cancer* **93**, 1244–1249 (2005).

⁵T. S. Deisboeck *et al.*, *Med. Hypotheses* **65**, 785–790 (2005).

⁶S. L. Stott *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **107**, 18392–18397 (2010).

⁷M. Cristofanilli *et al.*, *N. Engl. J. Med.* **351**, 781–791 (2004).

⁸J. C. Goeminne *et al.*, *Ann. Oncol.* **11**, 785–792 (2000).

⁹W. J. Allard *et al.*, *Clin. Cancer Res.* **10**, 6897–6904 (2004).

¹⁰S. Bustin and R. Mueller, *Clin. Sci.* **109**, 365–379 (2005).

¹¹V. Zieglschmid *et al.*, *Crit. Rev. Clin. Lab. Sci.* **42**, 155–196 (2005).

¹²F. Schüller and G. Dölken, *Clin. Chim. Acta* **363**, 147–156 (2006).

¹³S. Nagrath *et al.*, *Nature* **450**, 1235–1239 (2007).

¹⁴A. Bonnomet *et al.*, *Oncogene* **31**, 3741–3753 (2012).

¹⁵S. J. Tan *et al.*, *Biomed. Microdevices* **11**, 883–892 (2009).

¹⁶M. Hosokawa *et al.*, *Anal. Chem.* **82**, 6629–6635 (2010).

¹⁷Z. Chen *et al.*, *Surf. Interface Anal.* **38**, 996–1003 (2006).

¹⁸S. Zheng *et al.*, *Biomed. Microdevices* **13**, 203–213 (2011).

¹⁹Z. Liu *et al.*, *Biomicrofluidics* **7**, 011801 (2013).

²⁰S. C. Hur *et al.*, *Biomicrofluidics* **5**, 022206 (2011).

²¹A. A. S. Bhagat *et al.*, *Lab Chip* **11**, 1870–1878 (2011).

²²H. S. Moon *et al.*, *Biomicrofluidics* **7**, 014105 (2013).

²³J. Sun *et al.*, *Biomicrofluidics* **7**, 011802 (2013).

²⁴M. Alshareef *et al.*, *Biomicrofluidics* **7**, 011803 (2013).

- ²⁵F. Yang *et al.*, [Biomicrofluidics](#) **4**, 013204 (2010).
- ²⁶P. R. C. Gascoyne *et al.*, [Electrophoresis](#) **30**, 1388–1398 (2009).
- ²⁷V. Gupta *et al.*, [Biomicrofluidics](#) **6**, 024133 (2012).
- ²⁸S. Shim *et al.*, [Biomicrofluidics](#) **7**, 011807 (2013).
- ²⁹H. W. Hou *et al.*, [Sci. Rep.](#) **3**, 1259 (2013).
- ³⁰L. Weiss, [Adv. Cancer Res.](#) **54**, 159–210 (1990).
- ³¹J. P. Shelby *et al.*, [Proc. Natl. Acad. Sci. U.S.A.](#) **100**, 14618–14622 (2003).
- ³²H. Mohamed *et al.*, [IEEE Trans. Nanobiosci.](#) **3**, 251–256 (2004).