Capture of circulating tumor cells (CTCs) using a novel micro-device

S. Ranjan and Y. Zhang

Department of Bioengineering,
National University of Singapore,
9 Engineering Drive 1, Block EA #03-12,
Singapore 117576

ABSTRACT

Circulating tumor cells (CTCs) are metastatic cells that are detached from a tumor and are found in the blood of cancer patients. The presence of CTCs in the blood indicates the metastatic stage of cancer, so the detection of CTCs has been considered as a diagnosis and prognosis marker. Although some efforts have been made to isolate CTCs from blood, it is still challenging due to the rarity of CTCs in blood (approximately one in one billion of blood cells). Apart from some fundamental problems, like non-retrieval of cells from antibody-coated surface, long processing time etc., the main problem with these methods is the loss of cell-viability which impede further study. Here, we demonstrate a novel and efficient micro-device for capturing CTCs at high throughput besides retaining their vaibility. We have fabricated a unique membrane filter with an array of pores where each pore is surrounded by four dome-shaped microstructures. These microstructures extend extra support to cells to maintain their vaibility.

Keywords: microfluidics, circulating tumor cells, microstructures, porous membrane

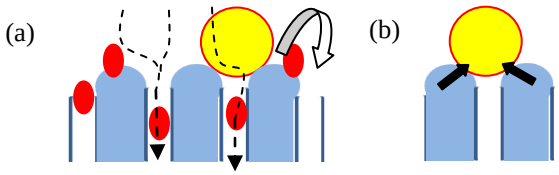
1 INTRODUCTION

Circulating tumor cells (CTCs) are metastatic cells that are detached from a tumor and are found in the blood of cancer patients. The presence of CTCs in the blood indicates the metastatic stage of cancer, so the detection of CTCs has been considered as a diagnosis and prognosis marker [1, 2]. The number of CTCs in blood has been found to be related with survival-rate [3]. Also, CTCs have spurred interest for understanding the biology of these cells by performing genetic-level analysis which can be helpful in the development of targeted cancer-therapy.

The major technical challenge in isolation of CTCs from blood is their rarity in blood samples (as low as one cancer cell in 10^9 blood cells). In past, efforts have been made to isolate and detect CTCs by using different methods like immuno-magnetic separation [3], using antibodies against cancer cells (like Ep-CAM) for capturing CTCs on micro-pillars [4] or by using physical traps [5]. The use of antibodies to trap cells makes it difficult to retrieve these cells for further studies. The trapping by using physical traps are slow and the efficiency of trapping is quite dependent on the flow-rate and hence control over flow rate is required by using additional equipment. Moreover, these techniques are limited by large processing time. Recently, membrane microfilter has been used as an alternative to isolate CTCs with less processing time and high efficiency [6]. However, cell viability is a concern for mambrane based isolation of cells. The cell viability is compromised in the membrane microfilter due to high hydrostatic pressure experienced by cells which is caused by the clogging of pores by cells. Moreover, such clogging makes it difficult to retrieve cells from the membrane. Recently, 3D microfilter has been developed to increase the cell viability by providing extra support to the clogged cells [7]. Although cell viability can be increased by such method, it complicates the fabrication process and it doesn't solve the problem of cell retrieval for further studies. Hence, there is need for another membrane based design to tackle these issues.

A unique porous membrane containing dome-shaped microstructures has been developed in this work for efficient isolation of CTCs without compromising the cell viability. This membrane has shown better cell retrieval compared to porous membrane without any dome-shaped microstructures. This unique porour membrane contains an array of pores where each pore is surrounded by four dome-shaped microstructures. These microstructures prevent pore clogging by cells and help in negating the pressure-drop (scheme-1a). Moreover, such microstructures may provide extra support to cells (scheme-1b). Overall, the cell viability is maintained and retrieval of cells becomes easier.

The porous membrane containing dome-shaped microstructures has been fabricated here by replica molding. A master mold is first fabricated by photolithography. Simple modification in the photolithography results in fabrication of dome-shaped microstructures [8]. Then, the membrane is fabricated from this mold by a simple method developed in this work. As a single mold can be used several times for membrane fabrication using PDMS, the developed method is simple and inexpensive. To prevent the membrane against deformation during high flow-rate filtration, the membrane is supported by uniquely designed flexible microstructures newly developed here. Overall, this method yields well-arranged array of pores which is better than the commercially available track-etched membrane which contains randomly placed pores with variations in pore



Scheme-1: (a) CTCs (yellow big circle) filtered by the membrane. Flow of fluid (dotted curves) and blood-cells (red dots) are shown in the scheme. The dome-shaped microstructures on the membrane keep CTCs un-trapped and negate the pressure-drop around the cell by allowing fluid to pass through. (b) Arrows show the reaction force from dome-shaped microstructures which provide extra support to cells and increase viability.

shape and size besides relatively low density of pores [9]. The replica molding approach is relatively simpler than other direct photolithographic methods as it doesn't require clean room facilities and use of expensive equipment often.

2 MATERIALS AND METHODS

Reagents and materials: SU-8 2050 photoresist and developer (MicroChem), PDMS (Sylgard-184, Dow Corning) were purchased. (Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane, Isopropyl Alcohol (IPA), CMPTX-live cell labeling dye were purchased from Sigma–Aldrich (Singapore). Cancer cells (MDA-MB-231) were purchased from ATCC (Manassas, VA). Basal DMEM cell-culture media and fetal-bovine serum (FBS) were purchased from Invitrogen (Singapore).

Master-mold fabrication: The master-mold was fabricated by modifying photolithography which has been published earlier by our group [10].

Device fabrication: (Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane was coated on the master-mold by vacuum deposition for 10min for easy release of PDMS membrane from it. A small drop of PDMS solution (base solution:curing agent ratio is 10:1) was put on the arrayed structures to cap them and was baked for 15min at 70°C in an oven. PDMS was then spin-coated at 1400rpm on the master-mold to cover channel-replica and was baked for 10min. The cap was then removed from arrayed structure and another layer of PDMS was coated at 3000rpm. A small piece of silicon-wafer was used as weight to remove excess PDMS and it was spun again to make this layer uniform. It was baked for another 60min. The sink-layer was aligned to porous region and was bonded by oxygen-plasma treatment. It was then submerged in IPA and was incubated for 30min at 70°C. The porous membrane along with sink-layer was automatically released from the master-mold. It was air dried and was sealed from the top (by a PDMS slab with an inlet and an outlet) by oxygen-plasma treatment.

Fabrication of sink layer: The master-mold for the sink was fabricated by spin-coating a layer of SU-8 on a silicon wafer at 2000rpm. It was soft-baked normally and was exposed through a photo-mask (with an array of opaque circles of 40µm diameter and center-to-center distance of 100µm between nearest circles, located in the sink channel) for 100S at a power of 7mW/cm^2. The PEB was performed by baking it for 1min at 65°C and it was transferred to the hot-plate with ramping temperature of 90-96°C. It was then baked for 15min at 96°C. It was cooled and developed normally. (Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane was vacuum deposited on this master-mold. PDMS (base solution + curing agent in ratio of 10:1) was poured on the master-mold and it was de-gassed in vacuum desicator. It was baked for 60min at 70°C. It was peeled-off from the master-mold and holes for outlets and inlet was punched in it.

Cell filtration: Cancer cell-line MDA-MB-231 was cultured in a DMEM cell culture-medium supplemented by 10% FBS in culture flasks. Cells were trypsinized and were counted using a hemocytometer. Appropriate amount of cells were pumped in the device at required flow rate by using a syringe pump. Cells were counted on the device by labeling them with CMPTX after cell filtration.

Cell Viability: For cell-viability studies, the device was sterilized by UV-light for 15min and by flowing 70% ethanol through channels. It was rinsed by 1X PBS for 15min. Then cell-culture media was flown in the device and it was allowed to stand for 30-60min before starting cell-based experiments. A CMPTX solution in 1X PBS for labeling viable cells was prepared. Cells were captured on the device. The cell-staining solution was flowed in the device and it was incubated for 15min at ambient temperature and fluorescence images were captured by using a fluorescence-microscope (Nikon, Eclipse, TE-2000-U, Japan). Cell viability was assessed by counting viable cells on the device using 'imageJ' software. For studying cell retrieval, cells were recovered by back-flow of fluid through the membrane.

3 RESULTS AND DISCUSSION

3.1 Device Fabrication

The microfilter device consists of a short microchannel connected to the porous membrane. The sample can be flowed in the membrane through this microchannel. The flow-through flows out in a sink attached below the membrane. The membrane can sag during fluid flow which may not be suitable for imaging. Thus, 'Doll-shaped' flexible microstructures were fabricated in the sink to support the membrane. This device has been successfully fabricated by the method mentioned here (Fig.1). Peeling-off the membrane from the master-mold was achieved by the solvent-based approach as described in the previous section. This approach allows easy release of the membrane from the mold. The membrane consists of a well-arranged array of 6µm pores separated by 15µm from each other. Each pore is surrounded by four dome-shaped microstructures (diameter of 10µm and height of 6µm).

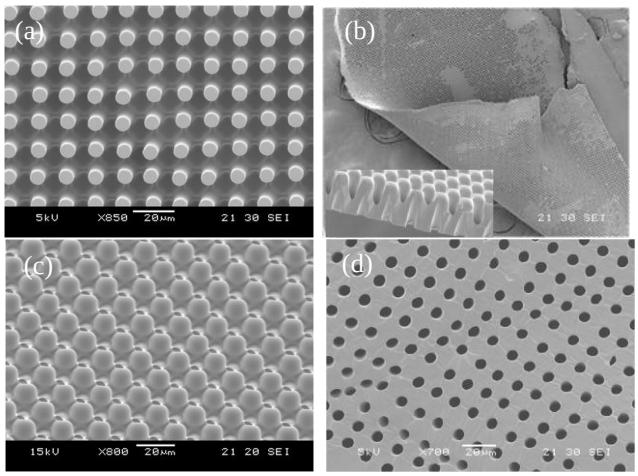


Figure1: The Porous Membrane: (a) the SU-8 mold fabricated by the modified photolithography, (b) the porous membrane fabricated from the SU-8 mold, the *inset* shows the side-view of the membrane, (c) the dome-shaped microstructures arranged around each pore, (d) the reverse side of the membrane showing an array of well-arranged pores.

3.2 Cell filtration

Cancer cells (10^4 cells/ml) were flowed in the device. The filtration efficiency was calculated as per the formula given below:

$$Filtration\ efficiency \equiv \frac{Total\ No.of\ cells - No.of\ cell\ in\ flowthrough}{Total\ No.of\ cells}$$

Filtration efficiency was obtained at different flow-rates and by manual delivery of cells (Fig.2a).The high filtration efficiency is achieved by this porous membrane. At high flow-rates some cells were obtained in flow-through which might have been caused due to flexible nature of PDMS. Further experiments were performed at 100µl/min as high filtration efficiency was achieved at this flow-rate.

3.3 Cell Viability and Cell Retrieval

The cell viability was studied for this porous membrane with dome-shaped microstructures and it was compared to the cell viability obtained for a porous membrane without any microstructures. Back-side of the porous membrane with dome-shaped microstructure was used as a porous membrane without any microstructures. This was done to keep all other parameters (like pore-size, pore-density etc.) same for both types of membrane.

High cell viability was observed for the porous membrane with dome-shaped microstructures compared to one without any microstructures (Fig.2b). The observed result is clearly due to dome-shaped microstructures. The high cell viability should have been caused due to extra support provided by dome-shaped microstructures as shown in scheme-1b. The increased cell viability due to extra support has previously been reported as well [7]. Thus, inclusion of dome-shaped microstructures is useful for maintaining high cell viability after filtration of cells. This can be useful for performing further functional studies on the isolated cells if cells can be retrieved from the membrane. Thus, comparative cell retrieval has been studied next for the porous membrane with dome-shaped microstructures and the one without any microstructures.

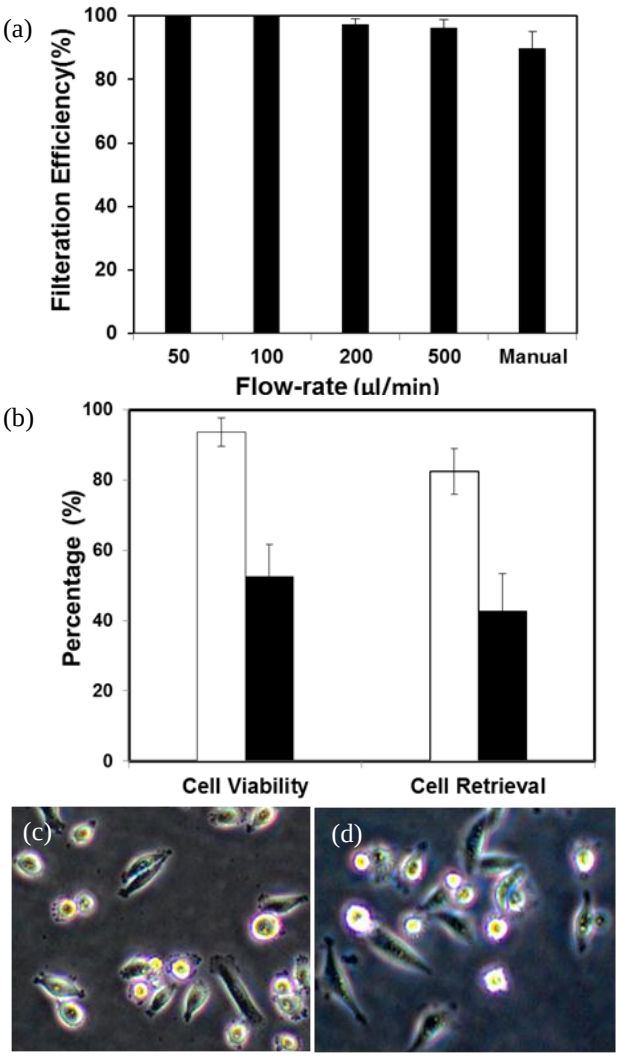


Figure2: (a) The filtration efficiency of the membrane calculated at different flow-rates. 1ml of media containing 10^4cells (MB-231) was flown in the device. The number of passed through cells and retained cells were counted. Filtration efficiency indicates the percentage of cells retained in the membrane. (b) Cell-viability and cell-retrieval were obtained for membrane with dome-shaped microstructures (white column) and for the membrane without any microstructures (black column). (c) Cells retrieved from the porous membrane with dome-shaped microstructures were cultured and compared to normal cell culture (d).

High percentage of cells were retrieved from the porous membrane with dome-shaped microstructures compared to the one without any microstructures (Fig.2b). This observed difference is also caused due to dome-shaped microstructures. Such microstructures do not trap cells and do not allow cells to get clogged in pores as compared to porous membrane without any microstructures. Thus, cells can be easily retrieved with high percentage of retrieval.

Next, the retrieved cells from this membrane were cultured to verify their viability and functionality. The retrieved cells were observed to be normal as compared to normal cell culture (Fig.2c &d).

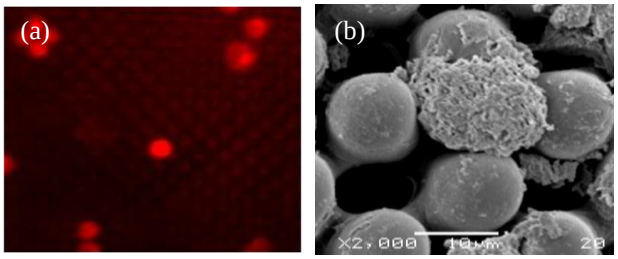
3.4 CTCs Isolation

To demonstrate that this porous membrane can be used for CTCs isolation, a small number of cancer cells (fifteen) were manually picked and were spiked in 1ml of buffer solution. This solution was flowed through the membrane device and number of isolated cells was counted. Figure-3 shows the labeled cells (Fig.3a) isolated in the membrane. SEM image of a cell isolated in the membrane can be seen in Figure3b. The table in Figure3c shows the different number of cells spiked in the buffer and the corresponding number of cells detected in the device.

The results here shows that the porous membrane with dome-shaped microstructures can be used for CTCs isolation as low number of cells can be isolated and detected.

4 CONCLUSION

The porous membrane containing dome-shaped microstructures have been successfully fabricated for high efficiency isolation of cancer cells. Cell viability and cell retrieval are found to be high for the isolated cells which can be useful for further functional studies of isolated cells.



(c)

Expected cells	15	18	16	13	15
Detected cells	14	15	13	12	12

Figure3: (a) Fluorescence image (live-cell labeling by CMPTX) of cancer cells filtered from media on the membrane, (b) SEM image of the cancer cell filtered by the membrane. (c) 15cells were picked manually and were diluted in 1ml of media. The media was flown in the device at a flow-rate of 100µl/min and cells were detected.

5 POSSIBLE IMPACT

The device can be useful as CTC-chip in clinical settings for cancer diagnosis, prognosis and possibly for drug selection. The device may work without the need of any special equipment (syringe pump) at high throughput. The same chip can be used for many types of cancer as CTCs are generally bigger and stiffer than blood cells.

ACKNOWLEDGEMENT

We acknowledge the National University of Singapore and the Institute of Materials Research & Engineering (Singapore) for their support.

REFERENCES

1. Maheswaran, S. and D.A. Haber, *Circulating tumor cells: a window into cancer biology and metastasis.* Current Opinion in Genetics & Development, 2010. **20**(1): p. 96-99.
2. Fernandez, M.J.S., et al., *Clinical relevance associated to the analysis of circulating tumour cells in patients with solid tumours.* Clinical & Translational Oncology, 2009. **11**(10): p. 659-668.
3. Cristofanilli, M., et al., *Circulating tumor cells, disease progression, and survival in metastatic breast cancer.* New England Journal of Medicine, 2004. **351**(8): p. 781-791.
4. Nagrath, S., et al., *Isolation of rare circulating tumour cells in cancer patients by microchip technology.* Nature, 2007. **450**(7173): p. 1235-U10.
5. Tan, S.J., et al., *Microdevice for the isolation and enumeration of cancer cells from blood.* Biomedical Microdevices, 2009. **11**(4): p. 883-892.
6. Zheng, S., et al., *Membrane microfilter device for selective capture, electrolysis and genomic analysis of human circulating tumor cells.* Journal of Chromatography A, 2007. **1162**(2): p. 154-161.
7. Zheng, S., et al., *3D microfilter device for viable circulating tumor cell (CTC) enrichment from blood.* Biomedical microdevices, 2011. **13**(1): p. 203-213.
8. Chee Tiong Lim, Y.Z., *Novel Dome-Shaped Structures for High-Efficiency Patterning of Individual Microbeads in a Microfluidic Device13.* Small, 2007. **3**(4): p. 573-579.
9. Kim, K.J., P.V. Stevens, and A.G. Fane, *POROSITY DEPENDENCE OF PORE ENTRY SHAPE IN TRACK-ETCHED MEMBRANES BY IMAGE-ANALYSIS.* Journal of Membrane Science, 1994. **93**(1): p. 79-90.
10. Lim, C., et al., *Fabrication of three-dimensional hemispherical structures using photolithography.* Microfluidics and Nanofluidics, 2009. **7**(5): p. 721-726.