Polymer-based biomedical microdevices for CTC diagnosis and keloid therapy

Xue, Peng

2015

Xue, P. (2015). Polymer-based biomedical microdevices for CTC diagnosis and keloid therapy. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/65571>

<https://doi.org/10.32657/10356/65571>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**POLYMER-BASED BIOMEDICAL
MICRODEVICES FOR CTC DIAGNOSIS AND
KELOID THERAPY**

XUE PENG

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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A thesis submitted to the Nanyang Technological University

in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

2015

Abstract

To complement the traditional silicon and glass-based microfluidic devices, polymer-based microdevices have attracted much more attention in recent years. Advantages of low cost, excellent mechanical property, low demands of facilities and reagents have further facilitated the development of polymer-based microdevices. Simultaneously, novel microfabrication techniques being developed enable the integration of microfluidic chips to achieve multiple analyses on the same chip, especially in the research fields of medical diagnostic assays and clinical therapy.

Cancer is a critical disease with high lethal rate nowadays. Diagnostics and therapeutics of cancer are important subjects, and any relevant creativity may change human lives in future. Polymer-based microfluidic microdevices have been extensively used for cancer relevant clinical applications.

For diagnostic application, a variety of dual-functional polydimethylsiloxane (PDMS)-based chips were developed to achieve both capture and elution of circulating tumor cells (CTCs). CTCs, which are derived from primary tumor and circulate to secondary site, are regarded as the cause of metastasis. Many methods have been applied for CTC isolation and enumeration so far. However, it remains a challenge to effectively elute the captured cells from the device for further cellular and biomolecular analyses. The presented dual-functional device showed ultrahigh sensitivity and specificity to capture and elute Hep3B CTCs with excellent reproducibility and stability, indicating its enabling capability for applications in cancer diagnostics.

For therapeutic application, a variety of polyethylene glycol diacrylate (PEGDA) and polyvinylpyrrolidone (PVP) based microneedle patches encapsulating drugs was designed and fabricated to achieve transdermal drug delivery. Briefly, keloids were selected as tumor model, and the inhibition effect was achieved by the release of 5-fluorouracil (5-FU) from the microneedle patch into tumor lesion. This study has provided evidence for effective inhibition of keloid fibroblasts *in vitro* and successful translation of this platform can likely result in a reduction in the cost of keloid treatment and an improvement in patient compliance. In future, a series of anti-tumor drugs may have great potential to be encapsulated into the microneedles and transdermal release can be triggered based on the pH, temperature and laser irradiation for tumor therapy.

Polymer based microdevices are still being developed and applied for cancer diagnosis and therapy. The development of platform is mainly dependent on two aspects: (a) synthesis of novel polymer: Polymer materials with improved mechanical property, higher level of biosafety and lower cost were expected to be invented to access clinical applications; (b) platform with well-design: polymer-based materials with excellent flexibility enable the achievements of platform with various designs based on the user requirements. As for the diagnostics and therapeutics of cancer, there still exhibit great opportunities to utilize polymer to design and fabricate novel microdevices. Additionally, multiple techniques, such as nanotechnology and medical assays, can be simply integrated into the microdevice to realize complex analysis on such miniaturized platform.

Acknowledgement

My deepest gratitude goes to my advisor Prof. Kang Yuejun, for his motivational encouragement and academic guidance throughout my graduate studies. His diligence and meticulousness has inspired me to be an independent researcher. I am forever grateful for this doctoral study opportunity he has offered me.

I would like to thank Prof. Xu Chenjie from Nanyang Technological University and Prof. Hui Kam Man from National Cancer Centre Singapore for providing me with many insightful ideas and training opportunities. I am also grateful to all the colleagues and friends who have offered me help in various ways: Dr. Wu Yafeng, Dr. Guo Jinhong, Mr. Chuah Yon Jin, Mr. Nishanth V. Menon, Dr. David Chenloong Yeo, Ms. Bao Jingnan, Mr. Shi Dongyuan, Dr. Huang Xiwei, Mr. Yang Cangjie, Ms. Liu Shiyang and Dr. Xu Zhigang. It is an honor to collaborate with these excellent people and it has been an enjoyable experience to learn from them. I am also grateful to technical and administrative staffs for their assistances in analytical instruments, in particular, Dr. Yu Shucong and Dr. Wang Xiujuan. My gratitude also goes to my former advisor Prof. Dai Zhifei from Peking University. His instructions and guidance have impacted me greatly in my pursuit of a scientific career.

I would like to acknowledge the NTU research scholarship from Nanyang Technological University to support my living and studying during my doctoral study.

Last but not least, I am foremost thankful to my beloved family members, especially to my mom and dad, for their everlasting caring, supports and encouragements throughout the journey. They helped form my personalities and built

up my confidence, and put here where I am. Thanks to my departed grandmother for keeping me on the right track since I was born, and I will miss her forever. Meanwhile, I deeply appreciate my soulmate Miss Zhang Lei, for sharing her life with me.

Vita

Xue Peng was born in Harbin, People's Republic of China. He obtained two Bachelor of Engineering degrees in July 2009, and one Master of Engineering degree in July 2011, from Harbin Institute of Technology, Harbin, China. In Aug 2011, he joined in Nanyang Technological University as a postgraduate student for pursuing a PhD degree in Chemical and Biomedical Engineering.

List of publications in refereed journals

1. Bo Hong[#], **Peng Xue**^{#,*}, Yafeng Wu, Jingnan Bao, Yon Jin Chuah, Yuejun Kang*. "A Concentration Gradient Generator based on a Paper-based Microfluidic Chip Coupled with Cell Culture Microarray for High-Throughput Drug Screening". Under review. (#co-1st author)
2. **Peng Xue**, Jingnan Bao, Yafeng Wu, Yilei Zhang*, Yuejun Kang*. "Porous Prussian Blue Nanocubes as Photothermal Ablation Agents for Efficient Cancer". *Journal of Nanoscience and Nanotechnology*, in press.
3. **Peng Xue**, Xuyang Zhang, Yon Jin Chuah, Yafeng Wu, Yuejun Kang*. "Flexible PEGDA-based Microneedle Patches with Detachable PVP-CD Arrowheads for Transdermal Drug Delivery". *RSC Advances*, 5, 75204-75209 (2015).
4. **Peng Xue**, Jingnan Bao, Yafeng Wu, Yilei Zhang*, Yuejun Kang*. "Magnetic Prussian Blue Nanoparticles for Combined Enzyme-responsive Drug Release and Photothermal Therapy". *RSC Advances*, 36, 28401-28409 (2015).

5. **Peng Xue**, Yafeng Wu, Jinhong Guo, Yuejun Kang*. “Highly Efficient Capture and Elution of Circulating Tumor Cells by Using a Dual Functional Chip Integrated with Herringbone Structure and Micropost Array”. *Biomedical Microdevices*, 17, 39 (2015).
6. **Peng Xue**, Karis Kar Ying Cheong, Yafeng Wu, Yuejun Kang*. “An In-Vitro Study of Enzyme-Responsive Prussian Blue Nanoparticles for Combined Tumor Chemotherapy and Photothermal Therapy”. *Colloids and Surfaces B: Biointerfaces*, 125, 277-283 (2015).
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13. Yafeng Wu, **Peng Xue**, Kam M. Hui*, Yuejun Kang*. “Electrochemical- and Fluorescent-Mediated Signal Amplifications for Rapid Detection of Low-Abundance Circulating Tumor Cells on a Paper-Based Microfluidic Immunodevice”. *ChemElectroChem*, 1, 722-727 (2014).
14. Yafeng Wu, **Peng Xue**, Kam M. Hui*, Yuejun Kang*. "A Paper-based Microfluidic Electrochemical Immunodevice Integrated with Amplification-by-polymerization for the Ultrasensitive Multiplexed Detection of Cancer Biomarkers". *Biosensors & Bioelectronics*, 52, 180-187 (2014).
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List of abbreviations

BSA	albumin from Bovine Serum
CTCs	circulating tumor cells
CT	computed tomography
CD	cyclodextrin
COC	cyclic-olefin copolymer
DMEM	Dulbecco's modified eagle medium
DEP	dielectrophoresis
ELISA	enzyme-linked immunosorbent assay
EpCAM	epithelial cell adhesion molecule
FDA	US Food and Drug Administration
FBA	fetal bovine serum
HMP	2-hydroxy-2-methyl-propiophenone
HPLC	high performance liquid chromatography
KF	keloid fibroblasts
LOC	lab on a chip
MNPs	microneedle patches
NF	normal fibroblasts
PDMS	polydimethylsiloxane
PEG	poly (ethylene glycol)
PEGDA	polyethylene glycol diacrylate
PMMA	polymethylmethacrylate

PP	polypropylene
PS	polystyrene
PVA	polyvinyl alcohol
PGA	polyglycolide
PLGA	poly(lactide-co-glycolide)
PLA	polylactic acid
PVP	polyvinylpyrrolidone
PBS	phosphate buffered saline
SEM	scanning electronic microscope
TGF	transforming growth factor
T _g	glass transition temperature
TPE	thermoset polyester
μTAS	micrototal analysis systems
USA	United States of America
3-MPTS	3-mercaptopropyl trimethoxysilane
5-FU	5-fluorouracil

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Chapter 1 Introduction

1.1 Polymer-based microdevice

The research field of biological and chemical analysis has been appealing to researchers, and much effort has been put into the miniaturization of relevant devices in recent years. Microfluidic platform, also known as lab-on-a-chip (LOC), and micrototal analysis systems (μ TAS) have been under extensive investigations, and latest discoveries have been considered revolutionary to the field of chemical and biological analysis in future. The development is mainly dependent on manipulating fluids in the channels with micron dimensions and designing devices with novel structures. Moreover, much more advantages can be attained through miniaturization compared to bulk devices, such as low cost, rapid analysis, tiny sample testing, parallel analyses, user-friendly interface and reduced waste generation.

At the beginning of microdevices occurrence at 1990s, glass had been firstly selected as the substrate material, and simple microfabrication techniques had been applied to fabricate glass microchips. Glass is an inert material and can tolerate a broad range of solvents. Additionally, excellent optical properties and biocompatibility had contributed to the extensive usage at that time. However, the system for manufacturing glass was too complicated with high cost, which eventually urged the researchers to develop alternative materials^{1, 2}.

Since 1995, polymers have emerged as the alternative material for the fabrication of microdevices. There are many advantages on using polymers, such as lower cost and simplified manufacturing process, comparing to traditional fabrication

based on glass and silicon. Meanwhile, researchers also pursue specific applications of polymer-based microdevices in terms of material properties^{3,4}.

1.1.1 Options of polymer

There have been many methods involved in manipulating polymers for microdevice fabrication, such as rapid prototyping. Simultaneously, the mechanical properties, such as storage stability, biocompatibility, roughness, hardness and optical characteristics, also impact on the final applications of these polymer-based microdevices. Hence, properties of the materials are critical factors that would determine the fabrication process and the ultimate application of the device.

Basically, polymers are large molecules comprised of continuous identical units. However, the chain length of polymers is highly differentiated in bulky materials, and it may influence the properties of bulky materials, melting temperature for instance⁵. The most critical parameter for polymer manipulation is the glass transition temperature (T_g). The polymers can transit to be solidified when the environment temperature goes below T_g , resulting in materials with high rigidity. Specifically, at temperatures above T_g , the polymers preserve the state with high viscosity which facilitates the molding process. Conversely, at temperatures below T_g , the morphology of polymers will be fixed during demolding. Generally, there are three categories of polymers based on the molding process. (1) Elastomeric polymers: The interconnection of monomer units is weak. The molecular chains can be easily stretched when an external force is placed. On the contrary, relaxation takes place when the force is being removed, and the polymer would recover to the original state.

(2) Thermoplastic polymers: The cross-linked polymer chains are weak in these polymers, and temperature plays an important role in determining the material state. At a temperature above T_g , the materials behave as liquid and the molding process can be conducted to shape the material into specific morphology. While, the shape will be fixed when being demolded below T_g . (3) Duroplastic polymers: The polymer is composed of strong cross-linked chains, leading to restrict shape deformation in these materials. They are obviously harder and stiffer compared to thermoplastics and elastomeric polymers. Moreover, at a temperature below T_g , the hardness increases very little compared to that above T_g ^{6, 7}. So far, there are numerous polymer materials that have been involved in micro-manufacturing process, such as polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), Polyvinylpyrrolidone (PVP), polystyrene (PS), poly(ethylene oxide) diacrylate (PEGDA) and polypropylene (PP), *et al*⁸. Mechanical property, chemical resistance, optical characteristics and storage stability are essential parameters that need to be evaluated for selecting the optimal polymer for different applications.

1.1.2 Microfabrication techniques

There are various methods that are engaged in the fabrication of microdevices, such as photolithography, wet etching, hot embossing, laser cutting, plasma etching and chemical etching⁹. Generally, the options of fabrication methods are dependent on costs, accuracy, processing speed and materials used.

Soft lithography is the most commonly used technique for elastomeric polymer molding. SU-8 is a revolutionary photoresist which possesses photo-active property,

and it is commonly used by patterning silicon wafer or glass substrate after being exposed to UV-light. Afterwards, PDMS is casted onto the master mold for further incubation at 70°C. After curing, the slice of PDMS with specific pattern can be peeled off from the mold for usage. The PDMS slice as-prepared can permanently bond to each other, or glass substrates as well as other plastics. This bonding process can be initiated by exposing PDMS to oxygen plasma ahead to bonding. There are many advantages of soft lithography, including fast processing, low cost, simple facilities required and safe handling. Despite the advantages, PDMS also preserves several shortcomings such as gas permeability and intolerance to corrosive organic solvents¹⁰.

Figure 1-1 Schematic illustration of typical soft-lithography technique for fabricating PDMS chip with specific pattern¹¹. Copyright 2012 Nature Publishing Group.

Hot embossing is another method for microchip fabrication. The process is fast, simple and with low cost. A slice of hard plastic is located against the master mold, and the setup is under a hydraulic squeezing. Afterwards, the setup is heated to the temperature above T_g and the status is retained for a specific time. Then, the temperature is cooled down below T_g to complete the hot embossing process. To avoid the replication errors induced by the different thermal expansion coefficients of substrates and master mold, the heating process should be as short as possible. Polymers, such as PMMA, PS and PP have been successfully replicated using hot embossing when heating temperature and thermal cycle is precisely controlled¹².

Figure 1-2 Schematic illustration of a typical hot embossing process including four steps, heating, embossing, cooling and demolding¹³. Copyright 2008 Springer.

Injection molding is a fabrication technique that is used in molding most thermoplastic materials into microdevices with dimensions from millimeter to centimeter. The raw material firstly melts in the cylinder and then is injected into the

master mold under driven pressure. The advantage of injection is the capacity to generate three-dimensional structures¹⁴.

Laser cutting is used for microdevice manufacturing based on the breakage of light-induced polymer backbone. The cutting process is dependent on photo-ablation, thermal degradation, or both. The ablation level is mainly attributed to the light absorption on the polymers. During the laser cutting, mask is not required and predesigned cutting path is achieved directly¹⁵.

1.2 Cancer diagnostics and therapeutics

Cancer is considered as malignant tumor, which involves abnormal cell growths with great potentials to invade and spread to other parts of the human body, and metastasis occurs frequently during terminal stages of cancer. There have been more than one hundred known cancers observed in humans. There are many causes of cancer, such as tobacco, obesity, unhealthy diet, lack in physical activity, and alcohol consumption. Additionally, ionizing radiations and environmental pollutions also contribute to the high morbidity of cancer. Moreover, genetic defects inherited are also involved in the cancer development. High fatality rate and serious symptom has made cancer as the most critical disease across the world¹⁶.

For the diagnosis, blood testing, computed tomography (CT) scans and endoscopy are mainly involved as the medical test for cancer screening. CT scan is a typical imaging test that uses X-ray to create 3D pictures of internal structures in human body. Blood testing is using facilities to test the relevant index of patient

blood which can indicate the tumor occurrence. Endoscopy is operated by using an endoscope to observe inside the human body. The endoscope has a tiny camera with light, and doctors can look inside the organs of body¹⁷. Meanwhile, there is a specific research area of circulating tumor cells (CTCs). When tumor cells metastasize within in the circulatory system, they are defined as the CTCs. Detection, characterization and separation of CTCs are critical for the diagnosis of tumor stage, metastasis and treatment effectiveness evaluation¹⁸⁻²⁰.

For the therapy, the primary treatments include surgery, chemotherapy, radiation therapy, hormonal therapy and gene therapy. Method option is based on the tumor type, location and cancer grade as well as patient wishes. Surgery is a direct method which can isolate the solid tumors to ensure a prolongation of survival. The entire tumor should always be removed during operation together with the lymph nodes affected. However, multiple surgeries are always required to completely remove the tumor tissue, and patients under severe conditions are not suitable for this therapy option. Chemotherapy is a treatment of cancer with drugs or agents that may be cytotoxic to tumors as well as normal cells. There are many categories of drugs including alkylating agents and antimetabolites²¹. The efficacy of tumor therapy is mainly dependent on the cancer type and the stage. It is demonstrated that chemotherapy may be useful to release the pain and reduced the size of tumor to ensure a possible surgery in future. Meanwhile, serious adverse side effects are inevitable during the treatment process. Radiation therapy is applied to improve the cancer symptoms by using ionizing radiation on patients. The principle is damaging the nucleic acids in order to inhibit the proliferation of tumor cells. Even though the

radiation beam is aimed at the tumor tissue from several angles of exposure, normal tissues are still partially damaged due to the radiation pollution²². Other therapies such as immunotherapy and alternative medicine are developed to substitute the traditional treatment, and many cancer therapies have not been approved from FDA (US Food and Drug Administration). Conclusively, there is still a long journey ahead to achieve cancer healing in future²³⁻²⁵.

1.3 Objectives

The objective of this thesis is to achieve cancer diagnosis and therapy by using polymer-based biomedical microdevice. In the diagnosis section (Chapter 3 and 4), PDMS microchip was designed and fabricated to achieve CTC isolation and enumeration *in vitro*. In the therapy section (Chapter 5 and 6), a polymer-based microneedle patch was developed to realize transdermal anti-tumor drug delivery *in vitro*. And keloids are selected as the tumor model for testing drug efficacy. Details are listed as follows.

1.3.1 Dual functional herringbone chip for CTCs isolation and elution

CTCs are a type of cell circulation in peripheral circulating system and can cause metastasis. Many devices have been developed for CTC isolation and enrichment. However, it is essential to elute the captured CTCs from the device for further biomolecular analysis for metastasis diagnosis. The overall objective of this project is to realize a dual-functional herringbone chip with high capture rate and elution rate of Hep3B liver cancer cells.

Specific aims and objectives

- (a) Design and fabrication of dual-functional herringbone chip.
- (b) Numerical study of flow behavior in the microchannel using finite element method.
- (c) Investigate the capture and elution of Hep3B CTCs by using PBS and blood sample based on this device.

1.3.2 Drug-loaded microneedle patch for keloid management

Microneedle patch is a novel transdermal microdevice for local drug delivery enhancement. It is a novel and creative idea to expand the application to keloid management. 5-Flourouracil is a typical drug which is used to treat small keloids intralesionally. As a versatile platform, it is challenging to effectively encapsulate 5-Flourouracil into the microneedle patch. The overall objective is to fabricate a flexible drug-loaded microneedle patch aiming at the noninvasive treatment keloid patients and investigate its potential clinical applications through characterization.

Specific aims and objectives

- (a) One-step fabrication of flexible drug-loaded microneedle patch.
- (b) Explore the morphology, mechanical properties, in vitro drug delivery and cytotoxicity.
- (c) Discuss the potential clinical application of this novel microneedle patch.

Chapter 2 Literature review

2.1 Methods for CTC isolation and elution

Cancer is universally recognized as the leading cause of deaths all over the world. Basically, cancer is caused by incontrollable growth of cells, which progressively invade tissues and organs around, and metastasis occurs frequently during terminal stages of cancer²⁰. When tumor cells metastasize within in the circulatory system, they are defined as the circulating tumor cells (CTCs). Detection, characterization and separation of CTCs are critical for the diagnosis of tumor stages, metastasis and treatment effectiveness evaluation^{18,26}. Traditional tumor cell detection and sorting techniques, including centrifugation, fluorescence method and chromatography are restricted in achieving high purity and specificity, devices derived from those techniques lacks intelligence and relies on personal expertise. Devices based on microfluidic technique have the advantages of tiny sample volumes, high throughput, fast processing, multifunctional detection and point-of-care detection, which provide with the opportunity for CTCs detection and characterization²⁷. In detail, a scale range from several to tens of micrometer is suitable for manipulation of single cells. Complexity of microfluidic patterning allows the realization of multi-functionality. Large surface-to-volume ration facilitates capturing cells based on affinity. Overall, there are four typical microfluidic platforms being applied for CTCs detection, including cell-affinity chromatography²⁸⁻³², magnetic-activated cell capture³³⁻⁴¹, size-based cell capture⁴²⁻⁵² and dielectrophoretic (DEP) method⁵³⁻⁵⁸.

2.1.1 Overview

Microfluidic devices are popularly used for CTCs isolation due to its compatible geometry in terms of cell size. As basic methodology for this topic, interaction between antibody-functionalized wall and antigen expressed on cell surface is commonly utilized for cell capture and enrichment. Antibody specificities and cell-wall collision mechanics are considered as factors that affect capture efficiency of CTCs. Additionally, the size of tumor cells is much larger than that of the surrounding cells, leukocytes particularly. Therefore, devices invented for screening CTCs based on size variation are also developed for CTCs isolation and enrichment.

CTCs surface biomarker consisted of aptamers and antibody is able to be used for cell capture at the presence of corresponding antibody. The ideal surface biomarker for CTCs capture is considered as over-expressed and not affected by microfluidic environment. One famous example for tumor cell marker is epithelial cell adhesion molecules (EpCAM), which is expressed on most gastrointestinal carcinomas. This biomarker is supposedly to be the target for cancer diagnostics and therapy. In the real immunocapture system, clarification of the specificity of antibody to a particular surface marker is essential for fundamental research. Therefore, cell type, expression density and location of antigen on the cell surface are critical to judge the specificity. Purity and efficiency are two important parameters to the performance evaluation of CTCs detection device. High efficiency allows effectively capturing CTCs from blood for clinical analysis and high purity promises downstream study of CTCs that are eluted from microdevice afterwards. Stable bond of receptor-ligand enhances the binding interaction and increase capture efficiency.

Simultaneously, purity can also be improved in high local flow rate without destroying the specific bond. In addition, it is essential to analyze the force of cell experiencing, depending on the combination of flow rate, fluid viscosity, device geometry and cell deformation. Mechanical separation is based on the larger size of CTCs compared to surrounding blood cells, especially leucocytes. This offers a possibility to use mechanical method to isolate CTCs from blood based on size variation. Therefore, filters with specific pore sizes are encouraged to be applied for capturing CTCs.

2.1.2 Cell-affinity chromatography

Cancer cells are considered to be captured on the walls of microdevice where specific high-affinity antibody is conjugated. In 2007, Nagrath *et al.* firstly reported a novel CTCs detection platform based on microposts patterned in PDMS microfluidic device (Figure 2-1a)²⁸. Microposts modified with specific antibody (anti-EpCAM) were used to capture CTCs selectively and efficiently under precisely controlled laminar flow conditions based on immunoreaction. In short, hydroxyl groups generated on PDMS surface and the glass slides are indirectly conjugated by amino groups in anti-EpCAM through three steps of chemical modification. After introducing the sample into the chip using a micropump, this platform identified CTCs derived from typical endothelial cancers in 115 of 116 (99%) samples from cancer patients, with a range from 5 to 1281 CTCs per ml. Additionally, CTCs were also clinically isolated from 7/7 patients with early stages of prostate cancer. Afterwards, an enhanced CTCs isolation device has been developed as the herringbone-chip, which improves the interaction between antibody-modified channel

wall and the surface of target CTCs (Figure 2-1b)²⁹. This 3-D herringbone pattern contributes to vortex mixing in each groove, and facilitates the capture of more tumor cells. Based on this device, CTCs were detected in 14 of 15 from patients with prostate cancer in the metastatic stage (386 ± 238 CTCs/ml). The microscopy of captured cells was obtained through fluorescent microscope based on fluorescent staining of live cells. Similarly, a 3-D nano-structured substrate modified with anti-EpCAM was applied to isolate CTCs and the capture rate based on this micron-size micropost is dramatically higher compared to traditional flat straight channel.

Figure 2-1 Cell-affinity chromatography techniques for CTCs isolation. (a) SEM image of a NCI-H1650 lung cancer cell captured on antibody modified microposts²⁸. (Copyright 2007 Nature Publishing Group) (b) Schematic illustration of herringbone structure that improves interaction between cell and functional surface²⁹. Copyright 2010 Proceedings of the National Academy of Sciences.

2.1.3 Magnetic-activated cell capture method

The principle of this technique is based on the interaction between specific antibody modified on magnetic beads and antigen on the surface of CTCs. Compared to other techniques; this method is capable of capturing CTCs but limited in recovery

afterwards. Moreover, local magnetic fields are required to be applied for CTCs manipulation. Hoshino *et al.* reported a novel detection platform for CTCs based on immunomagnetic reaction, benefiting from microfluidic concept and immunomagnetic assay (Figure 2-2a). Initially, anti-EpCAM was conjugated to Fe_3O_4 magnetic nanoparticles which were spiked into the whole blood subsequently. After running the sample, CTCs label by magnetic nanoparticles are considered to be isolated from the blood under the presence of magnetic field. For detection, fluorescent method by cell staining with anti-cytokeratin, anti-CD45 and DAPI is employed for differentiating captured CTCs from other cells. CTCs with density of less than 5 cells per ml were successfully detected by using this platform³³. Afterwards, a subsequent generation of magnetic-activated device is developed and considered as “double collection” based on placing two rows of side chambers for CTCs collection (Figure 2-2b). Capture rates of breast tumor cells with the number from 2 to 80 per ml can reach up to over 90% in blood sample tests. Moreover, it is still viable of captured CTCs in this device, which can be recovered easily for further biomolecular analysis and clinical applications³⁴.

Figure 2-2 Device based on magnetic-activation for CTCs capture. (a) Fe_3O_4 nanoparticles labeled CTCs flowing through the channel are captured in the presence of magnetic field³³. Copyright 2011 Royal Society of Chemistry; (b) Schematic illustration of the double collection channel structure and its working principle³⁴. Copyright 2012 Royal Society of Chemistry.

2.1.4 Size-based filtering for CTCs capture

Cell size is an intrinsic property for CTCs sorting without any necessity for biochemical reaction. Since the size of CTCs is much larger than the surrounding blood cells, leucocytes particularly, microfilter with pore size from 8 to 10 microns is popularly applied for CTCs enrichment. Zheng *et al.* invented a novel 3-D microfilter device for isolating CTCs from blood. This device is consisted of two separated layers of parylene membrane, on which pores (10 μm diameter) interlaced with each other precisely⁴². The misaligned bottom layer is designed for minimizing concentrated flow stress on the cell membrane (Figure 2-3a). Samples were prepared

by spiking 342 ± 58 MCF-7 cells in 1ml whole blood collected from healthy individuals. After filtration operation, capture efficiency was able to reach up to $86.5 \pm 5.3\%$, which is comparable to the result from traditional microfilter devices. Hydrodynamic force based microfilter was also developed for CTCs separation, which can isolate cancer cells with high-throughput (7.5×10^6 cells/s) (Figure 2-3b)⁴³. This cell isolating device can realize the recovery of CTCs-clusters after capturing, which are critical for further clinical analysis.

Figure 2-3 Size-based CTCs isolation from blood. (a) Filtration process for CTCs enrichment based on 3-D parylene membrane filter⁴². Copyright 2011 Springer; (b) A

hydraulic force based microfilter for CTCs isolation based on the difference in the lift forces and device geometric design⁴³. Copyright 2011 AIP publishing.

Other methods such as the DEP technique are also investigated for CTCs capturing or isolation. The DEP method is derived based on the electrical properties of cell membrane and cytoplasm. Series of epithelial and endothelial cancer cells have been successfully isolated from blood samples based on DEP with promising recovery rates. In future, multifunctional microfluidic devices are urgently required to be investigated and designed based on the physical and biochemical combined techniques to isolate the CTCs from heterogeneous environment with stable capture efficiency, high throughput and high recovery rate for commercialized clinical applications.

So far the study is limited on eluting and harvesting the captured cells from the herringbone chip for further clinical analyses, which can reveal the molecular characterization and further diagnosis of the metastasis stage. Therefore, a dual-functional herringbone chip is urgently required to realize the CTCs elution successively upon capture. In chapter 3 and 4, for the first time, a dual-functional PDMS-based herringbone chip is developed for Hep3B capture and elution. Hep3B is a typical hepatocellular carcinoma cell line and the number in patients' blood is essential for determining hepatocellular carcinoma metastasis stage. The herringbone chip was fabricated by using traditional PDMS elastomer and the wall of the inner channel surface was modified with anti-EpCAM. Afterwards, Hep3B cells were spiked into PBS and blood, respectively, before being introduced into the microchip by a precise syringe pump. The capture rate was calculated by using the fluorescent

microscopy after immunoreaction. Then, D-biotin was introduced into the microchannel to displace the site of biotinylated anti-EpCAM conjugation, followed by elution of unbound cells from the device. The present device enables the average capture rate of over 50% and average elution rate of over 50% respectively, demonstrating its potential applications for cancer diagnostics in clinical application.

2.2 Microneedle patch and keloid

2.2.1 Introduction of keloid

In daily life, cutaneous damages such as skin injuries are inevitable. Cutaneous wound healing is a complex process, in which scar formation could take place. The objective normal cellular interaction during wound healing is to restore skin integrity and function. However, degradation of this process can lead to scar formation excessively and abnormal scar will expand beyond the origin boundary of the wound and form tumorous keloid⁵⁹.

The first record of keloid dates back to 1700 BC printed in an ancient Egyptian document⁶⁰. Cheloide as a terminology derived from Greek was first proposed by Ailbert in 1806, which means “crab claw” to describe the expansion of keloid into normal tissue out of control. Throughout the history, dark-skin racial groups, such as African-American, Latin-American and Hispanics, are more probable to suffer from keloid. These groups are highly susceptible and more than 15% of these populations are at risk of growing keloid⁶¹⁻⁶³. Keloid occurs more frequently in the age between 11 and 30 year olds, but patients of other ages were recorded in clinical cases⁶⁴ as well. There are two pedigrees that are related to familial keloid, which are unrelated

locations on two separate chromosomes. However, there has not been enough evidence to identify any single gene which induces keloid occurrence so far⁶⁵. A typical keloid on shoulder is exhibited as Figure 2-4. The arrows show potential directions of skin stretching force that may result in the butterfly shape in this skin region⁶⁶.

Keloid is initially induced by a hypertrophic scar that is a skin with collagen and fibroblast overgrowth from the margin of origin scar. Injury in the dermis reticular layer is considered to result in the keloid formation. In normal skin, collagen fibers are freely patterned in disordered arrangement. However, abundant collagen bundles are significant in keloid, which creates nodule-like structure in dermal⁶⁷. Skin configuration deformity is inevitable for keloid and causes cosmetic defects. Other serious syndromes including ulcer and joint mobility limitation are significant in untreated patients. Moreover, keloid is considered to cause needle-like pain, and is erythematous and pruritic compared to hypertrophic scar. Keloid can grow beyond the origin scar gradually, and spontaneous regression is extremely rare in clinical cases.

Figure 2-4 Typical keloids on the shoulder⁶⁶. Copyright Elsevier 2008.

2.2.2 Pathology of keloid

Keloid is generally considered to be painful, erythematous and pruritic. However, the process of keloid development is poorly understood. It is commonly believed that insect sites, scratching, burns and skin trauma could result in the keloid formation. Most of the keloids form within the first three months after injury, whereas some even occur within one year⁶⁸. One of the most popular theories for keloid formation is the fibroblast dysfunction. At the early stage of keloid, the atypical fibroblasts overproduce type III collagen and will result in the deposition of extracellular matrix. Additionally, other growth factors including platelet growth factor, endothelial growth factor and transforming growth factor β (TGF- β) could express in a significant level⁶⁹. At the end of stage, collagen type III in the overgrown pathological tissue is substituted by collagen type I. Moreover, keloid fibroblasts exhibit insignificant apoptosis which can be explained as the down-regulated expression of tumor-related genes, such as p53.

Connexins are characteristic proteins in the formation of gap junction. However, a down-regulation of connexins is remarkable in keloids, indicating poor intercellular communication between the keloid fibroblasts⁷⁰. Therefore, a failure of exchanging inhibitory signal may result in the apoptosis of keloid fibroblasts being slower than the normal ones. Moreover, keloids are consisted of elongated and thickened collagen bundles based on histological study⁷¹. It was discovered that the metabolism in the keloid tissue is much more active than normal tissues in terms of oxygen consumption, which may result in keloid formation in this hypoxia environment⁷².

2.2.3 Traditional keloids treatment methods

Currently, many treatments methods have been developed for keloids management, including surgical excision, cryotherapy, laser removal, corticosteroids, and other adjuvant therapies⁷³. As a direct method, surgical excision is applied to remove keloids and keloid knots can be thoroughly removed by meticulous surgical operation. However, an obvious enlargement of final scar region could always occur after surgery, and larger keloid can be formed during recurrence with a rate from 20% and 85%^{74,75}. As first-line agents for keloid therapy, corticosteroids can reduce keloid fibroblasts proliferation effectively after intralesional treatment. However, patients always suffer dose-dependent side effects after long-term treatment, including injection pain, skin atrophy and telangiectasias⁷⁶. Low-dose external beam radiation is effective for keloid treatment based on inhibiting epithelial proliferation⁷⁷. However, side effects including postoperative fibrosis and serious cancerization of surrounding tissue occur from long period of treatment. Laser therapy is invented to ablate target tissue by using laser with mono-wavelength. But high recurrence and

side effects including purpura and hyperpigmentation associated with this technique can occur up to 24% of patients⁷⁸. Freezing agent is used to ablate target tissue in cryotherapy but may result in considerable pain and hypopigmentation⁷⁹. More adjuvant therapies are being applied for keloids treatment, including chemotherapeutic agents and immunomodulators. 5-flourouracil is a pyrimidine analog which serves as typical drug in chemotherapy. 5-flourouracil can interfere with TGF- β signaling and inhibit the expression of type I collagen in keloid according to previous report⁸⁰. In one pilot study, intralesional treatment of 5-flourouracil with a dosage between 25mg and 100mg were conducted on 28 keloid patients for as long as 12 weeks. The result showed that 5-flourouracil therapy had therapeutic effect on all patients with no recurrences reported⁸¹. Additionally, 5-flourouracil has much fewer side effects, such as scar height or erythema, compared to other intralesional steroids⁸². A significant height reduction of keloid tissue and decreased contractility of keloid fibroblasts can be observed compared to the control group after one month treatment.

Figure 2-5 Schematic illustration of methods on microneedle application for the enhancement of drug delivery into dermal. (a-d) Traditional methods of drug delivery based on microneedle; (e) A novel microneedle device based on PEGDA which includes a drug-loaded adhesive patch.

2.2.4 Microneedle techniques for biomedical application

Nowadays, almost all treatments of keloids are performed in clinics, which involve trained clinicians and require expensive resources from the healthcare system.

Most of the treatments are invasive and severe pain is caused to the patients. For instance, patients require meticulously local injection to the target tissue when they accept the 5-fluorouracil treatment. With the development of modern biomedical techniques, miniaturized and portable device provide alternative options for keloid treatment. Microneedle patch is one of the most attractive and novel platforms for transdermal drug delivery compared to traditional invasive injection methods and oral administration⁸³. For traditional hydrophilic macromolecules delivery, the outermost layer of epidermis and stratum corneum prevents the biopharmaceuticals with the molecular weight over 500 Daltons from permeating the inner tissues. On the contrary, microneedle platform offers potential capacity of delivering a broad range of therapeutic agents via minimally invasive route, comparable to the performance of hypodermic needles. Furthermore, since microneedles are in micro scale dimensions, they do not penetrate into dermis to stimulate the nerves and thus are considerably pain-free, which still remains as a major defect for traditional hypodermic needle⁸⁴. Microneedles can always be fabricated with various materials and the shape profile can be easily customized. So far, four types of microneedle-assisted drug delivery strategies have been reported: (1) solid microneedle to realize skin puncture with drug delivery, followed by treatment using a traditional transdermal patch (Figure 2-5a); (2) solid microneedles coated with drug powders to improve drug absorption, which has a low efficiency since drug loading on microneedles surface is difficult and majority of drugs are lost during penetration (Figure 2-5b); (3) drug-loaded microneedles are fabricated by polymerization for skin penetration and controlled drug release (Figure 2-5c); (4) hollow microneedles with interior microchannels as conduit for active drug

delivery, which however can be possibly blocked by epidermal layer.(Figure 2-5d)^{85,86}. Generally, microneedles are fabricated using silicon, metals and polymeric materials. In terms of strength, precision, rigidity and sharpness, metal and silicon microneedles are advantageous compared to other materials. Microneedles fabricated from PLA, PGA and PLGA post harsh processing difficulties due to the requirement of high temperature and organic solvent, which may inactivate the temperature-sensitive drug⁸⁷. Microneedles formed based on water dissolving polymer such PVP, PVA, maltose and amylopectin could result in hasty drug release into skin⁸⁸, which is not desirable for most clinical application that requires gradual and controlled drug release over a long period of time. Other techniques including X-ray lithography, UV lithography, soft etching and photopolymerization with long exposure time require advance and expensive fabrication instruments and hence may cost much for point-of-care applications.

In chapter 5, a flexible 5-fluorouracil encapsulated microneedle patch fabricated based on poly (ethylene glycol) discrylate (PEGDA) using a simple photopolymerization method is reported for microneedle fabrication⁸⁹.As a drug delivery system, PEGDA has attracted attentions for its excellent biocompatibility and nontoxicity. Although PEGDA is not biodegradable, nontoxic metabolites derived from it can be easily eliminated from internal environment via liver and kidney. It is demonstrated for the first time here that 5-flourouracil can be successfully loaded in PEGDA-based microneedle patch and can be release through capillary osmosis coupled with the swelling and degradation of PEGDA matrix (Figure 2-5e). This flexible microneedle patch aims at covering bumpy surface of keloid focus and

enhances the drug release in local tissues. In chapter 6, an improved microneedle patch with detachable arrowhead is reported to improve the microneedle performance during the application. Along with various advantages, the arrowhead loaded with drug could be embedded into the skin after the administration.

2.3 Functional polymers

In this thesis, three types of polymers were used for fabrication biomedical microdevices with different applications, which are PDMS, PEGDA and PVP.

2.3.1 PDMS

PDMS is a silicone based-elastomer which is attractive for fabrication of biomedical microdevices due to its desirable properties. Basically, it is transparent, gas permeable, stable under long-term storage, inert and cost efficient. Most importantly, it can be developed into structures with dimension of microns after simple manipulation^{10, 90-92}. The use of PDMS is largely expanded by the development of soft-lithography, such as replica molding on silicon mold, and solvent-based micro-molding. PDMS plays a role as elastomeric stamp to replicate the patterns on the substrate to create microstructures. As for biomedical applications, PDMS is biocompatible and nontoxic. Simultaneously, PDMS layer is transparent to allow the observation under the microscope. The resulting PDMS slice can be bonded to many different substrates⁹³. So far, PDMS has been used as biomaterial for manufacturing pacemaker, membrane oxygenators and other organ implants. Sylgard 184 (Dow Corning Corporation, Midland, MI) is the most popularly used brand of PDMS. The recommended mixing ration of elastomer and curing agent is at 10:1.

However, there still exists disadvantages with this polymer, such as functional molecules cannot be adsorbed to it covalently, which extremely impedes its biomedical applications. Fortunately, this drawback can be converted to advantage by simply modifying the surface with functional group to selectively immobilize biomolecules^{94, 95}. Bio-functionalization of PDMS can be categorized into two strategies: physical adsorption and covalent conjugation. The first strategy is simple, and interaction between PDMS surface and functional molecules is weak and instable. On the other hand, covalently modification provides a strong and stable interaction⁹⁶. It is demonstrated that hydroxyl groups (-OH) can be easily initiated on the PDMS surface, which can be further siliconized to conjugate protein or antibody. Specifically, PDMS surfaces can be easily treated by oxygen plasma or UV/ozone, to enable a hydrophilic surface by replacing methyl group by silanol group (Si-OH)^{97, 98}. Silanol group is essential for PDMS surface to be modified with enzymes, such as glucose oxide⁹⁹. The PDMS surface hydrophilization is achieved by plasma treatment and subsequent silanization, and glucose oxidase was immobilized to create a glucose sensor. PDMS surface treated by acidic solution containing hydrogen peroxide (H₂O₂) can also create silanol groups. However, the material transparency may decrease during the etching process without careful control¹⁰⁰.

2.3.2 PEGDA

Poly (ethylene glycol) (PEG) is a typical hydrophilic, biocompatible polymer that has been used for various biomedical applications^{101, 102}. The polymer is non-toxic in terms of molecular weights above 400Da, and is approved by FDA for clinical usage¹⁰³. PEGDA is synthesized by substituting terminal hydroxyl groups

with acrylates and a three-dimensional polymer network can be formed by crosslinking (Figure 2-6). The crosslinking is induced by many techniques, and photo-initiated polymerization is most commonly used for biomedical applications¹⁰⁴. PEGDA can form hydrogel very quickly, and cell encapsulation can be feasible in PEGDA on condition of selecting an appropriate photo-initiator. The hydrogel formation can be spatially and temporally controlled, and final shape is variable based on requirements¹⁰⁵. The mechanical properties of PEGDA hydrogel can be controlled by the polymer molecular weight, and the duration as well as laser intensity of photo-polymerization¹⁰⁶.

Figure 2-6 Chemical structure of PEGDA

2.3.3 PVP

PVP is a water soluble polymer formed by the monomer N-vinyl-2-pyrrolidone. It has been used in various industries, including cosmetics, pharmaceuticals, and food industry. PVP can be such widely used due to its intrinsic properties, including biocompatibility, low toxicity, storage stability, et al¹⁰⁷. PVP acts an important role as a tablet binder for oral drug delivery in pharmaceutical industry. The addition of PVP can improve the solubility of poorly soluble drugs during the degradation in human body¹⁰⁸. PVP polymer with the molecular weight less than 20,000 can be cleared by human kidney. The polymer with larger molecular weight is not metabolized within

the human body, which implies biological inert¹⁰⁹. The PVP has a great potential to be a structure material for the microneedle fabrication due to its rapid dissolution within the skin owing to its excellent water solubility¹¹⁰⁻¹¹⁵.

Figure 2-7 Chemical structure of PVP

Chapter 3 Dual-functional herringbone chip for detection of Hep3B CTCs

3.1 Introduction

Circulating tumor cells are derived from primary tumor site and transported to distant organ through circulatory blood system, which is known to cause metastatic cancer²⁰. The characterization of the CTCs is critical to evaluate the treatment efficacy and monitor disease progression due to the fact that the number of the CTCs in peripheral blood is proportionally correlated to the extent of metastases. The isolation and enumeration of CTCs is highly challenging, however, due to the extremely low cell density, normally 1 to 10 CTCs per ml of blood in cancer patients²⁷. The devices based on microfluidic technique have many advantages, such as tiny sample volume, high throughput, fast processing, and multifunctional detection, which provides the opportunity for rapid and efficient CTC detection and characterization for point-of-care applications¹¹⁶. The large surface-to-volume ratio of the micro-devices further facilitates reliable cell capturing using complex microfluidic patterning.

In 2010, an enhanced microfluidic mixing device was developed using a unique herringbone structure, which increased the interaction between the modified chip surface and the target human prostate cancer cells (PC3) based on cell-affinity chromatography²⁹. This 3-D herringbone design significantly enhanced the mixing within each groove, and facilitated cancer cell capture. For CTC concentration of 1,000 per ml, they reported capture rates that ranged from 40% under higher flow rate

(0.48 ml h⁻¹) to 80% under lower flow rate (0.12 ml h⁻¹). As a clinical trial using their device, CTCs were detected in 14 among the 15 patients who have prostate cancer at metastatic stage (386 ± 238 CTCs ml⁻¹). Although these devices have been applied for efficient CTC capture and enumeration, most of them can only realize the major function solely for cell capture. However the bound CTCs usually cannot be reversibly released after capture due to the strong interaction between antigen and antibody. For clinical application, there exists an urgent need for harvesting the captured CTCs for further analyses, such as enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), cytometric and electrochemical techniques¹¹⁷, which cannot be easily achieved in situ. These cellular and biomolecular characterizations of CTCs are essential for confirmation and in-depth understanding of the metastasis process, based on which medical diagnosis and treatment can be made to suppress the progression of tumor disease¹¹⁸. For this purpose, several common methodologies as mentioned above show some potential for on-chip cell elution and harvesting. For instance, the dielectrophoretic separation method can purify and elute the captured CTCs by simply adjusting the applied electric field⁵³. However this electrical method usually suffers from many negative effects on the cell physiology due to the imposed membrane stress, Joule heating, and thermal decomposition^{119, 120}. As another example, the immunomagnetic CTC detection method assisted by anti-EpCAM-coated nanoparticles can realize cell elution and harvesting by removing the magnetic field³³. Nonetheless, there is no report on how these conjugated nanoparticles would affect the cell physiology and

subsequent molecular analysis, neither on how to remove the magnetic particles from the cell membrane after elution.

Therefore a dual-functional chip is desired to enable the cell elution successively after capturing CTCs. In this work, for the first time, a dual-functional herringbone chip is developed for Hep3B cell capture and elution. Hep3B is a typical hepatocellular carcinoma cell line and its number density in patients' blood is essential for determining hepatocellular carcinoma metastasis stage. The herringbone chip was fabricated by using PDMS elastomer and the surface of the inner channel wall was modified with anti-EpCAM based on a previous report²⁹. Subsequently, Hep3B cells were spiked into PBS and whole blood, respectively, before being introduced into the microchip by a syringe pump. The capture rate was calculated by using the fluorescent microscopy after immunoreaction. D-biotin was then introduced into the microchannel to displace the site of biotinylated anti-EpCAM conjugation, followed by elution of unbound cells from the device¹²¹. In this study, we found that the capture rate for CTCs spiked into whole blood at density of 2,000 ml⁻¹ and 400 ml⁻¹ could reach 53% for perfusion rate of 1 ml/hour, which is comparable to that obtained in the previous work²⁹. In addition, we also investigated the CTC capture capability of this device under a wide dynamic range of the cell number density in the sample. In particular, we achieved acceptable capture rates for extremely low CTC concentrations of 100 ml⁻¹, 40 ml⁻¹, and 3 ml⁻¹, respectively, which are much more clinically relevant cell densities for early diagnosis of cancer and have not been reported by previous literature. More importantly, we have studied the elution rates under different CTC concentrations. The elution function based on the D-biotin

assisted competitive inhibition is a unique feature of this approach, and there is no previous report of similar study on CTC elution so far. The present CTC chip can achieve over 50% for both capture rate and elution rate, demonstrating its potential applications in clinical assay for cancer diagnostics.

3.2 Experimental section

3.2.1 Design of the herringbone-chip

The chip is composed of one common inlet, one common outlet and eight herringbone structure channels. The height of the herringbone structures is 45 μm ; height of channel space below herringbone structure is 50 μm ; width of chevron pattern is 50 μm . Gaps between the neighbor herringbones is 50 μm . The angle between the herringbone and the channel longitudinal axis is 45° . The herringbone grooves are patterned in alternate permutation to induce chaotic mixing and increase the total surface area to enhance rare cell capture from the sample. The mold was fabricated on a silicon wafer and the chip design is shown in Figure 3-1.

Figure 3-1 Schematic design of the microfluidic chip with three-dimensional geometry of the herringbone channels and their dimensions in horizontal and vertical cross-sections.

3.2.2 Chip fabrication

SU-8 negative photoresist was patterned on the silicon wafer to create a 3D mold including negative herringbone structure and flow channel using photolithography. PDMS microchannels are fabricated by rapid prototyping using the above mentioned mold with elastomer base and elastomer curing agent mixed at ratio of 10:1. The final chip was integrated by bonding the PDMS slice and a 1"×3" glass slide together after plasma treatment.

3.2.3 Channel surface modification with anti-EpCAM

The microchannels were treated with 4% (v/v) 3-MPTS (3-mercaptopropyl trimethoxysilane) in ethanol at room temperature immediately after chip fabrication. The chip was washed with ethanol thoroughly, followed by incubation with 10 mM GMBS (N-γ-maleimidobutyryloxy succinimide ester) for 30 min in ambient environment. The chip was eluted with PBS (pH=7.4) thoroughly and then treated with 10 $\mu\text{g ml}^{-1}$ NeutrAvidin biotin binding protein in PBS for 30 min at room temperature. Subsequently, the chip was incubated with 20 $\mu\text{g ml}^{-1}$ biotinylated anti-human EpCAM (with 1% BSA) for 30 min at room temperature. Then, the chip was washed with PBS thoroughly and ready for use. The procedure on immobilization of anti-EpCAM is shown in Figure 3-2.

Figure 3-2 Procedure of antibody immobilization on PDMS surface.

3.2.4 Hep3B CTC capture test

The setup includes three components: a syringe pump unit, a microchip core unit, and a waste collection unit. The number density of Hep3B cells were counted with a hemocytometer and stained with fluorescent dye CFDA-SE, and spiked into 1 ml of PBS or whole blood from healthy donors. Then, the sample was introduced into the prepared microchip at flow rate of 1 ml h^{-1} precisely controlled by a syringe pump for 1 hour, followed by rinsing with PBS buffer (pH=7.4) at flow rate of 4 ml h^{-1} to remove the unbound Hep3B cells and other blood cells from the chip. The number of captured cells was counted under a fluorescent microscope and capture rate was calculated accordingly. The overall process is illustrated in Figure 3-3.

Figure 3-3 Illustration of CTC capture process.

3.2.5 Hep3B CTC elution test

For elution test, 10 mM of D-biotin was introduced into the microchannels to block the binding sites of EpCAM antibody. After incubation for 30 min, the chip was washed with PBS manually for three times by simply using a pipette to remove the released Hep3B cells. The elution rate is calculated based on the ratio between the total number of the bound cells after and before the elution.

3.2.6 Boundary condition for simulation studies

In physics, Navier-Stokes equations are used to describe the motion of fluid substances. These equations are derived from fluid motion based on Newton's second law ($F=ma$), together with the assumption that the stress in fluid is the sum of a pressure term and a diffusing viscous term.

The solution of Navier-Stokes equations is the velocity field or flow field, which is the description of the fluid velocity at a specific point in time and space. The flow rate and drag force could be found once the velocity field is solved. The solution of Navier-Stokes equation is different from classical mechanics, the solution of which are trajectory of particle or a continuum deflection. Studying velocity field instead of position is more meaningful for fluid. The properties of Navier-Stokes equation are as follows:

Nonlinearity The Navier-Stokes equations are nonlinear partial differential equations in most real cases. Provided one-dimension flow and Stokes flow, the equation can be simplified from nonlinear differential equations to linear equations. Any convective flow including turbulent and laminar flow will result in the nonlinearity of Navier-Stokes due to convective acceleration. Laminar flow can be studied by building model as the conduit of viscous fluid through a tiny converging nozzle.

Turbulence Turbulence is time-dependent chaotic behavior commonly observed in real fluid flow, which is generally due to the inertia of the fluid: the convective acceleration and the culmination of time dependent. Moreover, laminar flow occurs where inertial effects are insignificant. The Reynolds number is used to quantify how

much effects of inertia on flow. It is believed that Navier-Stokes equations describe turbulence properly.

Combining conservation of mass and other boundary condition, Navier-Stokes equations may model fluid motion accurately to agree with real world observation. Navier-Stokes equations assume the fluid as a continuum, not moving at relativistic velocities. The result of real fluids composed of discrete molecules will be different from continuous fluids modeled by Navier-Stokes equation.

Using the rate of stress and rate of strain tensor, it is shown that the components F_j of a viscous force F in a nonrotating frame are given as equation (3.1).

$$\frac{F_j}{v} = \frac{\partial}{\partial x_j} \left[\eta \left(\frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} \right) + \lambda \delta_{ij} \nabla \cdot \vec{u} \right] \quad (3.1)$$

η is dynamic viscosity; λ is the second viscosity coefficient; δ_{ij} is the Kronecker delta; $\nabla \cdot \vec{u}$ is the divergence. For incompressible fluid, the divergence $\nabla \cdot \vec{u} = 0$. Considering η as constant in space, the vector form can be given as equation (3.2), where $\nabla^2 \vec{u}$ is the vector Laplacian.

$$\frac{F_{viscous}}{v} = \eta \nabla^2 \vec{u} \quad (3.2)$$

There are two other forces acting on fluid. One is the pressure force, as equation (3.3), where P is pressure.

$$\frac{F_{pressure}}{v} = -\nabla P \quad (3.3)$$

The other one is the body force, as equation (3.4).

$$\mathbf{F} = \frac{F_{body}}{V} \quad (3.4)$$

The Newton's law indicates that the total change in the momentum per unit volume is $\rho \frac{du}{dt}$; if only the fixed volume element is focused on, we will only obtain $\rho \frac{\partial u}{\partial t}$. In order to obtain the total change of momentum of the fluid element at volume element x , the fluid element should move out of x into the neighboring one at $x+\Delta x$, resulting in fluid mechanics nonlinear. Thus, the neighboring element being occupied by the fluid is fixed by the movement of fluid element itself. Thus, the total change in the fluid's velocity, based on one step differentiation, is given by:

$$\begin{aligned} \frac{du(t, x)}{dt} &= \frac{u(t + \Delta t, x + \Delta x) - u(t, x)}{\Delta t} = \frac{u(t + \Delta t, x + \Delta t) - u(t, x)}{\Delta t} \\ &= \frac{\partial \vec{u}(t, x)}{\partial t} + \vec{u} \cdot \nabla \vec{u}(t, x) \end{aligned}$$

The derivation of equation (3.5) is simply adding the three forces (3.2), (3.3) and (3.4) and equaling them to above Newton's law for fluid.

$$\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u} = -\nabla P + \eta \nabla^2 \vec{u} + \mathbf{F} \quad (3.5)$$

The vector equation of (3.5) is the Navier-Stokes equation.

The Navier-Stokes equation with no body force is shown as equation (3.6).

$$\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u} = -\nabla P + \eta \nabla^2 \vec{u} \quad (3.6)$$

It's essential to understand the meaning of each term,

Inertia section: $\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u}$; Divergence of stress: $= -\nabla P + \eta \nabla^2 \vec{u}$; $\frac{\partial \vec{u}}{\partial t}$ is unsteady acceleration; $\vec{u} \cdot \nabla \vec{u}$ is the convective acceleration; $-\nabla P$ is the pressure gradient; $\eta \nabla^2 \vec{u}$ is the viscosity.

For low Reynolds number, the convective acceleration is smaller than viscous term. Navier-Stokes equation can be simplified as equation (3.7).

$$\rho \frac{\partial \vec{u}}{\partial t} + \nabla P = \eta \nabla^2 \vec{u} \quad (3.7)$$

For low Reynolds number flow at low pressure, Navier-Stokes equation develops into a diffusion equation as equation (3.8).

$$\rho \frac{\partial \vec{u}}{\partial t} = \eta \nabla^2 \vec{u} \quad (3.8)$$

For high Reynolds number, the inertia term is larger than viscous term. Navier-Stokes equation can be simplified as equation (3.9).

$$\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u} = -\nabla P \quad (3.9)$$

In the absence of pressure force, Navier-Stokes equation can be simplified as equation (3.10).

$$\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u} = \eta \nabla^2 \vec{u} \quad (3.10)$$

Only the convective terms are nonlinear for incompressible Newtonian flow. The convective acceleration is resulted from a velocity change over position, such as the increasing speed of fluid entering a converging nozzle. If temperature effects can be neglected, the only other boundary condition required is the mass continuity

equation. Due to incompressible fluid, the continuity equation can be simplified as equation (3.11)

$$\nabla \cdot \vec{u} = 0 \quad (3.11)$$

These equations are commonly used in three coordinated systems: Cartesian, cylindrical and spherical.

For the simulation of flow in herringbone chip, the governing equations and boundary conditions are listed as follows:

Navier-Stokes equation:

$$\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u} = -\nabla P + \eta \nabla^2 \vec{u} \quad (3.12)$$

Continuity equation:

$$\nabla \cdot \vec{u} = 0 \quad (3.13)$$

Boundary conditions:

$$\text{Channel walls: } \vec{u} = 0, \vec{n} \cdot \nabla P = 0 \quad (3.14)$$

$$\text{Inlet: } v = 76 \mu\text{m/s}$$

$$\text{Outlet: } P = 0$$

where $\vec{u}$ is flow velocity; P is the pressure; η is the fluid viscosity; ρ is the fluid density.

3.3 Results and discussion

3.3.1 Chip fabrication and flow simulation studies

Since it is very challenging to directly measure the flow velocity inside the microchip, a finite element based numerical simulation was conducted to estimate and characterize the flow condition by using COMSOL Multiphysics 4.2a (COMSOL, CA, USA).

One typical section of the microchannel with 2 mm long herringbone structure (Figure 3-4a) was simulated for discrete streamlines (Figure 3-4b) and flow velocity (Figure 3-4c). The inflow velocity was set as $76 \mu\text{m s}^{-1}$ (corresponding to the actual perfusion rate of 1 ml h^{-1}). From the simulation results in Figure 3-4b, a very unique flow pattern can be observed that the herringbone structures on the channel ceiling split part of the main channel flow into the grooves periodically, and re-combine them with main flow at the end of the herringbone structure. Thus the cell-laden flow has a much larger interface area with the antibody-functionalized groove wall, where the average flow speed is $17 \mu\text{m s}^{-1}$ compared to that of $118 \mu\text{m s}^{-1}$ in the main channel as shown in Figure 3-4c. The flow inside the herringbone grooves is much slower than the main channel flow, which is highly favorable for the immunological interaction between the CTCs and the antibody-functionalized channel wall. In addition, from the 3-D streamlines in Figure 3-4, the groove flow direction has been deviated by 45° compared to the main channel flow, which effectively increases the total flow distance in the herringbone structure. The combination of these three features, flow splitting for large interface area, slower groove flow, and longer flow

distance, significantly contribute to the enhanced immunoreaction between the cell surface ligand and the immobilized antibody and thus the tumor cell binding in the herringbone structure. Therefore this herringbone chip is inherently much more advantageous than other chip designs with bare straight channels, leading to a much higher capture rate of the CTCs.

However the elution of CTCs is mainly dependent on the diffusion of biotin and the competitive surface reaction between the biotin and the antibody to block the binding sites for CTCs. The above three flow features that enhance cell capture do not contribute to promoting the diffusion of biotin or its surface reaction. Biotin is water-soluble organic compound with molecular weight of about 244, which is much smaller than biological cells such as CTCs. Therefore the diffusion rate of biotin molecules is much greater than that of biological cells, which implies that the limiting factor for enhancing the competitive surface reaction is the surface area. Meanwhile, the herringbone chip is characterized by a large number of microgrooves, which effectively increases the total surface area for the reaction between dissolved biotin and the immobilized antibody on the channel wall. After introducing the biotin as a competitor to active cell binding sites, the captured cells can be rapidly disassociated from the channel wall and the elution process can be simply achieved by washing with PBS. In this respect, the unique herringbone structure also helps the CTC elution, however, due to a totally different mechanism compared with cell capture.

Figure 3-4 Numerical simulation of the liquid flow in a herringbone chip: (a) 3-dimensional view of the channel geometry for computation with profile of herringbone structure; (b) 3-dimensional view of the simulated flow streamline, which occurs in the corresponding domain as shown in (a); (c) 2-dimensional cross-sectional view (in ZOX plane) of the simulated velocity field at three different locations.

3.3.2 Capture and elution study of Hep3B CTCs spiked in PBS

Figure 3-5 Microscopic images of captured CTCs before elution under bright field (a) and dark field (b); the remaining bound CTCs after elution under bright field (c) and dark field (d). (Scale bar: 100 μm)

CFDA-SE is a fluorescent tracer for long-term cell labeling. Green-fluorescence can be observed after cell staining under fluorescence microscope. CFDA-SE diffuses into cells and forms an amine-reactive compound. This product generates an observable fluorescence and binds to intracellular amine sources. This dye is advantageous in that local concentration of 10 μM is sufficient for staining with minimum cell death. After introducing the PBS spiked with 10^4 CTCs into the chip, microscopic images of the captured CTCs under both bright field and dark field were

taken as shown in Figure 3-5(a, b). The number of remaining bound cells after elution can be counted under fluorescent microscope through CFDA-SE staining as shown in Figure 3-5d. It is obvious that the Hep3B cells were successfully captured in the herringbone structure and green fluorescent dots in the dark field represent captured live tumor cells locating at the same position as the ones in the bright field. The capture efficiency and elution rate can be calculated by comparing the number of bound CTCs before and after elution with the total number of spiked CTCs.

The capture rates of CTCs spiked into PBS with different number density are demonstrated in Figure 3-6, which shows an average capture rate of 53.9% for the cell density ranging from 3 to 10^4 per ml. It was also noticed that, for the cell density of 10^5 per ml, the capture rate reduced to only 9.5%, which is due to the saturation of the binding sites between the immobilized antibodies and the tumor cells. This implies that the total number of the tumor cells perfused through the chip significantly exceeded the number of immobilized antibodies and hence depleted all the binding sites for extra cell capture. Nonetheless, 10^5 CTCs per ml is far from the realistic density of clinical samples anyway; while this result can still reveal important information for quantifying the active binding sites of the immobilized antibodies on a particular chip. On the other hand, for the more clinically relevant cell density of 3 and 40 per ml, the effective capture rate can reach 48.8% and 49.6% respectively, which is an acceptable level for isolation of extremely rare cells like CTCs. In addition, the lowest detection limit achieved by this device was 3 CTCs per ml, which is sufficiently sensitive for clinical applications. The measured capture rates are comparable to the previous report²⁹ and the results are consistent for five

repeated tests for each cell density. The results indicated that the herringbone chip coated with anti-EpCAM has significantly enhanced cell capture efficiently based on the interaction between the antibody-coated channel wall and the biomarker expressed on cancer cell surface. It is further proved that the microchannel fabricated based on herringbone structure has 3~4 times higher capture rate than the traditional flat channel²⁹. The overall processing time for 1 ml of sample takes only one hour, which is considerably rapid for clinical applications.

For harvesting the captured CTCs, D-biotin is introduced and will compete with the active reaction sites on NeutrAvidin that are originally bounded with anti-EpCAM. This competitive inhibition will deactivate the capture antibody and result in captured tumor cells being disassociated from the channel wall. The test results showed a high elution rate of more than 50% on average with the sample cell density from 3 to 10^5 per ml. For the more clinically relevant cell number density of 3 and 40 per ml, the elution rates reached up to 61.1% and 80.8%, respectively, which demonstrated a significant effect of D-biotin on antibody blockage. In a control experiment, we also tried cell elution solely by PBS washing without application of D-biotin. It was observed that only using PBS washing could not effectively elute the captured CTCs and the elution rate was as low as 1% for density of 1,000 CTCs ml^{-1} . Therefore it is the biotin-induced cell disassociation, rather than the flow induced shear stress, that enables the captured cells to be eluted from the chip. This excellent feature can be utilized for eluting and harvesting isolated CTCs efficiently from the chip for subsequent cellular and biomolecular analyses on cancer progression.

Figure 3-6 Capture and elution rates of CTCs spiked in PBS in different cell number density.

3.3.3 Capture and elution study of Hep3B CTCs spiked in whole blood

In order to evaluate the device performance on actual human blood, different numbers of CTCs were spiked into fresh whole blood samples from healthy donors and were tested for CTCs capture and elution using the developed chips. Compared to the previous results done with CTCs spiked into PBS, the same device demonstrated a similar CTC capture rate of more than 50% on average with the cell number density from 5 to 2×10^3 per ml of blood, as shown in Figure 3-7. With respect to more clinically relevant Hep3B number density of 5 and 30 per ml of blood, the effective capture rate could reach up to 50% and 83.3%, respectively. For the error bar going

beyond 100%, that is attributed to the slight errors in spiking concentration of tumor cells. It indicated that this herringbone chip was also capable of processing whole blood sample with a high capture rate. Even under the shear stress generated by the flow of 4 ml h⁻¹ PBS rinsing buffer, the interaction between the antibody and Hep3B was still strong enough to ensure the stable binding of the captured CTCs during washing process. On the other hand, the highly complex background in the circulating blood, such as erythrocytes, leukocytes, platelets, and other non-target cells, did not show obvious non-specific binding on the herringbone structure due to the weak expression of EpCAM on their membranes. Therefore, the herringbone structure modified by EpCAM antibody can specifically capture tumor cells without the inference from other cell types. After introducing D-biotin, the captured tumor cells were successfully disassociated from the channel wall with elution rate of more than 50% on average. For the CTC number density of 30 and 5 per ml of blood, the elution rate can be achieved as 54% and 75%, respectively.

Figure 3-7 Capture and elution rates of CTCs spiked in whole blood in different cell number density. Due to the human errors in spiking CTC concentration, the actual spiked cell number could be higher than the nominal value, which would lead to a capture/elution rate above 100% as shown in some of the error bars.

3.4 Conclusions

A dual-functional herringbone microfluidic chip was fabricated and utilized for capture and elution of Hep3B CTCs from PBS and whole blood samples. DSB-X biotinylated anti-EpCAM was covalently coated on the internal channel of this microdevice for CTCs capture. For elution process, D-biotin was introduced to block the antibody reaction site based on higher affinity of D-biotin binding to NeutrAvidin.

The number of the captured cells and eluted cells were counted under fluorescent microscope. The capture rate for Hep3B spiked in PBS and whole blood tested on this chip is comparable to the previous report. Moreover, in a detailed study on CTC harvesting, the captured Hep3B can be successfully eluted from the chip with an outstanding elution rate. More specifically, capture and elution rate of Hep3B was studied for a wide dynamic range of the CTC number density in the sample. The presented dual-functional device showed ultrahigh sensitivity and specificity to capture and elute Hep3B CTCs with excellent reproducibility and stability, indicating its enabling capability for applications in cancer diagnostics.

3.5 Declaration

Part of the work presented in this chapter has been published in the journal of *Microfluidics and Nanofluidics* (Copyright 2014 Springer).

Peng Xue, Kai Ye, Jie Gao, Yafeng Wu, Jinhong Guo, Kam M. Hui*, Yuejun Kang*.

"Isolation and Elution of Hep3B Circulating Tumor Cells Using a Dual-functional Herringbone Chip". *Microfluidics and Nanofluidics*, 16, 605-612 (2014).

Chapter 4 Hep3B CTC detection using microchip with herringbone and micropost arrays

4.1 Introduction

Circulating tumor cells (CTCs) are a source of potential cells derived from primary tumor and work as the precursors for formation of secondary tumors during metastasis. However, the isolation and enumeration of CTCs is still highly challenging, attributed to the extremely low concentration of CTC in the whole blood of patients with metastatic disease (1 to 10 CTCs per mL)²⁷. Thus, highly sensitive detection method is critical to precisely enumerate the CTCs in vitro. Currently, microfluidic devices have the advantages of high throughput, fast processing, low cost, tiny sample volume and the capability of multiplex detection, which provides the opportunity for rapid detection and characterization of CTCs for point-of-care applications.

In 2007, a microfluidic device integrated with antibody-coated micropost structure was developed for efficient and selective separation of CTCs from blood samples under precisely controlled laminar flow conditions²⁸. The capture rate reached up to 65% at the spiked concentration of 100 CTCs mL⁻¹ using non-small-cell-lung cancer (NSCLC) cells. Afterwards, an enhanced microfluidic mixing device was developed and integrated with a unique herringbone structure, which increased the immunoreaction between the chip inner surface and the target CTCs^{29, 122}. The reported capture rates were increased up to ~80% at spiked concentration of 1000 CTCs mL⁻¹ using PC3 prostate cells. In a clinical study using this device, CTCs were

captured in 14 of 15 patients who suffered from prostate cancer (median concentration = 63 CTCs ml⁻¹). Although these devices can realize the basic function of CTC isolation and enumeration, there still exists great potential to improve the performance of device with higher capture efficiency for clinical applications. Moreover, the capture rates of CTCs with various concentrations were not sufficiently demonstrated in previous studies. Most importantly, the majority of the existing devices can only realize the function of CTC capture without being able to reversibly release them from the device due to the strong antigen-antibody interaction. For practical clinical applications, there exists an urgent demand to harvest the captured CTCs for further cellular and biomolecular analyses, including quantitative PCR (qPCR), enzyme-linked immunosorbent assay (ELISA), and electrochemical detections et al., which cannot be simply achieved in situ¹¹⁷. These downstream analyses are critical for the evaluation of metastasis process and elucidating treatment strategies to suppress the tumor progression¹¹⁸.

Hep3B is a typical hepatocellular carcinoma cell line, in which the expression of epithelial cell adhesion molecule (EpCAM) was upregulated¹²³. The number density of Hep3B in circulating blood serves as the indicator to determine hepatocellular carcinoma metastasis stage. To further increase the capture efficiency of CTCs based on the platform introduced in Chapter 3, a dual-functional chip integrated with both herringbone structure and micropost array was developed for Hep3B cell capture and elution in this work. The interior channel surface of this PDMS-based microchip was covalently functionalized with anti-EpCAM²⁹. Hep3B cells were spiked into PBS before being infused into the chip using a digital syringe

pump. The CTC capture rate was calculated after the examination under fluorescent microscope. Afterwards, D-biotin was introduced into the microchip to displace the active site of DSB-X biotinylated anti-EpCAM conjugation, followed by eluting unbound CTCs out of the device¹²¹. The simulation results demonstrate that the advantages of herringbone structure and micropost array are mutually complementary in generation of amenable flow behavior for CTC isolation. The outstanding CTC capture and eluting capacity of this device was also verified under a wide range of cell number density in the PBS sample, indicating its great potential in clinical applications for cancer diagnostics and therapeutics.

4.2 Experimental section

4.2.1 Materials

3-mercaptopropyl trimethoxysilane (3-MPS), N-γ-maleimidobutyryloxy succinimide ester (GMBS), bovine serum albumin (BSA) was purchased from Sigma-Aldrich, Singapore. Neutravidin biotin binding protein was provided by Fisher Scientific, Singapore. Anti-human EpCAM (CD326) was purchased from Biomed Diagnostics, Singapore. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, D-Biotin, CFDA SE cell tracer kit and 1×PBS (pH=7.4) was obtained from Life Technologies, Singapore. Herringbone microchannel mold was provided by Bonda Technology Pte. Ltd., Singapore. Polydimethylsiloxane (PDMS) SYLGARD 184 silicone elastomer kit was purchased from Dow Corning Inc., USA. Photoresist SU-8 25 and SU-8 developer were obtained from Microchem, USA.

4.2.2 Chip design

The chip consists of one inlet, one outlet, and eight herringbone channels combined with micropost structure. The height of herringbone and micropost structures is $45\ \mu\text{m}$ and $30\ \mu\text{m}$, respectively. The width of chevron pattern is $50\ \mu\text{m}$. Gap between the neighbor herringbones is $50\ \mu\text{m}$. The interval between the centers of microposts is $150\ \mu\text{m}$. The angle between channel longitudinal axis and herringbone structure is 45° . The herringbone grooves with alternate permutation and micropost array are designed to induce chaotic flow for enhanced micromixing and to increase the total effective surface area for CTC capture. The 3-dimensional (3D) geometry and dimensions of the chip is illustrated in Figure 4-1(c).

Figure 4-1 (a) The final chip composed of eight microfluidic channels with common inlet and outlet. Food dye (red color) was used to demonstrate the flow conduits in the chip. (b) 3D structure of the device and the illustration of cell-surface interactions. (c) The top view and cross-sectional view of the herringbone grooves and micropost array.

4.2.3 Chip fabrication

The chip was assembled by stacking two layers of PDMS together. Briefly, a 3D micropost mold was fabricated by patterning SU-8 negative photoresist on a silicon wafer using photolithography. PDMS layers were fabricated by rapid prototyping using micropost mold and herringbone mold, respectively. Briefly, the PDMS elastomer base and curing agent mixed at the ratio of 10:1 were cast on respective molds and incubated in the oven at 70°C for 2 h. After plasma treatment, herringbone layer were precisely aligned and bonded with micropost layer.

4.2.4 Channel surface modification with antibody

To chemically modify the channel interior surfaces, the microfluidic channels were incubated with 4% (v/v) solution of 3-MPTS (3-mercaptopropyl trimethoxysilane) in ethanol at room temperature for 1 h. Afterwards, the chip was washed with ethanol thrice, followed by treatment with 100 μM GMBS (N-y-maleimidobutyryloxy succinimide ester) for 30 min at room temperature. After being washed with PBS, the microchannel was treated with 10 $\mu\text{g ml}^{-1}$ NeutrAvidin solution in PBS for 1 h at room temperature. Subsequently, the chip was filled with 20 $\mu\text{g ml}^{-1}$ DSB-X biotinylated anti-EpCAM solution containing 1% (w/v) BSA (bovine serum albumin) and incubated for 1 h at room temperature. Subsequently, the chip was washed with PBS thoroughly to remove the unbounded antibody. Then, the device was ready for use. The schematics on antibody immobilization are shown in Figure 4-2.

Figure 4-2 Schematics of antibody immobilization on PDMS surface.

4.2.5 Cell culture

Hep3B tumor cells were successively cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and a penicillin (100 U ml^{-1})/streptomycin ($100 \mu\text{g ml}^{-1}$) mixture. Culture flask seeded with cells was incubated in humidified atmosphere with 5% CO_2 at 37°C . The nonadherent cells were washed away after 48 h, and the adhered Hep3B cells were further expanded until reaching confluence. Afterwards, cells with good purity were removed from the flask using trypsin and are ready for the following test.

4.2.6 Flow capture of Hep3B cells

As shown in Figure 4-3, the platform comprises three components: a digital pump system (Legato 180, KD Scientific, MA, USA), a microchip core unit, and a waste disposal unit. Briefly, Hep3B cells with specific density were counted using a hemocytometer, followed by staining with fluorescent dye CFDA SE. Afterwards, the sample was prepared by spiking tumor cells into 1 mL 1×PBS. Subsequently, a syringe (BD syringe, US) loaded with sample was assembled on to the digital pump and the sample was pumped into the chip at the flow rate of 1 ml h⁻¹, 1.5 ml h⁻¹ and 2 ml h⁻¹, respectively, followed by rinsing with 1×PBS at flow rate of 4 ml h⁻¹ for 15 min to remove unbound Hep3B cells from the chip. The number of captured cells was counted under a fluorescence microscope (IX71, Olympus, Singapore). Capture efficiency was calculated based on the number of tumor cells retained in the device relative to the total number of spiked cells flowing through the chip.

Figure 4-3 Experimental procedure on CTC capture.

4.2.7 Elution of Hep3B cells

After cell capture and enumeration, 10 mM of D-biotin was introduced into the chip to dissociate the cells from the surface of microchannel. After incubation for 30 min, the chip was washed with PBS thrice manually to remove the dissociated Hep3B cells. The cell elution rate is evaluated based on the ratio between the number of bound cells after and before the elution.

4.3 Results and discussion

4.3.1 Numerical studies of fluid flow in microchannel

To elucidate the fluid flow behavior in the microfluidic channel, a finite element-based numerical simulation was performed to characterize the flow velocity using COMSOL Multiphysics 4.3a (COMSOL, Inc., CA, USA). The numerical model and boundary conditions for the computation were defined as follows.

Since the flow is pressure-driven only, the steady-state Navier-Stokes equation for incompressible flow is given as:

Navier-Stokes equation:

$$\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u} = -\nabla P + \eta \nabla^2 \vec{u} \quad (4.1)$$

Continuity equation:

$$\nabla \cdot \vec{u} = 0 \quad (4.2)$$

Boundary conditions:

$$\text{Channel walls:} \quad \vec{u} = 0, \vec{n} \cdot \nabla P = 0 \quad (4.3)$$

$$\text{Inlet:} \quad v = 76 \mu\text{m/s}$$

$$\text{Outlet:} \quad P = 0$$

where $\vec{u}$ is flow velocity; P is the pressure; η is the fluid viscosity; ρ is the fluid density.

The proof-of-principle simulation studies were based on a smaller version of the chip (2-mm-long) as shown in Figure 4-4a. The inflow velocity was set as $76 \mu\text{m s}^{-1}$ (corresponding to perfusion rate of 1 ml h^{-1}). From the simulation results of streamline (Figure 4-4b), a unique flow pattern deviated from the main stream direction is generated in the herringbone grooves. More specifically, the main flow was split into subordinate flows constrained within the grooves periodically, which were recombined with the main flow at the end of herringbone grooves. The cell-laden sample flow thereby has increased contact area with the antibody-functionalized groove wall of the herringbone structure, where the average flow rate is simulated as $18 \mu\text{m s}^{-1}$ compared to that of $111 \mu\text{m s}^{-1}$ in the main channel (Figure 4-4c). Moreover, the micropost structure also contributes to the increased inner surface area of this device. The flow inside the herringbone structure and near micropost is much slower than that in the main channel, which facilitates the immunological interaction between tumor cells and antibody-functionalized channel surface. Furthermore, considering the 3D streamline as shown in Fig. 4-4b, the flow direction in the groove has been deviated by 45° compared to that in the main channel, which apparently increases the relative flow distance in the herringbone structure.

Finally, the simulation results imply that the cells flowing near the top surface of microposts have higher chance to be drawn into herringbone groove (Figure 4-4c). The synergistic effects of increased interface area, slower flow in groove and near micropost, longer flow distance and greater chances of cells to be drawn into herringbone grooves contributes to higher binding efficiency of tumor cells on the surface of microchannel.

Figure 4-4 Numerical simulation of the fluid flow on the chip: (a) 3D view of the channel geometry for computation; (b) 3D view of the simulated flow streamline corresponding to the flow domain in (a); (c) stacked 2D cross-sectional view (in ZOY and YOZ planes) of the simulated velocity field at different positions.

4.3.2 Characterization of Hep3B capture and elution

Figure 4-5 Microscopic images of captured CTCs under bright field (a) and dark field (b); the remaining bound CTCs after elution process under bright field (c) and dark field (d).

The cell number was counted based on complementary microscopic examinations in both bright field and dark field. Green fluorescence was observed from the cells stained with CFDA SE, which is a typical fluorescent tracer for long term cell labeling. CFDA SE diffuses through plasma membrane and forms well-retained fluorescent conjugates. More importantly, this dye is non-toxic and allows

for bright and uniform cell staining. After running the sample loaded with 10^4 CTCs ml^{-1} at flow rate of 1 ml h^{-1} , microscopic images of the isolated CTCs under both bright and dark field were recorded as shown in Figure 4-5(a, b). It demonstrated that Hep3B cells were successfully captured on the microchannel wall, where the green dots in the dark field represented live tumor cells corresponding to the ones at the same position in the bright field. Fluorescence image of the remaining bound cells after elution was exhibited in Figure 4-5d. It was obvious that the majority of cells were dissociated from the channel wall and eluted from the device.

Figure 4-6 (a) Capture rates of CTCs spiked in PBS with different cell number density at the inflow rate of 1.0 ml h^{-1} , 1.5 ml h^{-1} and 2.0 ml h^{-1} , respectively. Data were shown as means $\pm$ SD ($n = 4$, $*p < 0.05$). (b) Mean and median elution rate of different bound cell number displayed as Box and Whiskers plots ($n=10$). The means are represented by +.

The capture rates of CTCs with specific number density under variable flow rates are shown in Figure 4-6. The flow rate plays an important role in determining the cell capture efficiency on the CTC-chip since it influences the duration of cell-

surface contact. At flow rate of 1 ml h^{-1} , the capture rate of CTCs was $\sim 70\%$ for the cell density ranging from 10 to 10^4 ml^{-1} , indicating a broad dynamic range of sample capacity. The results also implied that the active binding sites of immobilized antibodies were sufficient to capture the CTCs with number density up to 10^4 ml^{-1} , which was much higher than the regular density of clinical samples. Additionally, for lower ranges of cells density of 100, 50 and 10 ml^{-1} that are more clinically relevant, the effective capture rate reached up to 71.3%, 77.9% and 80%, respectively, which is an acceptable level for CTC isolation compared with previous reports^{28, 29}. The limit of detection achieved in this device was 10 CTCs per ml, which is a promising sensitivity for clinical applications. These results suggest that the anti-EpCAM-coated microchannel effectively promote the capture of CTCs based on the immunoreactions between the antibody and the biomarker expressed on cancer cell membrane. It was also proved that Hep3B CTC capture rates obtained using this device is $\sim 10\%$ higher than similar chip with herringbone structure only^{29, 122}. The typical processing time for 1 ml of sample took only 1 h, which is considerably rapid for actual clinical applications. However, as the flow rate was increased to 1.5 ml h^{-1} and 2 ml h^{-1} , the average capture efficiency reduced to $\sim 60\%$ and 40% respectively, indicating that the cells could not effectively bind to the channel wall compared to the condition at lower flow rate of 1 ml h^{-1} . More specifically, the time of cell-surface interaction was shortened under higher flow rate and the resultant shear force was too strong to maintain maximum cell attachment on the channel surface.

For harvesting of the captured CTCs, D-biotin was introduced to disassociate the cells from the channel wall based on competitively occupying the

immunoreaction sites on NeutrAvidin, which were originally bounded with anti-EpCAM. The results demonstrated a high elution rate of more than 55% with the captured cell number from 10^2 to 10^4 , as shown in Figure 4-6b. For more clinically relevant cell quantity from 1 to 100, the average elution rate can reach up to 69.4%, indicating a predominant effect of D-biotin on cell disassociation. Based on this unique feature, isolated CTCs can be simply eluted from the chip for further cellular and biomolecular analyses. Of course, the capture rate of CTCs could be further improved by adjusting the geometry of microchannel. For example, design parameters such as the channel height and the density of micropost could affect the cell capture efficiency and therefore need to be further investigated.

4.4 Conclusions

A dual functional chip integrated with herringbone structure and micropost array was designed and fabricated for capture and elution of Hep3B CTCs in PBS suspensions. DSB-X biotinylated anti-EpCAM was covalently conjugated on the microfluidic channel wall for CTC capture. The elution process was achieved by introducing D-biotin to block the antibody reaction site and dissociate the cells from microchannel. The capture rate for Hep3B tumor cells obtained using this device was improved notably compared to previous reports. Moreover, the captured Hep3B cells could be successfully harvested with a good elution rate for subsequent cellular and biomolecular analyses. This dual-functional device exhibits excellent stability and reproducibility, implying its promising application in cancer diagnostics and therapeutics.

4.5 Declaration

Part of the work presented in this chapter has been published in the journal of *Biomedical Microdevices* (Copyright 2015 Springer).

Peng Xue, Yafeng Wu, Jinhong Guo, Yuejun Kang*. Highly efficient capture and elution of circulating tumor cells by using a dual functional chip integrated with herringbone structure and micropost array. *Biomedical Microdevices*, 17, 39 (2015).

Chapter 5 Drug-eluting microneedles for keloid treatment

5.1 Introduction

Keloids are proliferative fibrous growths that result from excessive scarring response to skin trauma. They frequently persist at the site of injury, often recurring after excision and overgrow the boundaries of the original wound. Despite having no malignant potential, they cause itch, pain, restricted movement, cosmetic disfigurement and considerable adverse psychological effects. To control keloid activity, intralesional injections of corticosteroids and chemotherapeutic agents, surgical excision, cryotherapy, occlusive therapy, laser therapy, and radiotherapy have been used^{124, 125}. However, none of these methods can consistently achieve durable remission and patients are required to make repeated visits to the clinic, which not only increases the cost of medical treatment but also reduces the patient compliance¹²⁶. While intralesional injection of anti-metabolite compounds (5-fluorouracil, bleomycin etc.) have been shown to suppress the proliferation of keloid fibroblasts and lead to durable remission^{127,128}, administration of the drug is painful and is required to be performed in the clinic repeatedly. It is therefore highly desirable for the patients if there is an option to self-manage keloid lesions in a simple and cost-effective fashion. Such a treatment will reduce the economic burden to the healthcare system and can provide a means of treatment for patients who have limited access to standard treatments.

Over the past decade, microneedle transdermal delivery system has been shown to dramatically increase skin permeability to a broad range of compounds, such as

small molecular-weight drugs, oligonucleotides and proteins, by creating reversible microchannels in the skin¹²⁹⁻¹³¹. Compared to the conventional needles that are in millimeter or even larger dimensions, microneedles are generally shorter than one millimeter, thus are able to penetrate stratum corneum without contact with the nerves residing in the dermis. The decreased likelihood of causing pain, infection, or injury makes microneedles a great platform for self-administration of drugs¹³². In addition, microneedles can be fabricated in a scalable manner by adopting the microelectronics industry-based processes, thereby increasing their availability as well as affordability. Of note, most of the existing microneedle-based transdermal systems use microneedles for effective skin penetration through the formation of an array of conduits for fast delivery of drugs in a liquid medium. However, these methods are characterized by burst-release kinetics occurring immediately after skin penetration and are disadvantageous if controlled or sustained release is desired, such as in the treatment of keloids.

To address these challenges and inspired by the enabling microneedle platforms, we herein propose a microneedle device which could offer a simple, convenient, and cost-effective option for the self-management of keloids (Figure 5-1). As a proof-of-concept, we have designed and fabricated a microneedle patch pre-loaded with the anti-metabolite drug, 5-fluorouracil (5-FU), to prevent the proliferation of keloid fibroblasts (which are crucial for keloid formation). The microneedle array was fabricated on a flexible substrate and thereby will be able to conform to the curvy surface of the lesions. The drug delivery mechanism of this device is different from above-mentioned microneedle-based transdermal drug conveying systems. The

microneedles in this device are made of crosslinked poly(ethylene glycol) diacrylate (PEGDA) preloaded with 5-FU. There is no hollow structure in each microneedle dedicated as fluid conduit; instead, every monolithic microneedle encapsulates drug in solid state. After penetrating the stratum corneum, polymeric microneedles swell in the moist environment, opening the porous polymer network for the drug to diffuse into the skin tissue. This passive drug delivery modality demonstrates much more sustained release kinetics compared to those with direct trans-membrane diffusion in liquid. In addition, the microneedle patch can be easily peeled off from the skin after the desired amount of drug is released; the PEGDA will be removed such that it will not cause extra metabolic load for the patient's body to absorb and excrete this residual substrate material.

A few recent reports have shown evidence for the transdermal delivery of proteins, macromolecules, and organic dyes using similar conduit-free microneedle platforms^{133, 134}. However, there were no specific clinical problems being addressed in detail. Herein with this platform, the proliferation of keloid fibroblasts was completely inhibited *in vitro*. Furthermore, we found that periodic administration (at 12-hour intervals) of 5-FU loaded microneedles resulted in more than 3-fold decrease in fibroblast proliferation when compared to soluble 5-FU delivery. This indicates that cyclic rather than constant delivery of 5-FU through microneedles is more effective in inhibiting keloid fibroblast. Such a regimen of administration will also enhance compliance, as the microneedles can be put on only over bedtime and is not required in the day.

Figure 5-1 Schematic illustration of keloid treatment using drug-loaded microneedle patch.

5.2 Experimental Section

5.2.1 Materials

Poly(ethylene glycol) diacrylate (PEGDA, $M_n=250$), 2-hydroxy-2-methyl-propiophenone(HMP), NaAc and 5-FU were obtained from Sigma-Aldrich, Singapore. HAc and ethanol were purchased from Merckmillipore (Darmstadt,

Germany). All chemicals used were analytical grade and were used as received. Glass slides (1inch×3inch×1mm) and coverslips (22mm×22mm×0.19mm) were purchased from Leica Microsystems Pte Ltd, Singapore and Menzel Glaser (Braunschweig, Germany) respectively. Dialysis tubing cellulose membrane (D9777-100FT, MW=12313) was purchased from Sigma, Singapore. Phosphate buffer solution (PBS, 10mM, pH=7.4) and intercalating fluorescence dyes (PicoGreen®) was obtained from Life Technologies Pte Ltd, Singapore. Deionized water was collected from Millipore Sysnthesis A10 (Molsheim, France). Mask was printed from CAD/Art services, Inc (Bandon, USA).

5.2.2 Apparatus

UV irradiation was performed using OAI UV light source Model 30 (wavelength=360nm) obtained from OAI (San Jose, USA). The intensity of UV source was determined by OAI handheld UV intensity/power meter Model 308. HPLC measurement was conducted with Agilent HPLC-UV operating system (Agilent, Singapore). A SB-AQ chromatographic column (4.6×150 mm, 5 micron, Agilent, Singapore) was obtained as accessory for HPLC-UV operating system. Heating process is performed on EchoTherm Digital hotplate HP 40 series (Carlsbad, USA). The morphology of the microneedles was analyzed with a Hitachi 3500N scanning electron microscope (Hitachi, Japan). The mechanical compression test of microneedles was studied by using Instron 5565 Universal Testing Machine with 1KN loadcell. The elastic modulus and hardness of microneedles were performed on MTS Nanoindenter XP (Oak Ridge, US). Fluorimeter (Infinite® 200, Tecan) was used for measuring the fluorescence in cell proliferation assay.

5.2.3 Fabrication of the microneedle device

The microneedle device was fabricated with poly(ethylene glycol) diacrylate (PEGDA) through photopolymerization in two steps. The first step was for the fabrication of the substrate. Briefly, one glass slide (22mm×22mm) was used as substrate to support two untreated coverslips to form a gap. Another coverslip was carefully aligned on this gap to create a narrow cavity. Prepolymer solution, prepared by PEGDA, 0.5% w/w photoinitiator HMP (2-hydroxy-2-methyl-propiophenone), and 5-FU at certain concentrations (i.e. 0%, 0.1%, 0.3% or 0.5% w/w), was carefully introduced and spread into the cavity by capillary force. Then, the set-up was placed under UV light source, followed by a high intensity UV irradiation (wavelength = 360 nm, intensity = 17.0 mW cm⁻²) for 4 seconds. Subsequently, the supporting substrate was formed by photopolymerization and carefully removed from glass slide and dried in an oven.

After the substrate was ready, the second step was taken for the fabrication of microneedles. The set-up for microneedles fabrication was similar to supporting substrate except for the height of cavity and the different UV exposure time. Glass slides (3 inch × 1 inch) with thickness of 1 mm were placed on either side of another glass substrate (3 inch × 1 inch) to increase the cavity height. Then, the substrate was reversely aligned on the cavity, followed by introducing prepolymer solution into the cavity through capillary action. A patterned film photomask was designed in two dimensions using AutoCAD2012. The photomask bearing the patterns of microneedle array were precisely placed on top of the flexible substrate. Then, the set-up was irradiated under UV source (wavelength = 360 nm, intensity = 17.0 mW cm⁻²) for 10

seconds, which made the microneedles stiff for skin penetration. The microneedle array was washed by deionized water to remove uncrosslinked prepolymer and carefully peeled off from glass.

For the size of a single needle, our current facility allows for fabrication of microneedles with base diameter greater than 10 μm . The height of each needle is limited by the base diameter as microneedles with high aspect ratio (height/base diameter) could be subject to buckling and fracture under the required load for skin penetration. Therefore the base diameter was designed between 200 and 400 μm in order to fit the desired height of the microneedle ($\sim 1\text{ mm}$) to achieve a low aspect ratio about 2~5. On the other hand, the sharpness of the needle tip also depends on the base diameter during the masked photopolymerization process, in which the tips become sharper when the base diameter decreases. These results imply that the needles with larger base diameter have larger tips and average diameter, which may impede full needle insertion after penetration.

The packing density of the needle per unit area is a critical parameter that determines the rate of drug delivery per unit skin area, which implies that higher amount of drug can be delivered under higher packing density. On the other hand, the maximum packing density is limited by the base diameter of each needle, since per unit area on the patch can accommodate less number of needles that have greater base diameter. Therefore there is no technical lower limit for the packing density, while the max density depends on the needle base diameter. In this study, the needle density can be controlled at 30~300 cm^{-2} considering the rate of drug delivery and the needle size.

5.2.4 Mechanical test

Mechanical compression tests were conducted by using an Instron 5565 universal testing machine. A microneedle device (base diameter = 300 μm) was placed on the rigid steel base plate. A compressive force in the longitudinal direction of the microneedles was generated by a moving transducer at a speed of 0.4 mm min⁻¹. The initial distance from the cell load to the tips of microneedles was set at 0.5 cm. The force was recorded once the cell load touched the top surface of the microneedle tips. Four devices made from different batches were tested. A nanoindenter XP was used to determine the hardness and elastic modulus of the microneedle devices with 0%, 0.1%, 0.3%, or 0.5% w/w 5-FU loading. On each sample, three independent locations were randomly selected and tested using the built-in microscope of the nanoindenter XP. Each location is at least 300 μm away from each other to avoid any bias between measurements. Based on the indentation depth of 20 μm , the maximum load applied, and the slope of unloading curve at the turning point, hardness and elastic modulus were calculated.

5.2.5 Histological test

Porcine skin tissues were fixed in 10% formalin and embedded in Tissue Freezing medium (Leica Biosystems, Singapore) at -20°C. Sagittally oriented cryo-sections were made at 8 μm intervals on Leica CM1850 cryotome (Leica Biosystems, Singapore). Hematoxylin-Eosin (H&E) staining method was used to examine the general morphologic features under standard light microscopy. Four devices made from different batches were tested.

5.2.6 In vitro drug release

The experiments were performed by submerging the drug-loaded microneedle device or the dialysis bag in phosphate buffered saline solution (1×PBS, pH=7.4) at 37°C^{135, 136}. To mimic the real human internal environment, the microneedles patch sample (base diameter = 300µm) was immersed in a vial containing 25 ml of 10 mM PBS (pH=7.4) as release medium, and incubated at 37°C under stirring for 160 h. Additionally, 1 mL of 100 µg ml⁻¹ 5-FU stock solution was encapsulated in a dialysis bag as control experiment for drug release. 2 ml release medium was withdrawn from the vial for analysis and an equal volume of fresh PBS was added to replenish the release system at designated time moments. The released 5-FU from the microneedle device or from the dialysis bag was quantified through an Agilent 1100 series high performance liquid chromatography (HPLC, Agilent) fitted with a reversed phase column. The operation conditions of HPLC test are as follows: mobile phase (50 mM NaAc, pH = 4.6); flow rate (1 ml min⁻¹); UV detector (wavelength = 260 nm); temperature (25°C); sample injection volume (10 µl); time per run (8 min).

5.2.7 Cell Culture

Normal dermal fibroblast (NFS06) and keloid fibroblasts (KF12) were obtained courtesy of Prof. Toan Thang Phan, National University of Singapore. Both types of cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS, Merck-Millipore), 100U/ml penicillin G and 100µg/ml streptomycin (Invitrogen). The cells were maintained at 37 °C in a humidified environment containing 5% CO₂ and cultured to 80% confluence before passaging.

5.2.8 Cell proliferation assay

For proliferation studies, 5×10^3 cells cm^{-2} (1×10^4 per well) were pre-seeded 24 hours prior to commencement of the respective treatments (e.g. soluble 5-FU ($100 \mu\text{g ml}^{-1}$), blank microneedles, drug-loaded microneedle (0.1% w/w)) on 24-well plates. The proliferation rates of both normal and keloid fibroblasts were measured by estimating the total cell numbers. Quantification of DNA, was previously reported to be a reliable and high throughput means to quantify cell numbers^{137, 138}. This was applied by digesting cell pellets obtained at the respective time points using proteinase K ($50 \mu\text{g ml}^{-1}$, Sigma-Aldrich) dissolved in a buffer containing 100 mM dibasic potassium phosphate (pH 8.0) overnight at 37°C . To end enzyme activity, the samples were heated at 90°C and snap frozen (-20°C). The DNA solution was mixed with intercalating fluorescence dyes (PicoGreen®) and measured using a fluorimeter. A standard curve was carried out using known cell numbers and DNA weight to ensure that the fluorescence readings varied in a linear manner with DNA (cell number) concentration. Obtaining relative fluorescence units facilitated the fold expansion at the indicated time points.

5.2.9 Statistic analysis

Statistical analysis was carried out using one-way and two-way analysis of variance (ANOVA) using SigmaStat 3.5 software (Scientific solutions SA, Pully-Lausanne, Switzerland).

5.3 Results

5.3.1 Fabrication and characterizations

The microneedle device encapsulating 5-FU was fabricated with photoactive and biocompatible poly(ethylene glycol) diacrylate (PEGDA) through ultraviolet (UV) photopolymerization. The fabrication procedure is illustrated in Figure 5-2a and the details are described in the following “methods” section. The final device is a transparent and flexible patch that can be easily folded without damaging the microneedle arrays (Figure 5-2b). Considering that keloids often develop in ‘curved’ areas of the body, such as shoulders, upper back, chest, ears and the jawlines, which usually have relatively small radii of curvature (from a few to tens of centimeters), the flexibility in the microneedle patch will allow it to be applied to any potential lesion site. When examined with a scanning electronic microscope (SEM), all microneedles had a conical shape and were uniformly distributed on the substrate. Figure 5-2c shows an image of a representative device with microneedle density of 49 cm^{-2} , base diameter of $300 \pm 5\text{ }\mu\text{m}$, and height of $940 \pm 12\text{ }\mu\text{m}$. The microneedle packing density could be adjusted up to 300 cm^{-2} through the design of a photomask. The base diameter of each microneedle can also be tuned in between 200 and 400 μm . (Figure 5-3)

Figure 5-2 Fabrication and characterization of the microneedle device: (a) Fabrication procedure of the 5-FU loaded microneedles; (b) Image of a microneedle patch with a radius of curvature of 3.82 cm; (c) A scanning electron microscopy image of the microneedles containing 0.1% (w/w) 5-FU; (d) Mechanical compression test for the microneedles containing 0.1% (w/w) 5-FU; (e) Elastic modulus and hardness of the microneedles with 0%, 0.1%, 0.3%, and 0.5% (w/w) 5-FU loading. Statistical analysis using One-way ANOVA shows no significant

difference ($p>0.05$) in microneedle elastic properties within these 4 groups loaded with different drug concentrations (up to 0.5% w/w). Values are presented as Mean $\pm$ Standard Deviation, $n=3$.

A mechanical compression test was carried out to examine the force required to cause mechanical fracture of the microneedles using a universal test machine. When the microneedles started to fracture, there was a sudden force decline. As shown in Figure 5-2d, the microneedles fractured (indicated by an arrow) when the compression force reached 0.25 N per needle, which was 5 times greater than the force required for skin penetration (0.05 N per needle, Figure 5-4). This implies that this microneedle device is sufficiently strong to penetrate the epidermis of the skin.

Figure 5-3 Scanning electron microscope images of the microneedle devices with the same density (49 cm^{-2}), height ($920 \mu\text{m}$) and 5-FU loading (0.1% w/w) but different base diameters of each microneedle: (a) 200 (b) 300 (c) 400 μm . (Scale bar: 1mm)

Hardness (the resistance of a material to penetration and/or plastic deformation) and elastic modulus (the relative stiffness of a material against elastic deformation during penetration) represent the mechanical strength of the microneedle material used for this transdermal drug delivery device. As shown in Figure 5-2e, the microneedles had hardness of $45 \pm 11 \text{ MPa}$ and elastic modulus of $0.66 \pm 0.16 \text{ GPa}$, which were both much higher than the reported strength of the skin (hardness: $2.0 \pm$

0.5 MPa; elastic modulus: 90 ± 31 MPa)¹³⁹. It is also noted that the increased loading of 5-FU (up to 0.5% w/w) did not yield significant change in the hardness and elastic modulus of the microneedle device ($p > 0.05$, One-way ANOVA).

Figure 5-4 Porcine skin penetration efficiency of the microneedle device under a series of loading conditions.

5.3.2 Skin penetration with the microneedle device

A piece of fresh porcine skin sample was used in demonstrating the capability of the microneedle device in penetrating actual skin tissue. Porcine skin was selected due to its anatomical and physiological similarities to the human skin¹⁴⁰. The results (Figure 5-5a) showed that 100% of the microneedles on the same patch could penetrate the porcine skin with insertion force as low as 0.05 N per microneedle. A subsequent histological assay of the tested porcine skin verified that the microneedles had penetrated through the epidermis (Figure 5-5b). We further studied six

microneedle devices after 30 minutes of application to the porcine skin, and 100% of the needles were found to be intact (with no fracture).

With the knowledge that the device was able to penetrate the skin and, at the same time, bend flexibly without fracture, we tested its application onto the skin of the flexed elbow (Figure 5-5c). An adhesive tape was applied to secure the microneedle patch in place in the test. The device was shown to be able to conform to the curvature and is therefore anticipated to be applicable to various curved anatomical locations of the body.

Figure 5-5 Application of the microneedle device on skin tissues. (a) A piece of fresh porcine skin after being treated with the microneedle patch (specification of each microneedle: base diameter = $300\mu\text{m}$, height = $920\mu\text{m}$, containing 0.1% 5-FU) with insertion force of 0.05 N per needle or equivalent pressure of 4.1×10^6 Pa. (b) Histological assay of the pierced porcine skin. (c) The microneedle patch is able to bend flexibly when applied to the skin at the elbow joint.

5.3.3 Drug release kinetics from the microneedle device

In order to achieve a mechanistic understanding of this device, the drug release kinetics was investigated based on different drug loading concentrations in the microneedles: 5-FU at 0.1% ($8\mu\text{g cm}^{-2}$), 0.3% ($24\mu\text{g cm}^{-2}$) and 0.5% w/w ($40\mu\text{g}$

cm⁻²) (Figure 5-6a & Figure 5-6c). Free 5-FU (100 µg/ml) enclosed in a dialysis bag (MWCO = 12,313 Da) was used in the control test. As shown in Figure 5-6(b, d), 80% of the free 5-FU was eluted from the dialysis bag within one hour and almost 100% free 5-FU was eluted within 3 hours. In contrast, 5-FU encapsulated in microneedle devices was eluted at much lower rate. After gradual release in the first 12 hours that provided 49.7%, 55.0% and 65.4% drug elution from the devices loaded with 0.1%, 0.3% and 0.5% 5-FU respectively, the remaining 5-FU was eluted at an extremely slow pace with 8.4%, 10.6% and 18.3% release in the following 150 hours. Based on these results, this microneedle device is particularly suitable for sustained drug administration over 12 hours. In addition, the slower phase of 5-FU release after 12 hours, eluting 0.06 to 0.12% of drug per hour compared to 4.14 to 5.45% per hour in the first 12 hours, minimizes over-prolonged 5-FU exposure to the patient.

Figure 5-6 In vitro release kinetics of 5-FU in PBS from the microneedle devices with 0.1%, 0.3%, 0.5% (w/w) drug-loading, in which free 5-FU was the control: (a) the release kinetics for 160 hours; (b) the release kinetics for the first 24 hours (extracted from the dashed box in a); (c) cumulative drug release amount for 160 hours; (d) cumulative drug release amount for the first 24 hours (extracted from the dashed box in c).

Figure 5-7 Normalized drug release kinetics within the first 12 hours for devices with different drug pre-loading concentrations. Data are fitted with Peppas model ($M_t/M_\infty = kt^n$) using linear regression: $\ln\left(100\frac{M_t}{M_\infty}\right) = k_1 + n \ln t$, where M_t is the cumulative drug released, M_∞ is the amount of drug released as time approaches infinity, k is a constant incorporating characteristics of the macromolecular network system of the PEGDA substrate, $k_1 = 2 \times \ln 10 + \ln k$, and n is the diffusional exponent indicative of the transport mechanism.

A quantitative study of the drug release kinetics can help reveal the transport mechanism during drug elution. Since the drug release is insignificant after 12 hours, the analysis is focused on the first 12 hours. The normalized data of cumulative drug release from the microneedles preloaded with different drug concentrations were

fitted with Peppas model ($M_t/M_\infty=kt^n$) by linear regression (Figure 5-7). Since the PEGDA is a gel-based polymer and the release of the encapsulated 5-FU could depend on both dissolution and diffusion, the general power law model proposed by Peppas¹⁴¹ is employed to take account of both diffusion-controlled and relaxation-controlled transport mechanisms. Based on the model fitting results (Figure 5-7 and Table 5-1), the diffusional exponent $n=0.5$ for 0.1% loading concentration, which is consistent to the classical Higuchi model ($M_t/M_\infty=kt^{0.5}$) describing the transport mechanism dominated by Fickian diffusion; while $n=0.37$ and 0.41 for higher loading concentrations, indicating the anomalous transport mechanism that could be due to the conical shape of the microneedles and the swellable polymeric substrate.

Table 5-1 The linear regression fitting parameters of drug release data using the

Peppas model in Figure 5-7: $\ln\left(100\frac{M_t}{M_\infty}\right) = k_1 + n \ln t$

Drug dosage	Linear equation	n	r
0.1% w/w	$\ln\left(100\frac{M_t}{M_\infty}\right) = 3.19 + 0.5 \ln t$	0.5	0.992
0.3% w/w	$\ln\left(100\frac{M_t}{M_\infty}\right) = 3.54 + 0.37 \ln t$	0.37	0.984
0.5% w/w	$\ln\left(100\frac{M_t}{M_\infty}\right) = 3.33 + 0.41 \ln t$	0.41	0.990

5.3.4 Inhibition of the keloid fibroblast proliferation

Spindle shaped normal fibroblasts (NFs) exhibited a 7.4 ± 1.43 fold expansion over a 10-day culture period (5×10^3 cells/cm² seeding density) (Figure 5-8). However, keloid fibroblasts (KFs) appeared more granular and proliferated at a faster rate,

approximately 4-fold faster compared to NFs during the same culture period (21.8 ± 6.78 fold). The exposure of KFs to $100 \mu\text{M}$ 5-FU through solution delivery was able to completely ablate this proliferation activity, resulting in 0.26 ± 0.03 fold expansion at day 10 (Figure 5-8).

Figure 5-8 Growth kinetics of normal fibroblasts (NF) and keloid fibroblasts (KF) and treatment of 5-FU: (a) total cell numbers for the indicated group was quantified at the given time points. Whereas normal and keloid fibroblasts underwent typical sigmoidal growth kinetics, 5-FU ($100 \mu\text{M}$) inhibited the proliferation after day 2. (b) Representative images of NF, KF, and KF treated with 5-FU (soluble) after 10 days (scale bars: $500 \mu\text{m}$).

After the inhibitory effect of 5-FU on KFs was confirmed, we next evaluated the efficacy of the 5-FU-loaded microneedle device, which was compared to the soluble 5-FU and drug-free microneedle device for inhibiting KF proliferation over a 10-day culture period (Figure 5-9a). As demonstrated in Figure 5-9b, the 5-FU-loaded microneedle device showed similar efficacy as the free soluble 5-FU, significantly reducing the total viable cell numbers to below the seeding density (0.23 ± 0.05 fold expansion, $p < 0.05$). Unexpectedly, the drug-free microneedles also induced an inhibitory effect, with approximately 10-fold decrease compared to the positive controls (2.29 ± 0.09 fold expansion). To examine whether this inhibition of KF growth was due to any potential toxicity of the photoinitiator and/or uncrosslinked PEGDA, a drug-free microneedle device was fixed on the lid of the culture plate and submerged into the cell culture medium, while the microneedles were not in direct contact with the cell layer. As illustrated in Figure 5-9b (column C), the KF proliferation was not affected ($p > 0.05$), showing a similar rate of proliferation to the positive control that is free of any microneedle or drug. This further suggests that the microneedle device did not introduce soluble toxic substances. A further preliminary analysis of cell morphology showed that the KFs treated with blank microneedles appeared to have a decreased size and a high nucleus-to-cytoplasm ratio, while the control KFs were flat and spindle-shaped (Figure 5-9c). We are currently studying the mechanism of this phenomenon in which the physical presence of microneedles inhibited the proliferation of KFs.

Figure 5-9 Inhibition of keloid fibroblast (KF) proliferation with 5-fluorouracil (5-FU) and the microneedle device: (a) Schematic illustration of the inhibition experiments (w: with; wo: without); (b) Proliferation assay of KFs after 10 days under the presence of blank (no microneedles and no 5-FU: -/-), 5-FU-free microneedles that were incubated within the same wells without touching the cells (*/-), drug-free microneedles (with microneedles but without 5-FU: +/-), soluble 5-FU (no microneedle but with 5-FU: -/+), and 5-FU-loaded microneedles (with microneedles and 5-FU: +/+). (c) Representative images of untreated KFs (control) and KFs treated with blank microneedles on day 10. (Scale bar: 500µm) Values are Mean ± Standard Deviation, N=3, *: p<0.05.

5.3.5 ‘Patient-centric’ treatment with the microneedle device

Finally, we explored a potential therapeutic schedule for keloid treatment based on this microneedle device. Whereas continuous exposure to 5-FU (100 µM) effectively inhibits KF proliferation (soluble and microneedle delivery), adherence to this treatment is likely to be poor, given that continuous application of 5-FU through microneedles will interfere with the patient’s routine lifestyle. As revealed in Figure

5-6, the microneedle device released 50% encapsulated 5-FU in the first 12 hours, which is suitable for short-period drug administration (within 12 hours). Given that a typical patient has 8-12 working hours per day, this leaves 12 after-work hours which serendipitously will be suitable for drug administration (Figure 5-10a). To test this approach, a fresh microneedle device was applied to a KF monolayer every 12 hours, which was evaluated against a similar dosing schedule using soluble 5-FU delivery. Growth kinetics was compared at days 4 and 10, representing early and stationary growth periods (Figure 5-10b). Although soluble 5-FU delivery was able to reduce KF proliferation (6.9 ± 3.6 fold expansion), the extent of inhibition was even higher when microneedle-assisted delivery was applied (2.0 ± 0.4 fold expansion, $p < 0.05$, Two-way ANOVA). These results demonstrate that microneedle-assisted periodic 5-FU delivery enhances the inhibition effect on the proliferation of KFs, further validating the earlier findings under continuous delivery mode (Figure 5-9).

Figure 5-10 ‘Patient-centric’ treatment with the microneedle device: (a) Illustration of a ‘patient-centric’ dosing strategy. Microneedles are applied during periods of rest

(R), whereas application during working (W) periods will not be required; (b) Comparing the efficacy of periodic 5-FU delivery in ‘patient-centric’ dosing strategy. The proliferation of keloid fibroblasts (at days 4 and 10): control (–/–), soluble delivery (–/+), and microneedle delivery (+/–). Values are Mean $\pm$ Standard Deviation, n=3, *: p<0.05.

5.4 Discussion

In clinical practice, keloids are notoriously refractory to many modalities of treatment, including intralesional corticosteroid injection and surgical excision. Most of these treatments are invasive and cause significant pain. The current standard treatment of keloids with intralesional corticosteroid injection requires repeated treatment over prolonged periods (many months to many years) and administration by physicians or nurses is required. With intralesional injection of corticosteroid or 5-FU, the effect of the drug wanes progressively after the clinic visit and patients need regular repeated (typically monthly) injections to sustain the therapeutic effect. This results in a relatively high cost for treatment for the patients and society at large. Patient compliance to treatment is correspondingly reduced as well.

Given that keloids are often benign with no malignant potential and most patients seek treatment because of disfigurement and the association pain/itch, self-management of keloid would be very feasible. Central to this approach is to have a simple, convenient, and cost-effective device to inhibit the keloid fibroblasts and thereby reduce the size of keloid. Therefore, we designed and fabricated this microneedle device with PEGDA and 5-FU using photopolymerizable micro-fabrication technology. The designed microneedles in this study were 700 to 900 μm

in length, which are able to penetrate the epidermis but not reaching the deeper dermis where significant pain will be induced. Therefore, this device has a lower likelihood to cause pain, infection, or injury.

The pyrimidine analog 5-FU, widely used as an anti-metabolite, was adapted for keloid treatment in view of its efficacy (78% with more than 50% improvement), relatively low rate of recurrence (none at 24 weeks), and side effect profile (ulceration in 20%). Despite the extensive contraindications (anemia, pregnancy, infection, leukopenia etc.) of 5-FU garnered from oncology experience, its side effects during keloid therapy are comparatively minor, consisting of pain, hyperpigmentation and ulceration. In a typical clinical application, high (40~50 mg/ml) or low (1.4~3.5 mg/ml) doses are usually administered intra-lesionally at weekly intervals for longer than 2 months (at 40 $\mu\text{l}/\text{cm}^2$)¹⁴². According to these recommended weekly dosages and a cost analysis of the microneedle patches (Table 5-2), one microneedle patch (0.3% w/w, < US\$0.20) is required for low-dose applications, while less than 8 patches (0.5% w/w, < US\$2.00) are required for high-dose applications. In contrast, weekly clinic visit usually costs at least US\$90, including the drug, materials and injection labor. Therefore self-management using the microneedle patches is highly cost-effective compared to regular clinical keloid management by intralesional injection.

It is noted from the release kinetics (Figure 5-6) that about 30~50% of the drug, depending on the loading concentration, eventually remained in the microneedle device without complete elution. This implies that the encapsulated drug cannot be completely eluted and therefore the actual drug dosage needs to be doubled in order

to compensate for the entrapped drug. Considering the low-cost of 5-FU, the increased cost to produce microneedles of a higher loading concentration is marginal compared to the overall cost per microneedle patch (Table 5-2): from US\$0.18 to US\$0.20 when drug loading increases from 0.1% to 0.5% (w/w). Both key components of this device (PEGDA and 5-FU) have received FDA approval in previous formulations, which should expedite translation of the technology from bench to bedside. Finally, this microneedle device can be fabricated in a scalable manner and thereby is affordable for low-income patients.

Keloids are often stiff and tough due to the excessive synthesis of collagen in the lesion area. Previous studies suggest that keloid tissue is 3 times as tough as the normal skin as measured by tactile displacement sensing¹⁴³. It was also reported that the porcine skin is more resistant to penetration with a yield stress 2.5-fold as high as that of human skin¹⁴⁴. Since there was no available keloid tissue for testing, porcine skin model was used in this study. Based on above reported mechanical properties, it is reasonable to assume that the porcine skin has similar yield stress as keloid and therefore can be used to evaluate whether the microneedles have the requisite mechanical strength to penetrate into keloid lesions without fracture. The experimental data showed that the measured hardness of the microneedles were about 20 times (45 ± 11 MPa) greater than that of regular skin (2.0 ± 0.5 MPa). Subsequent histological assay confirmed that the microneedles were able to penetrate the stratum corneum, the toughest layer in both porcine and human skin, which further implied that the microneedles have sufficient mechanical strength to penetrate keloid lesions.

Although the exact biology behind keloids development is still unclear, it is generally accepted that increased proliferation of KFs with consequent synthesis of excess collagen is key to the development of keloids. Therefore, the first criterion in evaluating a device for the treatment of keloid is its ability to suppress proliferation of KFs. Here we examined two forms of treatments, namely continuous (Figure 5-9) and periodic exposure (Figure 5-10) using this device. Specifically, during continuous exposure, the device was placed on top of the KFs for a consecutive treatment period (e.g. 10 days) while the device was replaced with a new one after every 12 hours. The results showed that both of these two administration modes for microneedle-assisted 5-FU delivery could effectively prohibit the KF proliferation, although the continuous mode generates faster effect than the periodic ‘patient-centric’ dosing strategy.

It should be noted that the efficacy of such a microneedle device as an exclusive mode of treatment could be limited for some mature keloids, which overgrow their boundaries and become raised over the skin (1~2 mm). This may pose a challenge for this microneedle device (700~900 μm in height per needle) to reach sufficient depth within the dermis for effective delivery of 5-FU. In this respect, the microneedle patch may be more suitable as a post-excision adjuvant for patients who have severe symptoms. In addition, it is expected that application of microneedles at early stage of scar formation would be more effective than when thick late-stage keloids have developed.

Table 5-2. Cost analysis of microneedle patch with variable drug loading concentrations (calculation is based on 500 μ L prepolymer solution per microneedle patch (2 cm $\times$ 2 cm))

Material \ Drug dosage	0.1% w/w	0.3% w/w	0.5% w/w
PEGDA (209 SGD/500 mL)	0.209 SGD	0.209 SGD	0.209 SGD
5-FU (29.2 SGD/g)	0.015 SGD	0.03 SGD	0.045 SGD
HMP (312.5 SGD/250 mL)	$< 10^{-3}$ SGD	$< 10^{-3}$ SGD	$< 10^{-3}$ SGD
Total cost per patch (2 cm $\times$ 2 cm)	0.224 SGD (0.178 USD)	0.239 SGD (0.190 USD)	0.254 SGD (0.202 USD)
Total cost per cm ²	0.056 SGD (0.046 USD)	0.060 SGD (0.048 USD)	0.064 SGD (0.051 USD)
Total drug amount in microneedle patch	50 μ g/cm ²	150 μ g/cm ²	250 μ g/cm ²
Recommended weekly clinical dosage (40 μ l/cm ²) ¹⁴²	Low 56~140 μ g/cm ²		High 1600~2000 μ g/cm ²
Required microneedle patches	1		7~8

5.5 Conclusions

In summary, we have developed a 'wearable' microneedle device for transdermal delivery of an anti-metabolite drug (5-FU) to inhibit the proliferation activity of keloid fibroblasts (which are responsible for keloid scarring). This study has provided

evidence for effective inhibition of keloid fibroblasts in vitro and successful translation of this platform can likely result in a reduction in the cost of keloid treatment and an improvement in patient compliance. Future studies will focus on animal models and subsequent clinical trials will help determine the clinical efficacy and safety of this microneedle drug delivery system.

5.6 Declaration

Part of the work presented in this chapter has been published in the journal of *Technology* (Copyright 2014 World Scientific).

Peng Xue, David Chen Loong Yeo, Yon Jin Chuah, Yuejun Kang*, Chenjie Xu*. “Drug-eluting microneedles for self-administered treatment of keloids”. *Technology*, 2, 144-152 (2014).

Chapter 6 Detachable microneedle patches for transdermal drug delivery

6.1 Introduction

Pharmaceutical molecules, such as peptides, nucleic acids and antibodies, are important candidates for current therapeutics. However, most of these molecules are associated with issues of solubility and permeability when they are inducted through traditional routes. Therefore, large dosage of drugs are usually required to compensate inefficient delivery in order to achieve a remedy of desired efficacy, which further increases the medical cost and results in potential side effects due to toxicity^{145,146}. Although a huge number of drugs have been commercialized to date, the options of drug delivery are rather poorly limited. Oral formulations have been developed as the oldest and still the most popular method to deliver drugs nowadays. However, not all types of drugs could maintain their structural stability in the harsh environment of gastrointestinal tract¹⁴⁷. Alternatively, transdermal drug delivery has become a standard way for clinical therapeutics in modern medicine, through which the drugs are free from gastrointestinal degradation and thus able to maintain relatively higher plasma concentrations, and the patient compliance is also improved¹⁴⁸. However, conventional injection using hypodermic needles not only induce severe pain, bleeding and psychological stress, but also produces sharp and bio-hazardous wastes that are not ecofriendly¹⁴⁹⁻¹⁵¹. In addition, this precise medical operation needs to be administered by qualified healthcare professionals, which is not convenient and raises extra costs to the patients¹⁵².

To address these disadvantages of hypodermic needles, a new approach using microneedle patches (MNPs) has appeared in the field for minimally-invasive drug delivery¹⁵³. A typical MNP includes arrays of sharp projections in microscale size that are designed to penetrate the stratum corneum forming conduits for drugs to diffuse into the dermal circulation without stimulating the nerve endings, which is a pain-free experience for patients^{154,155}. MNPs can be used to deliver various types of drugs with a wide size range from small molecules to nanoparticles¹⁵⁶. Furthermore, MNPs have much lower manufacturing cost due to less complexity, and also enable self-administration by the patients. A large variety of materials, such as metals, silicon and polymers, can be used for fabrication of MNPs with various configurations. However, MNPs made of metal or silicon still generate non-degradable and bio-hazardous sharps after administration, which requires special treatment and disposal of medical wastes with additional cost. Moreover, these metal- or silicon-based MNPs usually do not contain drugs in the needle materials, while each microneedle encloses a hollow conduit for inducting liquid drug from an attached or external source. Therefore, such mode of delivery is rather passive and still requires intervention. On the other hand, MNPs made from biodegradable polymers can avoid the issues related to sharp bio-hazardous waste. More importantly, many drugs are able to be encapsulated in the polymer matrix of the inserted microneedles and can be released spontaneously via dissolution and polymer degradation in the physiological condition, leaving only the external MNP back layer to be disposed^{157, 158}. However, the backing layer needs to remain attached on the skin

until complete release of drugs, bringing discomfort and cosmetic concerns to the patients.

To achieve an improved drug release performance based on the microneedle platform in Chapter 5, we aim to revamp the polymer-based MNP devices with a novel design of detachable sharp tip on each microneedle in this work, so that the backing layer and blunt needle shafts can be easily separated from the inserted tips after application. The shaft-substrate complex and the detachable arrowhead tips are made from polyethylene glycol diacrylate (PEGDA) and polyvinylpyrrolidone-cyclodextrin (PVP-CD) respectively, both of which are approved biocompatible materials^{159, 160}. The PEGDA-based shaft-substrate complex is fabricated by one-step photolithography as described in a previous report¹⁶¹. Drug-loaded and detachable PVP-CD arrowhead microneedles with pyramid shape are precisely assembled onto the PEGDA backing shafts. This assembled MNP device provides desired mechanical strength to insert the arrowhead tips during skin penetration. Moreover, the flexible PEGDA substrate allows seamless adherence between the MNP and the lesion surface with any curvature. As a prominent feature, the backing shafts together with the patch substrate can be easily detached from the skin shortly after application, while the arrowheads remain inside the skin and release preloaded drugs continuously as PVP-CD gradually degrade and dissolve into the tissue (Figure 6-1). In the following, we demonstrate the detailed fabrication procedures of this MNP and a proof-of-concept application to delivery molecules of rhodamine B into a porcine skin.

Figure 6-1 Schematic illustration of applying the detachable microneedle patch onto the skin.

6.2 Experimental Section

6.2.1 Materials

PEGDA (polyethylene glycol diacrylate, average $M_n = 250$), PVP (polyvinylpyrrolidone, average MW = 10k), HMP (2-hydroxy-2-methyl-propiophenone), CD (hydroxypropyl-beta-cyclodextrin, average MW=1460) and rhodamine B (HPLC) were obtained from Sigma-Aldrich, Singapore. DI (Deionized) water was prepared from Milli-Q water purification system (Synthesis A10, Molsheim, France).

6.2.2 Preparation of backing substrate and microneedle shaft

The microneedle backing substrate and shaft were fabricated using PEGDA by photopolymerization. To fabricate the backing substrate, two coverslips (2.2 cm × 2.2 cm, SPD Scientific Pte Ltd, Singapore) were juxtaposed on a glass slide (1 inch × 3

inch, Fisher Scientific, Singapore) to form a gap in between (Figure 6-2). Another piece of coverslip was precisely aligned on top of this interval to form a shallow cavity. A prepolymer mixture containing PEGDA and photoinitiator (HMP, 0.5% w/w) was carefully injected into the shallow cavity through capillary action, followed by UV-laser irradiation (wavelength = 360 nm, intensity = 17 mW cm⁻²) for 4 s. Thus the backing layer was fabricated after rapid photopolymerization and was carefully peeled off from the coverslip. To fabricate the microneedle shaft array, the set-up was similar to that for the backing substrate but the depth of the gap was increased to 1 mm. Afterwards, the backing substrate was precisely aligned on top of the gap, followed by filling the cavity with prepolymer solution. A film photomask designed with transparent circle array was firmly held against the top side of the backing substrate (Figure 6-2). Each transparent circle has a diameter of 300 µm and the distance between the centers of two neighbouring circles is 500 µm. The subsequent UV irradiation for 12 s selectively exposed the prepolymer solution underneath the backing substrate, creating the needle shaft array. Finally, the shaft array sticking on the backing substrate was carefully peeled off from the glass substrate and rinsed with DI water to remove uncrosslinked polymer.

Figure 6-2 Fabrication procedure of PEGDA-based backing substrate and microneedle shafts.

6.2.3 Fabrication of final patch with detachable arrowheads

50 mg PVP, 50 mg CD and 0.01 mg rhodamine B were dissolved in 1 ml DI water, followed by sonication for 30 min and vortex stirring for 1 day. The prepared PVP-CD polymer solution containing rhodamine B was placed in a drying cabinet for 2 days before being used for molding. The PDMS mold was prepared by casting the PDMS prepolymer onto a customized steel master (Micropoint Technologies Pte Ltd, Singapore) with array of pyramid tips (Figure 6-3). After degassing in a vacuum box

and thermal curing at 70°C for 2 h, the crosslinked PDMS mold was peeled off from the steel master and ready for use. To fabricate the microneedle arrowheads, 100 μ L of PVP-CD polymer solution was firstly pipetted onto the PDMS mold. After being vacuumed at -91 kPa for 3 min, the system was centrifuged at 3000 rpm for 10 min at room temperature to force the polymer solution into the pyramid cavities and thus prevent the trapped air from forming void arrowheads. The residual PVP-CD polymer solution on the mold surface was carefully collected for future usage.

Figure 6-3 The fabrication procedure of detachable PVP-CD arrowheads and assembly of the final microneedle patch.

To assemble the final MNP device, the microneedle shaft array was carefully aligned on top of the PDMS mold cavities filled with PVP-CD polymer solution under a stereomicroscope (Leica MZ6). A slight force was applied on the backing substrate to ensure all the shafts submerged in the base of PVP-CD arrowheads for a

depth of about 200 μm . After drying in a desiccator at room temperature for 3 days, the arrowhead array comprising PVP-CD inclusion complexes with superior mechanical strength was created and attached on the needle shafts, forming the final assembled MNP with detachable arrowheads¹¹¹. The entire patch was removed from the PDMS mold and ready for skin test.

6.2.4 Mechanical test

A NanoIndenter XP (MTS Systems, Oak Ridge, TN) was used to measure the hardness and elastic modulus of the arrowheads of detachable microneedle patch. On each sample, three independent locations with the interval of at least 300 μm were selected and indentations were performed by using NanoIndenter XP. Based on the maximum load level and the indentation depth of 20 μm , hardness and elastic modulus were calculated accordingly.

6.2.5 Microscopic imaging

All images of the microneedle patch and administration sites on the porcine skin were captured using a stereomicroscope (Leica MZ6) with a digital camera (Leica DFC 300FX). The morphology of arrowhead tips was examined using a scanning electron microscope (SEM, Hitachi 3500N) at an accelerating voltage of 5 kV. Fluorescence image of microneedle insertion site was taken using a fluorescence microscope (IX71, Olympus, Singapore). For histological assay, fixation of the same skin tissue was conducted in formalin (10%). Before frozen section, the skin was embedded in tissue freezing medium (Leica Biosystems Singapore) at -20°C. Sagittal oriented cryo-sections were prepared at 8 μm intervals via Leica CM1850 cryotome

(Leica Biosystems, Singapore). The specific morphological features were examined using a fluorescence microscope under bright field.

6.2.6 In-vitro test of MNP insertion

The microneedle patches were tested on a porcine skin *in vitro*. Fresh skin samples were obtained according to Institution-approved protocols. Firstly, the sample was stabilized and flattened on a corkboard. The microneedle patch was then pressed onto the skin for 3 min manually using thumb pressure. To facilitate the detachment of arrowheads, the patch was slightly agitated against the skin before being peeled off from the application site. Images of the administered skin with released rhodamine B in the needle insertion sites were taken under standard light microscopy.

6.3 Results and discussion

It is essential that the microneedle shaft needs to have excellent mechanical property to facilitate the arrowhead tip to pierce into the skin. Metal material, such as steel, is commonly used to fabricate the shaft and the backing substrate of microneedle patches^{162, 163}. However, the biosafety of many metal materials is still unclear and metal-based backing layer is usually not flexible and adaptive to the arbitrary skin curvature¹⁶⁴. Meanwhile, PEGDA is a FDA-approved polymer material with acceptable biosafety for clinical use. Therefore, PEGDA-based microneedle shafts and backing substrate were fabricated following a simple photo-initiated polymerization method (Figure 6-2) as described in our previous study¹⁶¹. This PEGDA shaft-substrate assembly is transparent and flexible. Moreover, the microneedle patch can remain intact with a bending radius of curvature of 1.21cm,

which allows the patch to be fitted on any curved surface of body skin, such as nose, ear and articular surfaces that have small radius of curvature. Moreover, the PEGDA microstructure has been proved to have excellent mechanical strength with a hardness of 45 ± 11 MPa that is 20 times greater than that of a regular skin (2.0 ± 0.5 MPa)¹⁶¹, which ensures efficient penetration through stratum corneum the toughest barrier of skin. Meanwhile, the arrowheads possess the hardness of 67 ± 4 MPa and elastic modulus of 0.93 ± 0.07 GPa. The excellent mechanical property of arrowheads further facilitates the skin penetration during the administration. In addition, the packing density and the base diameter of needle shafts can be easily adjusted by photomask patterning. The shaft length can be also tuned by varying the cavity height and UV irradiation time.

The entire dosage of drug was encapsulated inside the arrowhead tips made of PVP-CD complex. PVP is a biocompatible and biodegradable material that has been approved for using as pharmaceutical excipient¹⁶⁵. In this study, PVP with molecular weight of about 10 kDa was used since it can be more efficiently cleared in the kidney during blood circulation compared to that of ~20 kDa. CD derived from starch has been commercialized in the pharmaceutical industry for surface coating of drugs¹⁶⁶. The CD was added into PVP forming PVP-CD complex in order to reduce the water absorption by the arrowhead tips and thus maintain their mechanical strength¹¹¹. Rhodamine B was pre-mixed in the PVP-CD complex as drug surrogate to demonstrate the drug encapsulation capability.

Figure 6-4 (a) The image of a final assembled microneedle patch with detachable arrowhead tips encapsulating rhodamine B; (b) A SEM image of the PVP-CD arrowhead tips.

Figure 6-4a shows the image of a final assembled MNP with detachable arrowhead tips. Each microneedle comprises a PEGDA shaft (base circle diameter = $300\ \mu\text{m}$, height = $800\ \mu\text{m}$) capped with a PVP-CD arrowhead (base square = $300\ \mu\text{m} \times 300\ \mu\text{m}$, height = $600\ \mu\text{m}$). The red colour of the arrowhead tips is due to the encapsulated rhodamine B. All the arrowhead tips have a shape of rectangular pyramid and are uniformly distributed on the base shafts. The density and the patterning of arrowheads can be adjusted by the designs of photomask and steel master. The PEGDA backing substrate is transparent and flexible, which facilitates the MNP administration. The scanning electron microscopy (SEM) revealed the detailed sharp morphology of each arrowhead tip (Figure 6-4b). It is observed that there are minor defects on some tip surface and some dried PVP-CD flakes at the base of the tips, which implied that the fabrication procedure needs to be further optimized. Despite of these imperfections, these PVP-CD microneedle tips are much sharper than those we previously fabricated using PEGDA only¹⁶¹.

To simulate the transdermal drug administration on a real skin, we applied a rhodamin B-loaded MNP with detachable arrowheads to a fresh porcine skin simply using thumb pressure. The patch substrate and needle shafts (Figure 6-5a) were peeled off shortly after application, leaving all the red arrowheads embedded inside the insertion sites (red dots in Figure 6-5b). It was found that the inserted PVP-CD tips quickly dissolved within a few minutes in the humid environment of the fresh skin sample due to the superior dissolvability of PVP. And these coloured dots could not be wiped off or removed by washing with DI water, indicating that rhodamin B were successfully released into the dermis. The histological assay of the pierced skin (Figure 6-5c) further confirmed that the arrowhead tips successfully penetrated the epidermis layer with an insertion depth of about 500 μm . This penetration depth was much shorter than the length of the entire microneedle, which was quite common due to the deformation of highly elastic skin as found in other similar studies¹⁶⁷.

Figure 6-5 (a) The peeled-off PEGDA substrate and shafts after application of MNP. Scale bar = 500 μm . (b) The insertion sites by the detachable arrowheads loaded with rhodamine B on a porcine skin. Scale bar = 500 μm . (c) Histological assay of the pierced skin after the PVP-CD tips were dissolved.

This self-administered microneedle patch provides a simple way of transdermal drug delivery for patients requiring hypodermic injection. The method is rapid, efficient and free from pain, bleeding and generation of bio-hazardous sharp wastes.

This MNP device can be obtained with an extremely low cost for convenient self-administration onsite or at home without the assistance from healthcare professionals. This polymeric patch can adapt to any lesion site owing to the good flexibility of the backing substrate. Additionally, the backing layer and the needle shafts can be easily peeled off and discarded shortly after application without causing any cosmetic concern to the patients. Moreover, the microneedle fabrication process is conducted under aqueous conditions at room temperature, which causes no damage to the fragile biomolecules during encapsulation. Therefore, many vaccines or other bioactive molecules could be incorporated into the polymer matrix for transdermal delivery, which has a great potential to treat aggressive skin and breast cancers¹⁶⁸. For further study, the detailed release kinetics against the type and concentration of various drugs from the PVP-CD complex can be investigated in vivo, which may elucidate the mechanisms and further optimize the actual therapeutic effect of this MNP device.

6.4 Conclusions

This study demonstrated the design and fabrication of a polymeric microneedle patch device with detachable and drug-loaded arrowhead tips for potential delivery of bioactive molecules into skin. This microneedle patch features good flexibility and convenient self-administration by patients without generating bio-hazardous sharp wastes. Shortly after skin piercing, the PVP-CD arrowhead tips are detached from the PEGDA shaft-substrate assembly and embedded into the skin for instant dissolution and release of the loaded molecules. This MNP device is expected to be a promising and eco-friendly tool for rapid, reliable, pain-free and convenient transdermal

delivery of diverse bioactive molecules in self-administered or clinical applications in the future.

6.5 Declaration

Part of the work presented in this chapter has been published in the journal of *RSC advances*. (Copyright 2015 Royal Society of Chemistry)

Peng Xue, Xuyang Zhang, Yon Jin Chuah, Yafeng Wu, Yuejun Kang*. “Flexible PEGDA-based Microneedle Patches with Detachable PVP-CD Arrowheads for Transdermal Drug Delivery”. *RSC advances*, 5, 75204-75209 (2015).

Chapter 7 General conclusions and future research

7.1 Contributions made by this study

Polymer based biomedical devices have been extensively involved in the field of biology, pharmacy and instrumentation *et al.* The devices should be customized designed and fabricated to fulfill the application requirements. The structure complexity of the systems varies in terms of perspectives, and the variability contributes to the advanced research into the biology and medical science. So far, various polymers, such as PDMS, PVP, PEGDA, are used as the alternatives of rigid polymers for the fabrication of biomedical microdevices with the advantages of low costs, flexibility, optical transparency and biocompatibility. Additionally, thermoplastic-based and photosensitive biomedical systems can be high-throughput manufactured using simple equipment and techniques. Meanwhile, cancer is considered as a critical disease and many researches have been devoted to this area with respect to diagnosis and therapy.

Enumeration of CTCs results is an important index for cancer diagnosis and for evaluation of cancer therapy effectiveness. Counting of CTCs using biomedical microdevices is extremely promising due to the low cost in clinical usage. In chapter 3 and 4, PDMS-based novel microchip with herringbone structures is reported and the operation performance is discussed. Not only was the CTC enumeration achieved, the captured CTCs can be eluted from the chip for further biological and chemical analyses as well. In a word, dual functions can be realized in such a tiny device profitably. PDMS is a commonly used polymer with excellent biocompatibility,

optical transparency and low cost. Moreover, this type of PDMS chips can realize mass productivity in accordance with commercial industry.

Polymer-based biomedical microdevices can also play an important role in cancer therapy. In chapter 5 and 6, polymer-based microneedle patches were designed and fabricated for keloid therapy. Keloids are tumorous tissues and their growth cannot be controlled without proper treatment. The principle of keloid treatment is based on efficient transdermal drug delivery using drug-loaded a microneedle patch. The treatment efficacy of this microneedle device is thoroughly demonstrated, and many other designs were raised to improve the performance of the microneedle patches. It is really a creative innovation for keloid treatment from now on, because the traditional treatment methods are not friendly and the recurrence rate is quite high. This study has attracted many interests from patients with keloids worldwide, and has been covered in many media reports, which indicates the importance of this study.

7.2 Directions for future research

Many more polymers should be developed for biomedical applications due to the advantages of mass production and other adaptable formulation changes. Additional to PDMS, thermoset polyester (TPE) as another elastomeric polymer is thermally formed by the polymerization of polyester and styrene based on UV irradiation or heating process. TPE has a higher elastic modulus compared to PDMS and it has great potential to be used as hybrid polymer microfluidic device for protein immobilization¹⁶⁹. Thermoplastics are densely cross-linked polymers which are stiffer than elastomers. Polystyrene (PS) is a polymer derived from vinyl-benzene monomers and favorable for being constructed as cell culture system. There exists

great opportunity to develop microfluidic systems using PS with high aspect ratios. Cyclic-olefin copolymer (COC) is optical transparent and desirable for usage with most chemical solvents. Polyurethane (PU) is an attractive polymer in microfluidic field due to the intrinsic stability and biocompatibility in fabricating microdevices. Zein is a novel biodegradable polymeric material derived from protein prepolymer for fabricating hybrid zein-glass devices. Actually, there is no “perfect material” since each preserves its own advantages and drawbacks. Hence, more attentions should be focus on the development of new polymeric materials or the improvement of the current ones.

On the other hand, as a follow-up study, the findings in my study can be helpful to further study polymer-based microdevices for cancer diagnosis and therapies or even development on the mass production of point-of-care devices. Specifically, two aspects will be more concentrated on, including the CTCs capture and elution with higher efficiency, as well as transdermal drug delivery using more powerful microneedle devices.

(1) For CTCs capture and elution, there still exist great demands in designing novel microdevices to achieve higher capture and elution rates, due to the extremely rare CTCs in patients’ blood samples. Additionally to geometry designs, other techniques such as acoustic wave and magnetic trapping can be integrated into the platform to enhance the performance of the devices. Moreover, there will be much more meaningful biochemical analysis by using the cells eluted from the microchip, qPCR for instance. Thus, there will be more indexes to evaluate the status of a patient by using just one blood sample.

(2) To achieve a perfect system, the CTCs capture and analysis is hopefully to be implemented on a single device, which means physicians could introduce the blood sample with tiny volume for once and obtain all correlative results directly without further operation. This assumption could save the patients a large amount of money, and also reduce the burdens of hospital and clinics.

(3) For transdermal drug releases for keloid treatment, more analysis needs to be conducted *in vivo*. A keloid scar tissue should be implanted onto the nude mouse, and the evaluation need to be made based on the status of keloid after the treatment with the microneedle patch. Furthermore, it would be more interesting to investigate the device performance on patients with keloids directly. Testing should be done dynamically in terms of the size of the keloid, the adverse side effects and patients' feelings. The variables such as the treatment periods per day, drug dosage, and microneedle density are required to be evaluated during the study. It would be more promising to improve the current device to be customized for commercial markets in the near future.

(4) Other polymer-based microneedle platforms are encouraged to be developed for more transdermal drug delivery applications, especially for the drug that could be easily invalidated through orally administration. High treatment efficacy, low cost and patients' comfort are factors, which need to be considered seriously during the device development.

In all, modern polymer-based microdevices have great opportunities in cancer diagnosis and therapy. The impact of this research could also broaden the

applications of improved and accessible platforms for the diagnosis and therapy of other critical diseases.

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