Development of disposable lab-on-a-chip system for point-of-care-testing

Yoon, Jaeyun

2017

Yoon, J. (2017). Development of disposable lab-on-a-chip system for point-of-care-testing.
Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/72724>

<https://doi.org/10.32657/10356/72724>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**DEVELOPMENT OF DISPOSABLE LAB-ON-A-CHIP SYSTEM
FOR POINT-OF-CARE TESTING**

YOON JAEYUN

SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

2017

**DEVELOPMENT OF DISPOSABLE LAB-ON-A-CHIP
SYSTEM FOR POINT-OF-CARE TESTING**

YOON JAEYUN

SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

Supervisor: Asst Prof. Yoon Yong Jin

2017

ACKNOWLEDGEMENTS

It has been an unforgettable journey completing my Ph.D. thesis. This work would never have been possible without the support and advice of many people. First, I would like to thank my supervisor, Prof. Yoon Yong Jin, for giving me this wonderful opportunity to pursue a Ph.D. in this promising field and guidance throughout this research course. It was a great experience for me to work in his group at Nanyang Technological University.

I am also grateful to my mentor, Dr. Park Mikyoung for her gracious help and motivation throughout my attachment programme in Institute of Microelectronics. It was a great privilege to work under her guidance.

I would also like to thank Prof. Shin Yong for his constant help and inspiration. I learned a lot from him throughout my research work. He encouraged me to enhance my critical thinking and problem-solving skills while working with him.

Great thanks to my lab colleagues at NTU for their contribution to this research work. And I also want to thank scientists and research engineers from the department of Bioelectronics for invaluable assistance and discussion.

Special thanks to the mechanical engineering department at NTU and Institute of Microelectronics, A-star for giving me the opportunity to study in their collaborative research project.

Last but not least, I want to express my deep gratitude to my beloved family and friends. I could never get here without their faith in me and endless support.

Table of Contents

Table of Contents	v
List of Figures.....	viii
Abstract.....	xix
Introduction.....	1
1.1. Background	1
1.1.1. Motivation	1
1.1.2. Microfluidic DNA based diagnostic system	2
1.1.3. POC RNA isolation technique	3
1.1.4. Lab on a chip system for nanoparticle separation for POC application.....	5
1.2. Thesis objective	6
1.3. Thesis outline	8
Literature Review	9
2.1 Introduction.....	9
2.2. LOC based approaches for DNA methylation analysis	10
2.2.1. Microfluidic sample preparation platforms for DNA methylation analysis	11
2.2.2. Biosensors for DNA methylation analysis	14
2.2.3. Biosensors for label-free DNA methylation detection.....	16
2.3. Microfluidic approaches for RNA isolation	21
2.3.1. Conventional RNA isolation techniques.....	22
2.3.2. Microfluidic RNA isolation techniques	22
2.4. Size based particle separation in microfluidic systems.....	25
2.4.1. Basic principles of dead-end membrane filtration	28
2.5. Conclusions.....	29
Lab On A Chip System For DNA Methylation Analysis	31
3.1. Introduction.....	31
3.1.1. DNA methylation as a cancer biomarker	33
3.1.2. Optical biosensor based on silicon microring resonator	34
3.1.3. Miniaturized LOC platform for DNA methylation detection	36
3.2. Working principle and device design	40
3.2.1. Working principle of LOC based DNA methylation analysis platform	40
3.2.2. Design of microfluidic sample pre-processing module for on-chip bisulfite conversion	46
3.2.3. Numerical simulation of mixing of two fluid flows in 3D mixer	49
3.2.4. Design of silicon microring sensor	55
3.3. Materials and Methods.....	58
3.3.1. Fabrication of microfluidic device for on-chip bisulfite modification of human genomic DNA	58

3.3.2. Fabrication of silicon microring resonator chip for methylation specific amplification and detection	62
3.3.3. On-Chip DNA methylation analysis	62
3.3.4. Sample preprocessing module.....	63
3.3.5. DNA methylation analysis by conventional End-point PCR and real-time PCR ...	65
3.3.6. Preparation of detection module	66
3.3.7. DNA methylation analysis on detection module	68
3.4. Results & Discussion	70
3.4.1. Optimization of bisulfite treatment	70
3.4.2. Characterization of microfluidic sample preprocessing module by Methyl-specific PCR	74
3.4.3. DNA methylation analysis using detection module.....	76
3.4.4. Quantitative analysis of DNA methylation status	81
3.5. Conclusion	84
A Disposable Microfluidic Platform for RNA Isolation	87
4.1. Introduction.....	87
4.2. Working principle	92
4.3. Materials and Methods.....	94
4.3.1. Device fabrication	94
4.3.2. RNA isolation from cancer cells on chip	94
4.3.3. Viral RNA isolation on chip	96
4.4. Results & Discussion	98
4.4.1. Numerical simulation	98
4.3.2. Polymer surface silanization	100
4.3.3. Optimization of disposable microfluidic RNA isolation chip.....	103
4.3.4. Characterization of disposable microfluidic RNA isolation chip	108
4.3.5. RNA isolation from various cancer cell lines and gene expression analysis	110
4.3.6. Detection of Dengue virus using microfluidic RNA isolation chip	114
4.4. Conclusion	118
Lab On A Chip System For Particle Separation.....	120
5.1. Introduction.....	120
5.2. Working principle of ultrasonic assisted nanoparticle separation	121
5.3. Materials and Methods.....	123
5.3.1. Chip design and fabrication	123
5.3.2. Ultrasonic assisted nanoparticle separation on microfluidic device	126
5.3.3. Characterization of Populations of Nanoparticles.....	127
5.4. Results & Discussion	128
5.4.1. Characterization of microfluidic size based NP separation with ultrasonic irradiation	128
5.4.2. Effect of surface treatment	131

5.4.3. Effect of orientation of ultrasonic treatment	136
5.5. Conclusion	140
Conclusions	142
Future Work.....	145
7.1. Multiplexing of LOC system for DNA methylation analysis.....	145
7.2. Integration of LOC system for DNA methylation analysis	146
7.3. RNA analysis on disposable microfluidic platform.....	146
7.4. Size based biomolecule separation on microfluidic LOC system with ultrasonic treatment	147
Bibliography	148

List of Figures

Figure 1 Fully integrated microfluidic molecular diagnostic platform.....	7
Figure 2 Overview of magnetophoretic bisulfite conversion process based on microfluidic droplet manipulation. Each droplet contains reagent necessary for bisulfite conversion. ¹³	13
Figure 3 Microfluidic platform with micro-pillar array for enrichment of hypermethylated DNA. Hypermethylated DNA can be captured onto the microfluidic surface via specific binding between hypermethylated DNA and MBD protein. ¹⁴	14
Figure 4 Electrochemical biosensor based DNA methylation detection. (A) The DNA methylation status of the p53 gene is recognized by the current signal of electroactive indicator interacting with negatively charged DNA backbone hybridized with PNA probe on electrode surface. (B) The DNA methylation status was quantitatively studied using the electrochemical biosensor. The current signal of [Ru(NH ₃) ₆] ³⁺ indicates DNA methylation level. ¹⁶	17
Figure 5 Nanowire-field effect transistor (FET) biosensor for ultrasensitive detection of methylated DNA. ¹⁷	18
Figure 6 After bisulfite conversion and asymmetric PCR of DNA sample from breast cancer cell lines, DNA is adsorbed onto gold layer of SPR sensor with affinity depending on adenine content, which indicates the methylation level by monitoring SPR spectral shift. ¹⁸	20
Figure 7 Silica based SPE devices. (A) The disposable silica cartridge consists of PDMS layers and silica membrane integrated into microfluidic system for automated DNA/RNA isolation. ²⁹ (B) Microfluidic device with silica bead packed within microchannel. Silica beads are coated with chitosan to avoid the use of chaotropic reagent for binding. ³⁰	23

Figure 8 DNA methylation in cancer cells: This aberrant methylation of cytosines in CpG Islands has been known to be associated with cancers, therefore, this can be used as biomarkers for cancer diagnosis.....	34
Figure 9 Bisulfite conversion of DNA. Cytosines are converted into uracils in the presence of bisulfite, while methylated cytosines remain intact.....	36
Figure 10 Working principle of sample pre -processing module. Human gDNA in bisulfite solution was driven into the module for on-chip bisulfite conversion. Bisulfite conversion was carried out in microchamber at 70 °C. On-chip Bisulfite conversion gives rise to sequence difference by converting unmethylated cytosine to uracil while methylated cytosine is unaffected. After conversion, subsequent desulfonation and purification processes were performed within microchannel before elution. Bisulfite converted DNA sample was collected through an outlet in TE buffer and used for analysis on detection module. ⁹²	42
Figure 11 Detection module based on silicon microring sensor chip. The inset shows microring structures functionalized with either Methyl or Unmethyl forward primer. After bisulfite conversion in sample preprocessing module converted DNA was loaded onto sensor chip in RPA solution containing Recombinase, reverse primer and Polymerase.....	44
Figure 12 The detection of DNA methylation on detection module (silicon microring sensor)	45
Figure 13 Schematic of sample pre-processing module design (Top view). Inset figure shows three-dimensional structure of split and recombine (SAR) micromixer. ⁹²	47
Figure 14 Photograph of the fabricated device. Bisulfite conversion chamber is visualized with red dye on the left-hand side. Mixing between two fluids is visualized within micromixer region. Two streams from inlet I and inlet II are mixed thoroughly in 4 mixing units of three-dimensional micromixer as they proceed towards DNA Capture microchannel, shown as purple.	48

Figure 15 The mixing result at initial flow velocity 3.2 mm/s at the inlet boundary in three-dimensional micromixer	52
Figure 16 The mixing result at a flow velocity of 3.2 mm/s in modified tesla micromixers.	52
Figure 17 2D cross-sectional plots of lamination in the first mixing unit.	53
Figure 18 Numerical simulation results of 3D mixer (Red) used in the sample processing module and tesla mixer (Blue) for comparison of mixing performance with varying Re from 0.1 to 100.....	55
Figure 19 Layout of silicon microring sensor array. The sensor array consists of four racetrack shaped microrings. ⁷⁸	56
Figure 20 Fabrication of sample pre-processing module. The sample pre-processing module was built with multiple layers of PMMA sheets by laser cutting process. The bottom layer consists of a blank glass slide (piranha cleaned) for DNA binding.	60
Figure 21 Sample pre-processing module is mounted on Peltier thermoelectric cooler for feedback temperature control.	61
Figure 22 Workflow of DNA methylation analysis using Lab-on-a-Chip system	63
Figure 23 Conventional PCR results for optimization of temperature and molarity condition. (A) Human gDNA (1 μ g) isolated from MCF-7 cells was used as a template. Gel electrophoresis of MS-PCR products amplified using template treated on sample pre-processing module under Low MT or High MT condition. (M= Methyl-specific primer, W=Wild-type primer and L= 50 bp DNA ladder) (B) After 240 min of incubation, serious loss of DNA samples detected was observed under both low MT and high MT condition. (C) Conversion efficiency using the sample pre-processing module as a function of the reaction time of bisulfite conversion. Incubation time varied from 5 min to 4 h. Conversion	

efficiency was analyzed by obtaining the normalized intensity of products from MS-PCR. 73

Figure 24 Characterization of sample pre-processing module for on-chip bisulfite conversion by Methyl-specific PCR. (A) Comparison of conversion efficiency. Genomic DNA from MCF-7 (1 μ g) was used as an input. The normalized intensities of amplicons from MS-PCR by M primer (Blue) and W primer (Red) set were compared. The inset shows gel electrophoresis results. (B) Quantitative analysis of DNA methylation using a varying amount of input DNA after conversion by on-chip and conventional techniques. (1 μ g, 500 ng, and 100 ng from left). Average normalized intensity was obtained from gel electrophoresis results. 75

Figure 25 Analysis of on-chip bisulfite modified DNA by detection module, MS-PCR (Methylation specific PCR) and real-time PCR. (A) Workflow of bisulfite modified DNA analysis using detection module. (B) DNA methylation analysis of bisulfite treated DNA by the detection module. Resonant wavelength shift was measured during amplification on detection module functionalized with either M or U primer. (C) Analysis of bisulfite treated DNA by MS-PCR. Bisulfite treated gDNA from MCF-7 by using proposed method(I) compared to untreated (No bisulfite conversion) gDNA from MCF-7(II); M=methylated, U=unmethylated, W=Wild-type specific primer set, respectively. (D) DNA methylation analysis of bisulfite converted DNA (BS converted) by real-time PCR. Amplification with methylated specific primer (M+BS converted), unmethylated-specific primer (U+BS converted) of RAR β , and NTC (No template). 79

Figure 26 Repeatability of LOC based DNA methylation detection system (sample pre-processing & detection module). (A) Long-term stability of grafting primers on silicon microring resonator (SMR) for storage possibility of the detection module. (B) Reusability of the detection module. All experiments were performed using the same SMR chip for detection of the bisulfite converted gDNA. The used chip was washed with NaOH to remove all bound molecules on the surface. (C) Reusability of sample pre-processing module. All experiments were performed using the same sample pre-processing module for bisulfite

conversion of gDNA from MCF-7. The used chip was washed with dH₂O to get rid of all bound molecules on the glass substrate. The DNA-modified was analyzed by MS-PCR using the methyl-specific (M) primer.80

Figure 27 Quantitative study of DNA methylation. Human genomic DNA (1 µg) was extracted from serial dilutions of MCF-7 including RARβ mutation with Jurkat cells (RARβ wild-type). The ratios of MCF-7 cells were 0%, 1%, 10%, 50% and 100%, respectively. Human gDNA (1 µg) extracted from the mixture was bisulfite converted in sample pre-processing module and DNA methylation was quantitated by the detection module. Error bars shows standard error of the mean. (A) Resonant wavelength shifts during amplification on detection module up to 30 min. Dotted line (100% *) represents resonant wavelength shift using bisulfite converted DNA of the sample with 100% MCF-7 and unmethyl-specific primer. (NTC) (B) Resonant wavelength shifts as a function of the ratio of MCF-7 cells. The presence of DNA methylation was successfully detected from a sample containing MCF-7 as little as 1 %. The shaded section represents mutation detected by LOC technique. The dashed line shows detection limit determined by resonant wavelength shift of bisulfite converted DNA from a sample with 0 % MCF-7 cell and unmethyl-specific primer).83

Figure 28 DNA methylation analysis of bisulfite converted DNA by real-time PCR. Human genomic DNA (1 µg) was extracted from serial dilutions of MCF-7 including RARβ mutation with Jurkat cells (RARβ wild-type). Extracted DNA was bisulfite converted under the same condition as on-chip detection. The mutation was detected from a sample containing MCF-7 no less than 10 %. The dashed line represents cut-off value for detection of bisulfite converted DNA determined by threshold cycle of a sample with 0 % MCF-7 cell. (NTC= No template)84

Figure 29 Schematic of Lab-On-a-Chip system for rapid and simple RNA isolation. Chemical lysis was carried out, and the lysate was injected onto the chip with DMA as a binding agent. Before loading, the lysate was treated with DNase and Proteinase K to remove gDNA and protein. As cell lysate is loaded onto the microfluidic device, DMA/RNA conjugates were captured onto amine-modified

polymer chip surface. As the binding reaction is pH dependent, the pH level of the sample was monitored and maintain ~pH 8. After incubation for RNA binding, contaminants and cell debris were removed during washing step while retaining RNA-DMA complex on the surface. After washing, 10 mM sodium bicarbonate (pH 10.6) was used as an elution buffer. Above pH 10, RNA was released from the surface and collected in the elution buffer. Total RNA can be utilized for subsequent downstream analyses.93

Figure 30 RNA isolation process using the disposable microfluidic platform. Cells were lysed and homogenized off-chip using chemical lysis buffer. Then the lysate was mixed with proteinase K and DNase, as well as DMA, followed by sample loading on a microfluidic platform. The lysate was incubated for 10-20 min to complete gDNA & protein removal and binding reaction between DMA-RNA complex and microfluidic channel surface. After incubation, thorough purification is required to remove residual cell debris and impurities before elution. RNA was eluted by increasing buffer pH level thereby reversible reaction occur to release RNA from the chip surface. RNA isolated from LOC system was analyzed for downstream application.95

Figure 31 Numerical simulation of multi-vortices in the microfluidic RNA extraction chip. (A) Fluid flow field calculation by computational fluid dynamics simulation. Contraction-expansion array (CEA) geometries were used in the microfluidic RNA extraction chip. (B) Simulation results in a horizontal plane (x-y plane) at different Reynolds numbers ($Re = 7.4, 15, \text{ and } 37$) illustrate the vortex at the entrance of the expansion area at a high flow rate due to flow detachment. (C) Velocity field calculation (y and z components) in a cross-sectional plane (y-z plane) shows four counter-rotating vortices near the entrance of the contraction area. 100

Figure 32 Water contact angle measurement on the polymer surface. The contact angles were measured on the intact surface and after oxygen plasma treatment, APTES treatment respectively. APTES treatment was performed either at room temperature (3-1) or 65 °C for 1h. 102

Figure 33 Optimization of disposable microfluidic RNA purification chip. (A) Comparison of total RNA purification efficiency on APTES treated the surface with and without DMA. In order to investigate the RNA recovery rate of the assay, 1 μ g of total RNA extracted from HEK-293 cells was injected with DMA as a binding agent(Black) or without DMA(Grey). RNA recovery rate was determined by absorbance at 260 nm using a spectrophotometer. (B) Purity of each sample was determined by ratio A260/A280 104

Figure 34 Effects of DNase and Proteinase K treatment on relative gene expression level. 5 μ l, 10 μ l and 20 μ l of DNase and Proteinase K were added into HEK-293 cell lysates, respectively and RNA was extracted from the assay for expression level analysis. EGFR expression levels relative to a housekeeping gene (18S rRNA) were determined by real-time quantitative RT-PCR. 107

Figure 35 Characterization of disposable microfluidic RNA isolation chip. (A) Effects of input PBS volume during washing step on gene expression level. Total RNA was isolated from 10^6 HEK 293 Cells. Before elution 1ml (LOC) and 2 ml (LOC*) of PBS was used as washing buffer for on-chip RNA isolation. C_T values of reference gene (18S rRNA) were investigated. C_T values of RNA extracted using on-column method is included for comparison. (B) Quantitative analysis of extracted RNA. Total RNA was purified from serial dilutions of HEK-293 cells (10^6 to 10^2 cells) using microfluidic RNA isolation chip and analyzed by real-time quantitative RT-PCR..... 109

Figure 36 RNA purification from various cancer cell lines and gene expression level analysis. RNA was isolated from MCF-7, T24 and Caco-2 cells (3×10^5 cells each) using the microfluidic RNA isolation chip and on-column method. (A) Real-time quantitative RT-PCR of eluted RNA from different cancer cell lines. 5 μ l of eluted RNA from each assay was used as a template. Amplification curves for a housekeeping gene (18S rRNA) are shown (B) Comparison of threshold cycles between microfluidic RNA isolation chip and on-column method. Using the amplification data from A, threshold cycles are compared 112

Figure 37 RNA isolation from various human cell lines and gene expression level analysis. (A) RNA was isolated from HEK 293 (1×10^6 cells each) using the microfluidic RNA extraction chip and on-column method. To analyze gene expression level, real-time quantitative RT-PCR of eluted RNA from different human cell lines was carried out by using 5 μ l of eluted RNA from each assay as template and threshold cycle data of target gene(EGFR) were compared. (B) RNA was isolated from HEK 293, MCF-7, T24 and Caco-2 cells (3×10^5 cells each) using the microfluidic RNA extraction chip and on-column method. From the threshold cycle data of 18S rRNA and EGFR, relative gene expression levels were determined..... 113

Figure 38 Comparison of viral RNA isolation efficiency. In order to compare virus lysis efficiency, Dengue-1 virus supernatant was mixed with chaotropic or non-chaotropic (LB1 and LB2) lysis buffer and loaded onto silica spin column. (Left) Eluted RNA from the spin column was used as a template for real-time RT-PCR for quantitative analysis. Threshold cycle of LB2 is comparable to that of chaotropic reagent from a commercially available kit (chaotropic method). Using non-chaotropic lysis buffer (LB1, LB2) viral RNA extraction from Dengue-1 virus was performed on the chip and eluted RNA was quantified by real-time RT-PCR. (Right) As a result, improvement in amplification was observed using LB2 on chip (LOC1) as compared to LB1 on chip (LOC2). 115

Figure 39 Detection of Dengue virus from eluted viral RNA using microfluidic RNA extraction chip. Viral RNA extraction from three different dengue virus serotypes. The utility of microfluidic RNA extraction chip in virus detection was investigated by using three different dengue virus serotypes (DENV-1, DENV-2 and DENV-4). Virus supernatant was mixed with lysis buffer containing DMA and processed on microfluidic RNA isolation chip. (A) The eluted viral RNA samples were analyzed by real-time RT-PCR. (B) For comparison, conventional on-column isolation was conducted. RNA integrity was also qualitatively assessed by conventional RT-PCR and the following electrophoresis..... 117

Figure 40 Schematic of a microfluidic platform for nanoparticle separation. Track-etched polycarbonate membranes with different pore size were embedded to

capture target molecules. Particles larger than cut-off of the first membrane will remain on the membrane (waste), while target molecules penetrate the first molecule with large pores and capture in microchamber underneath the second membrane with the lower cut-off. Captured molecules are collected in elution buffer by applying negative pressure to microfluidic networks connected to a syringe pump. In order to enhance recovery rate by detaching particles on fouled membrane surface, ultrasonic transducers were incorporated with the microfluidic platform.....	122
Figure 41 Effect of ultrasonic treatment during NP separation	123
Figure 42 Fabrication of microfluidic nanoparticle separation device. (A) Each PDMS layers were patterned by standard soft lithographic technique. Membrane filters were embedded in the interface between the bottom layer and middle layer using uncured PDMS prepolymer. Left reservoir served as a sample loading well. (B) A microfluidic nanoparticle separation device with sample loaded in sample reservoir.	125
Figure 43 Microfluidic device for on-chip nanoparticle separation consists of three PDMS layers. Membrane filters with different cutoff size (15 nm, 30 nm) were sandwiched between the bottom layer and middle layer in the case of 20/ 50 nm nanoparticle separation. Particles larger than cut-off of the first membrane remain in sample loading reservoir, and the rest can penetrate the membrane. Particles and buffer solution can go through the second membrane with smaller cut-off (15nm), while the target size particle (20nm) stay in chamber region between those two membrane filters.....	127
Figure 44 60 nm Au NP filtration using membrane filter with 80 nm pore size cutoff embedded in the microfluidic size based particle separation device. (A) The absorbance was measured using the feed, filtrate, retentate sample collected after filtration without ultrasonic irradiation. (w/o US) (B)The absorbance results were compared to the case with ultrasonic irradiation (w/ US). (C) In the presence of ultrasonic irradiation, 70.6 % of input 60 nm particles were successfully collected while 21% of input particles were collected without ultrasonic treatment.	130

- Figure 45 Comparison of particle removal rate under different surface condition (wet/dry). Under the dry surface condition, particles irreversibly formed cake layer near membrane filter surface yielding 65 -70 % recovery rate. On the other hand, most of the input particles are collected when membrane filter remains wet during the filtration. 133
- Figure 46 Fractionation of 100nm polystyrene NP from polydisperse mixture solution by using ultrasonic assisted microfluidic particle sorting. Dynamic light scattering (DLS) spectra were obtained from filtrate (Green) and a mixture containing 1:1 polydisperse 100nm and 400 nm particles (Black). Light scattering spectra of a stock solution of 100nm and 400 nm were obtained for comparison. (Red and blue respectively) Size distribution of filtrate shows that size of particles collected in the filtrate is close to 100 nm stock solution. 135
- Figure 47 Effect of the orientation of ultrasonic treatment on recovery rate. Ultrasonic wave with 40 kHz, 40Vp-p output voltage generated by ultrasonic transmitter was focused onto membrane filters either at the permeate side or feed side of the membrane. (A) Layouts of the microfluidic systems for particle filtration for ultrasonic treatment from the permeate side (left) and the feed side (right) of the membrane filters. The distance between the transducer and membrane remains 300 μm for the experiment. (B) Absorbance spectra of 60 nm Au NP collected in the filtrate solution by ultrasonic treatment from the permeate side (left) and feed side (right) of the membrane filters were compared with the absorbance using an original stock solution of 60 nm Au NP. 137
- Figure 48 Volumetric flow rate of sample filtered using microfluidic particle separation system as a function of filtration time. Samples containing 60 nm Au NP were processed using the microfluidic system with ultrasonic irradiation from the feed side (green), permeate side (blue) or without ultrasonic treatment. As a result, a decrease in flow rate was observed in three cases. However, the highest flow rate was achieved with ultrasonic irradiation from the feed side, and the steady flow rate was observed after 600 sec of the filtration process. For the case of the ultrasound treatment from the permeate side, it showed the same level of fouling as the case of no ultrasound treatment, which indicating that the

ultrasonic irradiation is not effective when the ultrasonic waves propagate from the permeate side of the membrane filter.....	139
---	-----

Abstract

With the steady growth in microfluidic technologies, substantial advance in Lab-on-a-Chip system has been observed, which enables miniaturization and integration of complex functions that had been routinely performed by hand in the traditional analytical processes into micro-scale small devices. One of the most active applications of these technologies is Point-of-care (POC) diagnostic device that can rapidly deliver the diagnostic outcomes to patients. POC diagnostic system provides the users with many benefits over the traditional diagnostic methods such as straightforward and easy-to-use interface, portability, less sample and reagent consumption, etc.

This thesis presents novel microfluidic LOC techniques which are potentially suitable for POC diagnostic testing. Of the three technologies described in this thesis, the first two of LOC methods are to address current issues regarding nucleic acid analysis in POC testing. The first is LOC system for the detection of DNA methylation by combining on-chip bisulfite conversion and detection on a silicon biophotonic sensor. By performing bisulfite conversion reaction, an essential sample processing step in DNA methylation detection, within the microfluidic platform, it was able to overcome limitations that existing DNA methylation methods are facing, such as DNA degradation, long sample processing time, low amplification efficiency, etc. This novel method has great potential as a useful tool in epigenetic biomarker studies for both research and clinical use. The second approach is LOC system for RNA isolation. High-quality, intact RNA from various biological samples can be isolated within the disposable microfluidic platform for downstream diagnostic analysis with reduced time and minimal hands-on processes. Gene expression studies, as well as detection of infectious disease, were performed using the isolated RNA from LOC system to show

the feasibility of proof-of-concept. This novel microfluidic approach offers a new paradigm of RNA isolation that can be potentially used in many diagnostic applications at point of care.

The last LOC technique described here is to enhance nanoscale biomolecule separation by integrating dead-end filtration and ultrasonic treatment into small-scale microfluidic platform which aims to perform particle separation as a preparative process in POC diagnostic testing. The functionality of this novel LOC based technology was confirmed by demonstrating size based gold and polymer nanoparticle separation.

Introduction

1.1. Background

1.1.1. Motivation

Over decades, mortality of human diseases such as cancers has been decreasing with the advance in technology and medical science. However, incidence of cancer, as opposed to this trend, are expected to further increase by 70 % to 22 million new cases over next two decades, according to the World Cancer Report 2014.¹ In addition, the rapid growth of interest in health care and increasing average life expectancy have led to growing demand for medical checkup to screen for various diseases such as cancers, diabetes, and infectious diseases, etc. However, in places where the resources are insufficient (developing countries, rural areas), current health screening system, which mostly relies on testing in the centralized lab, hinders its accessibility and frequency. And also, patients who are less mobile have difficulties in visiting health care facilities in regular intervals. Accordingly, this has led to a huge demand for Point-of-Care testing (POCT) systems for efficient diseases monitoring and treatment. Since the testing can be carried out near the patient, the diagnostic process is simple, rapid, and cost-effective. In resource-limited settings, the high cost of health screening is also one of the key factors that hamper the regular checkup for early stage detection of diseases. The turnaround time plays an important role in the clinical decision-making process for successful treatment. To address these unmet needs, a number of studies in developing miniaturized devices have been conducted, which can perform analysis of diseases outside the laboratory.

Microdevices containing several functional elements in micrometer scale (10 nm ~ 0.1 mm) such as micromixers, microchannels, micropumps, microfilters, and microheaters

are miniaturized platforms that can be used for POCT. Microdevices, also referred to as Lab-On-a-Chip (LOC) devices, can offer the diagnostic results using a low amount of reagents and buffers, consuming less power with little contamination of the sample. And it also enables automation of processes by integrating a few laboratory functions on a small scale single device. Therefore, it can meet the requirements of the new healthcare system for people in regions that lack resources (i.e. electricity, storage facilities, trained personnel) and less mobile patients. In this thesis, development of Lab-On-a-Chip system that allows for rapid, simple, and inexpensive Point-of-Care testing will be discussed. It will primarily involve the identification of methods for 1) Manipulation of the fluid flow of bio-samples (e.g. blood, urine) to integrate the functional components on a single microfluidic device, which can potentially replace existing diagnosis processes based on centralized laboratory testing, 2) realization of easy sample handling with minimized sample contamination and 3) enhancement of the sensitivity of the sensor for detection.

1.1.2. Microfluidic DNA based diagnostic system

Over past two decades, DNA-based genetic testing has moved towards developing miniaturized and automated platforms to monitor DNA biomarkers in clinical settings. Despite its high sensitivity and specificity, conventional DNA-based biomarker testing technologies cannot meet the requirements of Point-of-Care clinical diagnostics, which has led to a transition to portable and miniaturized DNA testing platforms. This is mainly because its usage is primarily concentrated in centralized laboratories and hospitals equipped with sophisticated diagnostic tools and expensive instruments. In particular, microfluidic lab-on-a-chip (LOC) system has provided simple and rapid micro-scale analysis environment in cancer diagnostics with high sensitivity, high throughput and low reagent consumption. Due to these benefits, a number of publications and research

works regarding LOC systems for DNA biomarker detection have been reported. State-of-the-art microfluidic-based biosensors for genetic testing or rapid diagnosis of infectious diseases use various physiochemical detection methods such as electrochemical, optical, electrical and physioelectric, etc.

Even though the majority of existing cancer biomarker screening techniques are focused on genetic analysis, there has been growing demand for miniaturized LOC system that can perform epigenetic biomarker detection in clinical settings. Epigenetic alterations of genome, such as DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing are known for regulating gene expression that does not involve changes in the nucleotide sequence, which possibly leads to cancer development and progression.^{2,3} Therefore, development of analytical systems for POC epigenetic testing is emerging as an important issue. In order to realize nucleic acid based epigenetic testing for POC clinical diagnosis, a sample-in-answer-out, fully integrated and automated analytical system, which reduces the risk of contamination by simplifying the working process and eliminating the need for the high-cost instrument is preferred. In this thesis, one of epigenetic analysis in POC application, DNA methylation detection will be discussed.

1.1.3. POC RNA isolation technique

Nucleic acid testing is one of the most common applications of miniaturized diagnostic system, which has been employed as a useful tool for monitoring diseases and determining the suitable treatment method in a very short period. As a result, there has been a significant advance in POC nucleic acid testing. However, the majority of these techniques rely on the use of DNA as a biomarker for disease diagnosis. Inherent issues associated with the use of RNA as a biomarker such as low stability, high degradation due to RNase activity makes RNA analysis more challenging than DNA analysis and

precautions are needed for sample handling to avoid RNA degradation. As RNA analysis plays an important role as well in the diagnosis of genetic diseases like cancer, quantitative studies of gene expression and infectious disease, development of a miniaturized diagnostic system based on RNA testing is unavoidable to realize POC RNA testing.

To realize such system for POC application, a few LOC techniques that can perform RNA testing have been developed, however, the majority of these techniques are concentrated to detection of target RNA which entails off-chip RNA isolation. Conventional RNA isolation methods, such as phenol-chloroform based extraction⁴, Oligo-dT based affinity chromatography⁵ and silica column based purification cannot be easily integrated into a compact and miniaturized platform that is needed in POC testing due to their complexity. Lack of reliable LOC based RNA isolation techniques has limited the use of isolation technology for RNA testing to research purpose which can be performed in laboratories. Therefore, development of LOC RNA isolation method that facilitates the RNA handling is necessary to realize solid RNA analysis platform for Point-of-care application for both research and clinical use.

In order to overcome the challenges involved in RNA isolation, there have been a few attempts to bring RNA extraction onto microfluidic chip assay. But these techniques are not widely applicable for POC application due to technological limitations such as contamination of final eluent with chaotropic agents, incompatibility with certain cell types or complexity in the manufacturing of platforms. To solve the problems current techniques are facing, I developed a disposable microfluidic platform for RNA isolation which can be potentially used for both research and clinical use. In this thesis, demonstration of RNA analysis using the sample prepared on the disposable

microfluidic platform for gene expression analysis as well as infectious disease detection will be discussed.

1.1.4. Lab on a chip system for nanoparticle separation for POC application

In biomedical research, size based particle separation plays a major role in sample preparation process as well as detection of target species. As the size of analytes in biological research range from angstrom to millimeter scale, separation mechanism of choice depends on their dimensions. During the past decade, many size based separation techniques have been established for biomedical application.⁶ Enormous progress has been made, and size based separation technologies move towards the miniaturized platforms to meet the requirements for point of care testing.^{7,8} However, the use of the miniaturized LOC based separation techniques is limited to sub-micron scale⁹⁻¹² due to the technological limitation, which resulted in inefficient separation that causes large sample loss, diagnostics misinterpretation as well as poor detection limit. To obtain insights into the various biological system in nanometer scale, development of robust and reliable size based particle separation technique is emerging as an essential prerequisite for nanoscale measurement.

For nanoscale biomolecule sorting, several size-dependent nanoparticle separation techniques such as technology based on external fields application, physical sieving and size selective precipitation have been carried out in biomedical research. Unfortunately, commonly used current technologies for nanoparticle separation were still not sufficiently efficient in selectively sorting particles based on their size or molecular weight for proper POC use due to their complex hands-on processes and incompatibility with downstream analytical devices. To eliminate the need for bulky and sophisticated instrument and large input material and minimize the complexity of the device operation, miniaturized and compact platform for size-based separation, which can be

used as a cost-effective preparative method is more favorable compared to one performed in the centralized laboratories.

In this thesis, a microfluidic lab-on-a-chip system with embedded ultrasonic transmitter for efficient particle sorting will be discussed. This novel LOC system can perform rapid size based nanoparticle separation by dead-end filtration, which is suitable for POC application. This novel microfluidic LOC system holds great potential as a useful tool that provides many advantages over other microfluidic size-based technique such as simple fabrication, easy end-user interface and high recovery rate of target size NP.

1.2. Thesis objective

As discussed above, the use of molecular diagnostics is still limited to laboratory based research due to their complexity, low efficiency and labor intensive processes. Challenges associated with the use of conventional diagnostic medical technologies have been a driving force behind the development of microfluidic diagnostic technologies. To address the technological issues regarding the current laboratory based diagnostic processes, novel microfluidic LOC systems are presented in this thesis.

As nucleic acid testing is the most powerful tool for rapid and early diagnosis of many diseases, realization of reliable diagnostic platform for NAT that can be practiced near patient environment is of great interest in POC application. To perform fully integrated NAT on microfluidic platform, development of multiple functional units such as biosample processing, DNA assay, RNA assay is necessary. Therefore, I present a new microfluidic approach for DNA based epigenetic screening system which is relatively unexplored. It is often useful to be able to detect multiple diseases simultaneously using the same specimen from patient, therefore, RNA detection as well as DNA assay is essential. Therefore, I present a RNA isolation module based on microfluidic LOC

system that can provide high quality RNA from various biosamples. To ensure high sensitivity in downstream analysis, cell enrichment of target cell subpopulation is also very important. Pre-concentration of target size cells of interest can greatly improve assay performance in downstream analysis. As a cell enrichment tool, microfluidic size-based particle separation system is suggested. Those microfluidic LOC systems presented in this thesis will be able to configure fully integrated system that provides simultaneous NAT investigating multiple diseases using both RNA and DNA.

Figure 1 Fully integrated microfluidic molecular diagnostic platform.

This allows for miniaturization of instrument and integration of multiple functionalities on microfluidic devices, as well as reducing analysis time, contamination, and the amount of reagents needed. These disposable microfluidic LOC systems are fabricated with inexpensive material by simple and rapid fabrication processes. And the system will be user-friendly by automating manipulation of sample solution and buffers.

1.3. Thesis outline

In this thesis, novel microfluidic LOC systems for POC diagnostic testings will be discussed. Chapter 2 describes the literature review of LOC systems for 1) DNA methylation analysis, 2) RNA isolation and 3) microfluidic particle separation. Chapter 3 describes an epigenetic analysis, DNA methylation analysis, on LOC system for POC cancer diagnosis. LOC based bisulfite conversion, an essential preparative process, followed by detection using optical biosensor will be discussed. Chapter 4 describes the principles of RNA isolation on a disposable microfluidic device for genetic analysis and infectious disease diagnosis. Chapter 5 presents size-based nanoparticle separation and investigation of the main factors that affect the separation performance. Chapter 6 & 7 summarize the features of each system described in previous three chapters and conclude with future works associated with the development of more comprehensive LOC systems for POC diagnostic testing based on the techniques described in Chapter 3 – 5.

Literature Review

This chapter presents a review of the Lab on a chip systems that have been developed for molecular based diagnostics at Point of Care settings. For molecular testing, the LOC systems have been designed to perform sample preparation on microfluidic platforms such as size based sample separation, cell lysis, purification as well as optical detection of the sample on the chip. This chapter presents a review of various LOC technologies for epigenetic analysis, genetic testing based on RNA analysis and microfluidic size based particle separation techniques as a preparative process to enhance a sensitivity in a subsequent analysis.

2.1 Introduction

Lab-on-a-chip technology has been extensively utilized to build a miniaturized diagnostic platform especially for point-of-care testing. LOC is typically based on microfluidic technology that can handle a minute amount of liquid by taking advantages of microscale channel, which makes it easier to manipulate, transport and chemically process the fluid in automated manner. LOC technology has experienced a great advance for the past two decades with the aid of molecular biology along with microfluidics. Molecular diagnostics which normally requires laboratory or bulky instruments can take place on a small chip. Among many applications of LOC technology, the majority of LOC studies so far has been focused on diagnostic platforms that is based on molecular testing for this reason.

One good example of LOC based molecular testing is nucleic acid testing (NAT). The greatest advantages to using NAT is its high sensitivity and specificity, which is crucial for effective diseases monitoring for genetic (e.g. Cancer) and infectious disease diagnosis. LOC platforms offers a lot of laboratory functions such as cell separation,

nucleic acid isolation, chemical reaction and detection on a single microscale chip by integrating them with microfluidic network.

To optimize each functional unit, modular approach has been widely taken. Modular approach subdivides an LOC system into smaller units so each unit can serve as an independent device. A modular approach is advantageous giving the system more flexibility in design as well as high level of customizability to create a novel diagnostic platform with lower cost for the development of LOC system by simply adding or removing the individual modules. In this thesis three LOC system with distinct function necessary for molecular diagnosis (**DNA Methylation analysis system, RNA isolation system and particle sorting system**) are presented which can be potentially utilized in point of care testing. Each LOC system can be used independently or incorporated with other functional unit depending on purpose of use. (e.g. Genetic studies, Epigenetic analysis, Infectious disease detection etc.)

In the first section of this chapter, I present a variety of lab on a chip based approaches for DNA methylation analysis and epigenetic testing for cancer diagnosis. This section has subsets describing microfluidic platforms developed for sample preparation and pretreatment and biosensors for DNA methylation detection. In the second section, I present microfluidic approaches for RNA isolation for genetic testing and infectious diseases. Lastly, in the third section, I present existing Nano/micro particle sorting techniques using LOC platform with different separation principle. At the end of this chapter, the section identifying the existing research gap and describing the novelty of the LOC systems proposed in this thesis is included.

2.2. LOC based approaches for DNA methylation analysis

LOC based approaches have revolutionized molecular diagnosis by offering platforms that require only a minute amount of sample to run diagnostics, drug delivery, single

cell level analysis and immunoassay since the small sample loss may lead to distorted measurement results. LOC based approaches have been demonstrated extensively for the genetic diagnosis and extended their application to epigenetic studies recently, due to increasing demand for epigenetic analysis in early stage cancer diagnosis. DNA methylation analysis is particularly suitable application of LOC based approaches since the hypermethylated DNA, which is known to be related to abnormal gene silencing that may lead to cancer development, in typical clinical samples is very rare. Hence, the ability to manipulate small volume of sample of microfluidic technology can make huge enhancement in sensitivity and through-put while reducing the cost and analysis time. In this thesis, I present a novel LOC based approaches for DNA methylation analysis. The LOC platform presented in this thesis consists of two modules; 1) The microfluidic platform for the sample preparation and DNA enrichment, which is crucial in DNA methylation analysis, 2) The detection module based on biophotonics sensor. Therefore, I reviewed various microfluidic platforms developed for sample preparation processes and the LOC based detection methods in the following section.

2.2.1. Microfluidic sample preparation platforms for DNA methylation analysis

Development of microfluidic based sample preparation methods is essential to bring DNA methylation analysis processes on the miniaturized platform that is suitable for the point-of-care application. The sample preparation method depends on the mechanism of DNA methylation detection. Among a variety of DNA methylation detection methods, bisulfite based analysis is the current gold-standard technique in benchtop application. This is mainly due to its high resolution (single base pair level) and repeatability. However, due to challenges involved in bisulfite conversion of DNA during the process such as intricate purification and incubation steps, application of

bisulfite conversion to microfluidic sample preparation has not yet been vigorously pushed forward so far.

One of recent researches on microfluidic sample preparation based on bisulfite conversion is reported by Stark *et al.*¹³ (Fig. 2) The whole process of microfluidic bisulfite conversion in this paper is operated by droplet based magnetophoresis. The microfluidic channel has multiple micro-chambers interconnected by microfluidic network and chemical reactions and purifications steps are carried out in each chamber sequentially. DNA immobilized on the silica coated magnetic beads are delivered to laterally along the microfluidic channel by applying magnetic field. Beads with DNA can be transported to subsequent droplets for deamination, desulfonation and sequential washing steps to complete bisulfite conversion on chip. By taking advantage of reduction in sample volume by constraining the reagents within a droplet for sample preparation steps, the processing time and reagent consumption can be greatly reduced and the sample manipulation is streamlined.

Figure 2 Overview of magnetophoretic bisulfite conversion process based on microfluidic droplet manipulation. Each droplet contains reagent necessary for bisulfite conversion.¹³

However, this microfluidic bisulfite conversion platform needs further quantitative studies since the processing time is not explicitly specified in the paper. And the use of this technique is limited to manual loading and recovering, therefore, restructuring and rearranging of current microfluidic design is necessary to build integrated LOC system for DNA methylation analysis in further studies.

Due to low amount of hypermethylated DNA in biosamples, enrichment and pre-concentration of hypermethylated DNA prior to detection is critical to enhance sensitivity and detection limit in subsequent detection processes. Microfluidic platform can serve as a useful tool for the solid phase enrichment of hypermethylated DNA by leveraging its ability to capture DNA on its microchannel surface. De *et al.*¹⁴ employed specific binding between Methyl-binding domain (MBD) protein and hypermethylated DNA to capture hypermethylated DNA on MBD functionalized microfluidic surface.

(Fig. 3) The silicon microfluidic platform consists of many of micro-pillars to increase surface area where abnormally hypermethylated DNA from cancer cell-lines can be possibly captured. The captured hypermethylated DNA is enriched by elution in a reduced final elution volume. The MBD protein based microfluidic hypermethylated DNA enrichment method could achieve rapid enrichment/purification of hypermethylated DNA, 28-fold enrichment factor with 71 % of recovery efficiency.

Figure 3 Microfluidic platform with micro-pillar array for enrichment of hypermethylated DNA. Hypermethylated DNA can be captured onto the microfluidic surface via specific binding between hypermethylated DNA and MBD protein.¹⁴

However, this MBD protein based enrichment method showed very high level of non-specific capture of non-hypermethylated DNA, which is presumably due to non-specific binding between the silicon surface and non-hypermethylated DNA. This may cause false positive results in further measurement of DNA methylation status. Surface treatment to deposit blocking layer is necessary to address the issue regarding the non-specific binding.

2.2.2. Biosensors for DNA methylation analysis

As one of the aims of this thesis is to develop a DNA methylation assay based on biosensor as a detection module, various biosensors those have been used for DNA methylation analysis are reviewed in this section.

Conventional methods for DNA methylation analysis can be classified into three categories based on their differentiation mechanism. To differentiate methylated DNA of a gene of interest from unmethylated DNA, the sample can be pretreated by bisulfite conversion, restriction enzyme digestion, or affinity enrichment. As reviewed in section 2.2.1., bisulfite conversion is the most widely used method for DNA methylation detection, which selectively converts unmethylated cytosines to uracils while methylated cytosines remain intact so that the DNA methylation status can be detected by sequence difference. The restriction enzyme digestion methods employ restriction enzymes, HpaII or MspI. By selective digestion depending on methylation status of cytosine, gene-specific methylation analysis is carried out using the digested DNA sample. Typically, the pretreated DNA samples undergo amplification via PCR and detected by different techniques such as Methylation specific PCR (MS-PCR), Combined Bisulfite Restriction Analysis (COBRA), bisulfite sequencing, or microarray analysis. One of well-established techniques that is successfully applied to commercially available assay is microarray based analysis. Illumina Infinium assay analyzes DNA methylation of human genomic DNA samples by whole-genome amplification of bisulfite converted DNA followed by fragmentation and hybridization of the targets to methylation-specific DNA probes.¹⁵ These conventional methods of DNA methylation analysis have led to remarkable scientific breakthrough in DNA methylation studies. However, due to inherent limitations of these techniques, the use of conventional methods has been limited to centralized laboratories. Typically, detection processes involved in conventional DNA methylation analysis are time-consuming and labor-intensive. And the results are easily influenced by the operator and the sample quality. Moreover, the DNA sample is vulnerable to contamination during complicated sample treatment and amplification. Hence, development of novel method

of DNA methylation detection that can streamline the protocol and perform rapid detection of DNA with minimized human intervention is required to realize POC DNA methylation analysis.

Biosensors offer many advantages as a detection platform for DNA methylation analysis. Typically, biosensors enable miniaturization of DNA methylation assay and detection of DNA methylation biomarkers by utilizing a variety of recognition element specific to target (hyper -methylated DNA) for quantitative and qualitative measurement of DNA methylation level. And biosensors can be easily integrated with microfluidic platform, which is potentially practical and desirable to build the fully integrated DNA methylation monitoring system for POC application. The biosensors for DNA methylation analysis can be classified by detection mechanism into a few categories: 1) electrical, 2) electrochemical and 3) optical. Those biosensing techniques can also be classified according the use of labeling: 1) label-free and 2) labeled detection methods. Labels have been used to allow the biosensors higher specificity and sensitivity in detection. Fluorescent and nanoparticle based labelings are the most commonly used detection scheme. However, due to complex labeling processes and end-point data acquisition, label-free methods are preferred as a detection scheme for POC DNA methylation analysis.

2.2.3. Biosensors for label-free DNA methylation detection

A few of label-free DNA methylation detection methods employ electrochemical biosensing scheme. Electrochemical biosensors have been widely used as a diagnostic tool due to their simplicity, rapidity and high sensitivity. The use of an electroactive indicator such as Methylene Blue (MB) or $[\text{Ru}(\text{NH}_3)_6]^{3+}$ transduce the methylation status of the target gene in biosamples through measurement of the current signal, which allows for detection of analyte near the sensor surface. In one study reported by Wang

et al., label-free DNA methylation detection was demonstrated electrochemically using the peptide nucleic acid (PNA) probe immobilized on the glassy carbon electrode (GCE) with the choline ($\text{HOCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3\text{Cl}^-$; Ch) monolayer.¹⁶ (Fig. 4)

Figure 4 Electrochemical biosensor based DNA methylation detection. (A) The DNA methylation status of the p53 gene is recognized by the current signal of electroactive indicator interacting with negatively charged DNA backbone hybridized with PNA probe on electrode surface. (B) The DNA methylation status was quantitatively studied using the electrochemical biosensor. The current signal of $[\text{Ru}(\text{NH}_3)_6]^{3+}$ indicates DNA methylation level.¹⁶

The surface of Ch/GCE was modified by gold nanoparticles to increase effective surface area. For detection of methylated cytosine, DNA sample was bisulfite converted to induce sequence difference. The PNA probe on the electrode can selectively capture hypermethylated DNA, whereby the DNA methylation status of the gene is recognized by the current signal of $[\text{Ru}(\text{NH}_3)_6]^{3+}$ indicator that can form binding by electrostatic force with negatively charged DNA backbone bound to the electrode. This label-free electrochemical biosensor showed highly sensitive (pM level) analysis of methylation status of p53 gene in case of fully methylated DNA. However, both unmethylated and partially methylated DNA show low binding efficiency, which makes it difficult to detect single base mismatch by measuring current signal of the electrochemical indicator.

Another method of label-free DNA methylation detection is based on electrical biosensors. There have been a few attempts using electrical biosensors such as field

effect transistors (FETs) or nanopore to detect methylated DNA by means of changes in capacitance, impedance or conductance in the presence of target molecules. Electrical biosensors typically exhibit higher sensitivity while reducing analysis time as compared to electrochemical ones. FETs are one of sensing platforms that have been often utilized to electrically analyze biomolecules without labeling. Recently, the application of FET biosensors is expanding into DNA methylation analysis. Typically, binding of the biomolecules with receptor such as antibody, oligonucleotides that specific to the target induces changes in the electrostatic potential at the surface of the semiconductor, which results in a change in conductance of the FET channel. (Fig. 5) One of DNA methylation detection methods based on FET is presented below.¹⁷

Figure 5 Nanowire-field effect transistor (FET) biosensor for ultrasensitive detection of methylated DNA.¹⁷

The methylation status of the promoter region of p16^{INK} gene was analyzed using nanowire-FET. The off-chip enrichment of target DNA was performed prior to detection and captured onto silicon nanowire surface functionalized with anti-5-methylcytosine antibody, which is specific to methylated cytosine. By measuring changes in current, the presence of the methylated DNA is recognized. This method can achieve low detection limit, however, the use of anti-methylcytosine antibody has limited its

application to the semi-quantitation of the total amount of methyl-cytosine in the sample, which makes it impossible to perform sequence-specific methylation analysis.

Lastly, optical biosensors based approach is another attractive label-free alternative that can possibly replace current laboratory based DNA methylation assays. Optical biosensors measure the changes in absorption, emission, reflectance or resonance of incident light traveling within the nanostructures of devices that offers highly sensitive detection of biomolecules bound to sensor surface with the aid of receptors. Optical biosensors are also employed for fluorescence assays, however, many researches reported recently combined optical biosensing technologies with label-free recognition method to leverage its simplicity and rapidity. One of the most actively utilized optical detection methods is based on surface plasmon resonance (SPR) detection. SPR detects changes in refractive index at the surface of a sensor that typically consists of a thin gold coating on top of glass substrate. A portion of the incident light couples into the thin gold coating, whereby surface plasmon wave is generated along the surface of the gold layer. The changes of refractive index at the surface is induced by binding of biomolecules, therefore, target molecules can be detected by monitoring the changes in refractive index optically, which can also be applied to direct detection of DNA methylation. Sina *et al.* has reported a label-free DNA methylation detection by employing SPR biosensor.¹⁸ (Fig. 6) The single strand-DNA amplicons of bisulfite converted sample are bound onto gold layer of SPR sensor with different affinity determined by adenine content. Taking advantage of higher affinity of adenine towards gold, unmethylated DNA exhibit higher adsorption level that results in higher SPR spectral shift. As with other optical biosensors, such as silicon microring resonator (SMR), SPR enables label-free and real-time detection of DNA methylation.¹⁸

Figure 6 After bisulfite conversion and asymmetric PCR of DNA sample from breast cancer cell lines, DNA is adsorbed onto gold layer of SPR sensor with affinity depending on adenine content, which indicates the methylation level by monitoring SPR spectral shift.¹⁸

Comparison of conventional and current state-of-art label-free DNA analysis methodologies is summarized in Table 1.

Table 1 Comparison of conventional and current state-of-art label-free DNA analysis methodologies

Assay	Detection principle	Assay time	Detection limit	Quantitative	Advantages	Limitations
MS-PCR ¹⁹	Bisulfite conversion+ Amplification	>3h	0.1-1%	No	-Gold standard -Very high sensitivity/ specificity -Reliability	-False positive -Cannot integrated into microfluidic system
Methylight ²⁰	Bisulfite conversion+ fluorescence-based real-time PCR	>2h	0.1%	Yes	-Very high sensitivity -Low false positive	Limited to detection of fully methylated/unmethylated alleles
Combined bisulfite restriction analysis (COBRA) ²¹	Bisulfite conversion+ PCR+ restriction enzyme digestion	5h	3%	Semi-Quantitative	-Very high sensitivity/ specificity -Reproducibility	-Limited to detection of specific restriction region -Time consuming processes
DNA microarrays (Illumina)	Bisulfite conversion+	3d	Single base resolution	Yes	-High resolution	-Expensive

	Fluorescence based amplification on microarrays				-Very high throughput	-Time consuming processes
Photoelectrochemical immunosensor ²²	Electrochemical (Bi ₂ S ₃ nanorod)	<1d	3.5×10 ⁻¹⁴ M	Yes	-High resolution base mismatch)	-Relatively low sensitivity compared to PCR based techniques.
Cyclic voltammetry (CV) ¹⁶	Electrochemical [Ru(NH ₃) ₆] ³⁺	-	18 pM (Fully methylated)	Semi-Quantitative	-Simple, rapid	
Si Nanowire FET biosensor ²³	Electrical (anti-5-methylcytosine antibody)	>2h	2.5×10 ⁻¹⁹ M	Yes	-Low detection limit	-Semi quantitation -Not applicable to sequence-specific analysis
SiN Nanopore ²⁴	Electrical (MBD1 proteins)	-	Single dinucleotide resolution	Semi-quantitative	-Simple	-Relatively low sensitivity
SPR ¹⁸	Optical (Affinity adsorption between gold coating-DNA)	~40 min	5 pM	Yes	-High sensitivity	-Time consuming off-chip pretreatments (Bisulfite conversion, PCR)
Piezoelectric biosensor ²⁵	Mechanical (Quartz-crystal microbalances)	<1h	20 nM	Yes	-High reproducibility -Cost effective -Rapid detection	-Limited to specific frequency range operation (~50MHz)

2.3. Microfluidic approaches for RNA isolation

Miniaturized diagnostic systems those are capable of performing nucleic acid test (NAT) have been developed by taking advantage of microfluidic technologies. Due to its high sensitivity and specificity, the use of NAT has been increasing for diagnosis of infectious diseases as well as genetic diseases. However, the pretreatment of biosamples, such as whole blood, tissue, saliva, serum or urine, prior to NAT for collection of nucleic acids is labor-intensive, involving multiple sample loading, purification, recovery steps during which large sample loss and sample contamination occur. Despite a huge progress in detection techniques and analytical tools, NAT is carried out in laboratory by sending the specimen from patient because of inefficient pretreatment processes. To address this issue, tons of nucleic acid (DNA, RNA) preparation methods based on microfluidic technologies have been recently reported. Microfluidic nucleic acid preparation methods enable automated sample processing with reduced preparation time and lower the risk of contamination during the processes. And they greatly reduce sample and reagent consumption to obtain the required amount of input nucleic acid to

run subsequent analysis. However, most of microfluidic techniques recently reported for sample preparation (lysis, nucleic acid isolation) are focusing on DNA rather than RNA. This is mainly due to challenges involved during RNA isolation, such as high level of degradation of RNA results from RNase digestion. To address the increasing demand for reliable diagnostic system that can be used at clinical setting, stable and reproducible RNA isolation method is desirable. In the next section, the state-of-art microfluidic nucleic acid preparation methods are reviewed focusing on RNA isolation.

2.3.1. Conventional RNA isolation techniques

Conventional methods of RNA sample preparation have employed a few isolation techniques such as phenol-chloroform-based extraction,⁴ Oligo-dT-based affinity chromatography,^{26,27} and silica-based purification.²⁸ A great advance has been made in those RNA isolation techniques exhibiting a high yield and quality. However, translation of those techniques to automated platform is quite challenging since they require multiple purification processes as well as centrifugation. And contaminants-carryover is one of those issues regarding the use of the conventional methods based on phenol and chaotropic reagents. Therefore, a novel RNA isolation method that enables automated on-chip processing based on microfluidic technologies are necessary.

2.3.2. Microfluidic RNA isolation techniques

Since microfluidic RNA isolation remains relatively unexplored as compared to DNA isolation, there have been very few researches reported in literature due to the issues aforementioned. Microfluidic RNA isolation techniques recently reported are typically isolate RNA from biosamples via various lysis scheme (chemical, mechanical, thermal and electrical) and capture RNA strands by using solid phase substrate or matrix adjusting buffer condition to enhance RNA affinity. Then the RNA bound to the substrate goes through washing in continuous stream to remove cell debris as well as

reagents used for lysis. Finally, RNA was eluted in a small volume for downstream analysis.

Silica-based solid-phase extraction (SPE) is one of the most well established microfluidic approaches used for nucleic acid preparation. RNA isolation has been presented using SPE techniques in recent literatures. Silica is structured into microfluidic channel as a membrane, bead or matrix. When the sample (lysate) containing RNA is loaded onto microfluidic platform, RNA is captured onto silica structure with the aid of chaotropic salt. Other residual impurities such as protein from the lysate pass through the structure and are discarded as a waste during washing process. As shown in Fig. 7A, silica membrane is simple to integrate into microfluidic platform by inserting the membrane in between PDMS layers with spacing that can hold membrane without leak.²⁹ This microfluidic device works in the similar manner as silica column that is employed in a large number of commercially available nucleic acid purification kit. However, by using microfluidic network, the whole purification process can be automated and streamlined requiring less manual handling as compared to the column method. As with column based method, this microfluidic SPE technique uses alcohol to enhance nucleic acid binding. Alcohol (ethanol, isopropanol) is potential PCR inhibitor that affect the enzymatic reaction in downstream analysis.

Figure 7 Silica based SPE devices. (A) The disposable silica cartridge consists of PDMS layers and silica membrane integrated into microfluidic system for automated DNA/RNA isolation.²⁹ (B) Microfluidic device with silica bead packed

within microchannel. Silica beads are coated with chitosan to avoid the use of chaotropic reagent for binding.³⁰

Hence, a few alternatives have been devised to avoid using alcohol. Microfluidic oligo(dT) affinity purification techniques bypass the use of alcohol by employing the selective binding between poly (A) tail of mRNA and oligo(dT). The photo-initiated methacrylate-based porous polymer monolith (PPM) in microfluidic channel functionalized with oligo(dT) or paramagnetic beads coated with oligo-dT's were used to capture and trap RNA taking advantage of their large surface area.^{31,32} The use of magnetic beads exhibited low RNA isolation efficiency and required extra handling process. PPM can be easily integrated into microfluidic format and offers simple operation. However, the use of this technique relies on poly-A tail of mRNA, therefore, is limited to the analysis of the gene expression of eukaryotic cells.

Another microfluidic approach bypassing the use of chaotropic agents has been developed that can perform RNA isolation in an aqueous solution.³⁰ (Fig. 7B) Chitosan has been known to interact with DNA by forming DNA-Chitosan binding in solution depending on ionic strength of solution. This technique can also be used for RNA binding onto microchannel via surface modification of silica particles or microchannel walls with chitosan prior to RNA isolation. The buffer condition was adjusted to establish favorable ionic strength for RNA binding. Since chaotropic agent is not required in this process, it enhances quality of isolated RNA lacking the PCR inhibitors commonly used in SPE based RNA isolation. Since the RNA-chitosan binding depends on the buffer pH, adjustment of buffer pH enables reversible RNA binding. RNA is adsorbed to the positively charged chitosan under low pH condition (Capture mode) and the binding can be reversed at the pKa of the amino group above which RNA is released from the silica substrate (Recovery mode). This method achieved a higher RNA

recovery rate (71%) than the silica-binding method. However, a long processing time is required for the preparation of chitosan-coated silica and its integration into the microchip prior to isolation. Therefore, development of LOC system is desirable, which can offer simple and rapid RNA isolation without need for complicated pre-preparation process.

2.4. Size based particle separation in microfluidic systems

Size based particle separation has become essential in many biological research and environmental applications.³³ In order to provide unbiased and accurate insights into the biological process or phenomena, a number of detection techniques require isolation of target species of interest from solution during sample preparation. Since the dimension of biological structures ranges from angstrom to millimeter scale, separation method varies depending on the target size. Due to growing the need for robust and reproducible size based particle separation technique, many particle separation techniques have been devised and reported. Especially, significant efforts have been put into the development of miniaturized size-selective particle sorting method for point of care application such as Lab-on-a-chip particle sorters.^{7,34} However, the use of the majority of current miniaturized size-selective particle sorting techniques are limited to sorting sub- micron scale macromolecules such as cells, viruses, bacteria due to technological limitations and restrictions.

For nanoscale biomolecule separation, several size-dependent nanoparticle separation techniques have been employed. Based on the separation mechanism, these techniques fall into three categories; one based on the use of external fields (centrifugal^{35,36}, electrical^{37,38} and magnetic³⁹), physical barriers for sieving⁴⁰, and size-dependent precipitation⁴¹. One of most widely used methods that are based on the application of external field is field flow fractionation (FFF). FFF is a particle separation technique

taking advantage of field exerted to a solution flowing through a channel. The field is perpendicular to the fluid flow, which results in separation of particles suspended in the carrier solution, due to the difference in traveling speed depending on their size and molecular weight. Despite its variations and versatility, it has not been widely used as a size based nanoparticle separation method due to its low throughput and large input material required. Alternatively, size dependent precipitation method has been devised. Size-dependent precipitation occurs when the stability, physical or chemical properties change depending on the surface chemistry of the nanoparticles. Sieving is another alternative for size selective fractionation of nanoparticles. Chromatography and membrane filtration employ physical barriers such as a column or physical hole to control elution and retention depending on the size of particles. Unfortunately, these traditional size based nanoparticle separation techniques result in huge sample loss, therefore, large input material is required. In addition, bulky instrumentation is involved in generating external field during the separation process. Due to the complexity of these technologies, there has been increasing demand for the development of miniaturized and compact platform for size-based separation, which can be used as a cost-effective preparative method in point of care application.

There have been many microfluidic approaches to bring the size selective nanoparticle separation into miniaturized platforms. One of the most common separation mechanism utilized in microfluidic platform is membrane based filtration because of its high resolution and straightforward procedure.⁴²⁻⁴⁴ Gaborski *et al.*⁴⁰ demonstrated nanoparticle fractionation using porous nanocrystalline silicon membranes with resolution as high as 5 nm, which can be potentially applicable to Lab-on-a-Chip system for dead-end filtration. However, the use of membrane filtration typically suffers from low efficiency associated with clogging and formation of cake layer near membrane

filter⁴⁵ that leads to low flow rate, sample loss and high fluid resistance.⁴⁶ Additionally, in order to realize nanoscale particle separation, fabrication of nanopore or sieving structure involve complicated and time-consuming microfabrication process. To circumvent this limitation, “filter-free” microfluidic separation techniques have been developed. Microfluidic approaches based on electrophoresis, field-flow fractionation (FFF)⁴⁷ and centrifugation³⁶ have been devised and implemented for size based nanoparticle separation and fractionation. However, these techniques still rely on external fields that usually needs for the bulky instrument to manipulate particle motion. And most of passive separation techniques requires expensive fabrication to build microstructures. For a point-of-care application, lab on a chip system that can be realized with simple fabrication process, providing a user-friendly interface enabling easy sample handling, yet with high resolution with little sample loss is necessary. For these reasons, lack of reliable size selective nanoparticle separation is a bottleneck in analyses of nanoscale biomolecules such as ribosome profiling, which requires size selective separation of monosome from polysome molecules.⁴⁸

As membrane filtration can provide the most straightforward processing as well as high resolution, it has many advantages over filter free alternatives. Hence it can be a promising size selective nanoparticle separation tool by solving problems associated with membrane fouling during the process. As a part of its effort, many publications and research works have shown improvement in membrane filtration by utilizing ultrasonic treatment of membrane filter. However, the majority of works were focused on bulk processing for industrial purposes such as waste water processing, food processing and production of ultrapure water.^{49,50} Typically, they employed ultrasonic bath which requires high energy consumption and large input material. It may not be suitable for biological application where available input material is low.

To realize simple and rapid size selective nanoparticle separation for point-of-care application, I developed lab on a chip system based on dead-end filtration by incorporating commercially available track-etched membrane filter with different pore size cutoff. Sample containing polydisperse nanoparticle was processed in the microfluidic platform with embedded membrane filters. To overcome the limitation of dead-end filtration, which is clogging and membrane fouling due to the formation of cake layer near membrane surface that leads to low flow rate and damage of membrane and sample loss, I introduced ultrasonic treatment by integrating ultrasonic transmitter with membrane filtration system on-line. Ultrasonic assisted membrane filtration was modified to bring it into a miniaturized microfluidic platform.

2.4.1. Basic principles of dead-end membrane filtration

Typically, in dead-end membrane filtration, the input fluid flow is perpendicular to the membrane surface, which is called “feed” solution. Driven by pressure in filtration system along the channel, fraction of the fluid pass through the membrane pores, which is called “filtrate” or sometimes “permeate”. And a fraction of the input remains on the membrane surface, which is called “retentate”. To quantify the performance of membrane based filtration, permeates and retentates are collected separately and investigated by various analysis methods. When the target nanoparticle is metal such as gold or silver, one way to quantify the performance is absorbance measurement. For small gold nanoparticles (~30 nm), the surface plasmon resonance effect induce the absorption maximum at 526 nm wavelength. For relatively large gold nanoparticles (60 nm), the absorption maximum is induced at 540 nm wavelength. Hence, the UV-Vis spectrometer is useful tool for identifying the size of the gold nanoparticles. By measuring the absorption from the collected filtrate and retentate samples, it is able to quantify the fraction of target size nanoparticle in each sample. From the absorption

measurement data, the performance of the dead-end membrane filtration system can be determined.

2.5. Conclusions

Despite huge advances in technologies for DNA methylation analysis, the use of these analytical methods is still limited to laboratory based research due to their complexity, low efficiency and labor intensive processes. Those biosensor techniques reviewed above have been developed to address the unmet requirement to build epigenetic analysis platform for POC application. However, biosensors based DNA methylation analysis that have been recently reported needs integration with microfluidic system to automate pretreatment processes and sample retrieval, thereby realizing the fully integrated system that can be easily operated at clinical setting. Therefore, in this thesis, integration of state-of-art biosensor technique and microfluidic system is presented to address the current gap and address challenges that conventional and current biosensor based DNA methylation.

As with DNA based nucleic acid testing, demand on gene expression analysis and disease diagnosis using RNA is also increasing for rapid monitoring of infectious disease and genetic disease. To detect multiple diseases simultaneously using the same specimen from patient, RNA detection as well as DNA assay is necessary. Therefore, I present a RNA isolation module based on microfluidic LOC system that can provide high quality RNA from various raw biosamples.

To realize reproducible nucleic acid testing, size based cell separation is pre-requisite. Among many novel microfluidic platform reported recently, filtration using dead-end membrane is the most straightforward and simple method to use as a cell separation methods. By addressing the current challenges surrounding dead-end membrane filtration, such as large sample loss and clogging, a novel method presented in this thesis

is capable of performing high resolution size based separation in nanometer scale. By combining ultrasonic transducer with microfluidic system, this novel LOC platform can possibly serve as a sample pretreatment module for target size cell enrichment.

Each module presented in this thesis (DNA methylation detection, RNA isolation, Size based particle separation) can be used stand-alone or in conjunction with other systems establishing fully integrated diagnostic system for POC application.

Lab On A Chip System For DNA Methylation Analysis

In this chapter, I present a Lab-on-a-Chip platform that can perform DNA Methylation profiling which is based on bisulfite conversion reaction on the microfluidic chip surface in a continuous fluid flow. A myriad of techniques for the detection of DNA methylation have been devised, however, bisulfite-conversion based methods offer the most comprehensive information of genome-wide DNA methylation pattern with high accuracy and specificity. Despite its advantages, current bisulfite-conversion based techniques are not suitable for clinical use due to long processing time, labor intensive treatment and purification steps involved. To address this issue, I developed the microfluidic-based diagnostic platform that consists of bisulfite pretreatment module and label-free, real-time detection module that employs silicon microring resonator functionalized with methyl-specific probes. Methylated DNA in genomic DNA sample isolated from MCF-7 cells was efficiently pretreated and analyzed by the novel LOC system with reduced processing time (within 80 min) compared to conventional Methylation-specific PCR (3 to 24 h). This novel technique can detect as little as 1% mutation present in mutant (methylated) / wild-type (unmethylated) cell mixture with high sensitivity, whilst providing an efficient diagnostic tool for simple, fast and quantitative evaluation of DNA methylation pattern for clinical applications.⁵¹

3.1. Introduction

Over past two decades, tremendous efforts have been put into developing miniaturized and automated platforms to monitor cancer biomarkers in clinical settings. In particular, microfluidic lab-on-a-chip (LOC) system has provided simple and rapid micro-scale analysis environment in cancer diagnostics with high sensitivity, high throughput and

low reagent consumption. And also, biocompatibility of medical devices has been improved by employing biocompatible materials, thereby reducing non-specific interaction between biomolecules and the surface of the LOC system during the process. Especially biomedical grade polymers such as PDMS and thermoplastics (PMMA, Polycarbonate, PET, etc) greatly facilitate fabrication of a variety of structures for medical devices while ensuring improved biocompatibility, as well as durability than traditional materials like silicon and glass.⁵² Due to these benefits, a tons of LOC systems for genetic and proteomic testings of cancer-related biomarkers have been reported. State-of-the-art microfluidic-based biosensors for genetic and proteomic testings include capillary array electrophoresis (μ -CAE), label-free DNA hybridization technique measuring surface charge changes using nanowire-based transistors⁵³ or carbon nanotubes⁵⁴, label-free detection of DNA molecule using silicon photonic sensors^{55,56}, electrochemical impedance spectroscopy⁵⁷ and nanopore-based detection⁵⁸.

Although genetic and proteomic analyses account for the majority of existing cancer biomarker screening techniques, the demand grows for miniaturized LOC platforms for epigenetic biomarker detection in clinical settings.⁵⁹ Epigenetic modifications of genome, such as DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing are known for regulating gene expression without alteration in the gene nucleotide sequence that possibly leads to cancer development and progression.^{2,3}

Especially DNA hypermethylation at CpG islands, regions with a high percentage of CpG dinucleotides, in a promoter region of genes causes aberrant silencing of tumor suppressor gene that possibly results in cancer formation and progression. DNA methylation generally occurs at an early stage in carcinogenesis, which makes DNA methylation attractive as a biomarker for early cancer diagnosis and prognosis,

determination of appropriate treatment and prediction of response to the chemotherapeutic agent. There are a few international scientific organizations such as Human Epigenome Consortium (HEC), a worldwide collaboration among cancer researchers, and NIH Roadmap Epigenomics Consortium, which now aim to create a large-scale mapping of human DNA methylation. This move reflects increasing necessity of epigenetic analysis of cancer for research and clinical uses.^{60,61} Hence, development of reliable and robust diagnostic tool for analysis of DNA methylation status is of great interest in epigenetic biomarker studies to meet the requirements for both research and clinical use.

3.1.1. DNA methylation as a cancer biomarker

As cancer is one of World's leading causes of death, there is a growing interest in how to detect cancer in the early stage for successful treatment. Although the method for detection of cancer depends on the type of cancer and cancer stages, in most of the cases, biopsy is the “gold standard” for definite detection of cancers. However, the procedure is invasive, uncomfortable and relatively expensive. Among many candidate methods that can potentially replace the current gold standard, one of the promising techniques is DNA methylation biomarker test. It is a promising biomarker that enables detection of cancer determined by methylation status of DNA from the cancer cell by using blood or urine samples from the patient. It can be a powerful tool in cancer diagnosis, providing a non-invasive procedure and allowing early stage cancer detection with high sensitivity.^{62,63}

Within the DNA sequence, there is a region called CpG Island, and this region contains a number of cytosines located beside guanine (CG blocks on sequence)(Fig. 8). In a cancer cell, cytosines within this CpG islands tend to be methylated unlike cytosine on DNA from normal cells. And this aberrant methylation of cytosines in this region has

been known to inhibit normal gene expression of the gene and be associated with cancers.⁶⁴ Especially this DNA methylation is very sensitive and gene-specific, so this can be used as biomarkers for cancer diagnosis

Figure 8 DNA methylation in cancer cells: This aberrant methylation of cytosines in CpG Islands has been known to be associated with cancers, therefore, this can be used as biomarkers for cancer diagnosis

3.1.2. Optical biosensor based on silicon microring resonator

Optical biosensors for DNA detection have overcome the drawbacks of assays based on fluorescently labeled molecule detection. Various approaches using biophotonic sensors have been studied such as surface plasmonic resonance (SPR)^{65,66}, interferometers^{67,68}, grating⁶⁹⁻⁷¹ and resonant cavities⁷²⁻⁷⁵. Among these optical biosensors, silicon-on-insulator (SOI) biosensors based on microring resonators, which is fabricated by standard CMOS technology that allows for low-cost mass production, have been widely used for biosensing due to easy integration with electronics, and the ability to multiplex on a single chip. The biosensor provides label-free detection of DNA, real-time and quantitative monitoring with high sensitivity and specificity.⁷⁶ By combining it with a microfluidic module, it can reduce the time for sample preparation and detection. In this project, I adopted silicon biophotonic sensor as a key component

of a novel point-of-care diagnostic platform for the detection of cancer biomarkers. I investigated the working principle of silicon biophotonic sensor and its application.

Typically, biosensing mechanism behind silicon microring resonators is a change in refractive index. Briefly, in a microring resonator, light propagating along waveguides is confined within the waveguides due to total internal reflection of light. Total internal reflection (TIR) occurs when light strikes the boundary of silicon waveguides and is not able to refract through the medium boundary. TIR only occurs under the condition that the angle of incidence is larger than the critical angle, and the refractive index of waveguides is higher than the medium surrounding them. Light travels along the boundary of the ring between the high (e.g. Si) and low refractive (e.g. SiO₂) index media. Light traveling along a linear input bus waveguide is coupled to the ring-shaped resonator located adjacent to a linear waveguide. Typically, a gap between the ring cavity and a linear waveguide is sub-micrometer. For circulating light to add constructively in the ring resonator, the ring circumference must be an integer multiple of the light wavelength. Those wavelengths that meet this condition are called ring resonant wavelengths. This is given as follows for a single-ring ring resonator:

$$2\pi r n_{eff} = m \lambda_m \quad (1)$$

where n_{eff} is the effective refractive index of the waveguide material, r is the radius of the ring resonator, m is mode number and λ is the initial resonance wavelength. Changes in refractive index induced by binding of biomolecules (proteins⁵⁶, nucleic acids⁷⁷, aptamer⁷⁸, telomerase⁷⁷) lead to resonant wavelength shift that can be monitored in real time using optical measuring systems.⁷⁹ The wavelength shift is proportional to the mass change of analytes on the surface of resonators enabling label-free biosensing. Shin *et al.*⁵⁵ demonstrated the detection of bladder cancer biomarker in urine samples by using silicon biophotonic sensor. It has shown that the targets are firmly captured by

complementary probes compared to nonspecific probe. By using immobilized probes, the target sequence is distinguishable with high sensitivity via hybridization between probe and target in a sequence-specific manner.

3.1.3. Miniaturized LOC platform for DNA methylation detection

Amongst a variety of technologies, bisulfite conversion based methylation specific PCR is considered the “gold standard” in DNA methylation studies. Bisulfite conversion technique takes advantage of the alteration of the gene sequence that results from chemical reactions, which alters unmethylated cytosine to uracil in highly concentrated sodium bisulfite solution while methylated cytosine remains intact. (Fig. 9)

Figure 9 Bisulfite conversion of DNA. Cytosines are converted into uracils in the presence of bisulfite, while methylated cytosines remain intact.

Bisulfite conversion based methods allow for genome-wide DNA methylation profiling with high accuracy. However, the challenges associated with the integration of bisulfite conversion technique into miniaturized on-chip assay such as long processing time, labor intensive treatment process involved, and need for time-consuming purification steps was a major barrier to wide application to automated biosensing

technology. Moreover, harsh condition during bisulfite conversion such as long incubation of target templates at high temperature may cause a high degree of degradation of the templates, which would lead to a biased analysis of DNA methylation status. To circumvent such limitations in combining the bisulfite conversion technique with biosensor device, “bisulfite-free” methylation detection methods have been studied as alternatives. Recently Maria *et al.* demonstrated DNA methylation analysis by fluorometric methylation assay based on a microfluidic platform that exploits a restriction endonuclease digestion method.⁸⁰ Hairpin-shaped DNA oligonucleotides with a single CpG methylation site within the recognition sequence of methylation-sensitive HPAII endonuclease were used. Lim *et al.* reported methylation pattern profiling based on binding of a fluorophore-tagged methyl-CpG-binding domain protein to the sDNA segment stretched onto nano-channel.⁸¹ The detection of binding pattern achieved single-gene relevant resolution.

Most of the biosensors for DNA methylation analysis that have been published in the research literatures are based on endonuclease digestion⁸²⁻⁸⁴ (e.g. electrochemical sensor-enzyme digested target hybridization⁸⁵, or affinity enrichment^{14,81} (e.g. nanowire field effect based biosensor anti-5-methylcytosine antibodies¹⁷, solid-state nanopore combined with labeling of DNA with MBD protein⁸⁶), as these techniques can deliver qualitative and quantitative assessments of DNA methylation pattern efficiently combined with other biosensing techniques.

Recently, a method for direct detection of methylation of the synthetic oligonucleotide has also been reported, which is based on electrochemical detection using nanofilm.⁸⁷ However, typical resolutions of those “bisulfite-free” techniques are lower compared to bisulfite conversion based techniques. In practice, bisulfite conversion is the most reliable method to obtain single-base pair resolution information. In addition, coverage

of endonuclease digestion based and affinity enrichment based techniques vary according to the distribution of potential cleavage sites/affinity targets.⁸⁸ Furthermore, those techniques require relatively high purity and quantity of input DNA, which may limit the real-world application. Therefore, in most of the DNA methylation analyses, bisulfite conversion based techniques are still the most prevailing technology, as they can offer the most comprehensive information of DNA methylation pattern despite its limitations such as sample loss due to degradation during treatment. Many recent studies demonstrated that miniaturized biosensing platform based on bisulfite conversion is a promising tool for highly accurate DNA methylation analysis in clinical settings.

A few of array techniques based on bisulfite conversion have been successfully established for DNA methylation analysis. For example, Illumina Infinium assay analyzes DNA methylation of human genomic DNA samples by whole-genome amplification of bisulfite converted DNA followed by fragmentation and hybridization of the targets to methylation-specific DNA probes.¹⁵ Despite their multiplexing capabilities and high throughput of current DNA methylation analysis techniques on the basis of bisulfite conversion, application of these techniques to point-of-care application is still challenging work due to labor-intensive chemical treatment steps required, inefficient and time-consuming processes, considerable sample loss and potential contamination of samples during transferring templates during the process which cannot be avoided when off-chip treatment is involved. Also, most of the techniques simply utilized off-chip bisulfite-converted DNA and demonstrated microfluidic analytical scheme.⁸⁹⁻⁹¹ Hence, development of LOC system that incorporates bisulfite conversion process and detection protocol is necessary to achieve simple and fast, nonetheless, accurate near-patient DNA methylation biomarker test.

In this chapter, I present the achievements which is focused on 1) the development of a simple and rapid Lab on a Chip system for analysis of DNA methylation status based on bisulfite conversion and 2) integration of a single microfluidic channel based on bisulfite conversion (sample pre-processing module) and an isothermal solid-phase amplification/detection (detection module).

The microfluidic bisulfite pretreatment module was fabricated with multi-layered Poly(methyl methacrylate) (PMMA) by laser ablation processing to reduce fabrication time and facilitate fast prototyping. Bisulfite pretreatment module was able to deliver on-chip bisulfite modification reaction, an essential prerequisite of DNA methylation analysis with high reproducibility, while significantly reducing analysis time from 16 h by a conventional method to 50 min.

Next, on-chip isothermal amplification techniques were used as a detection method. DNA pretreated in sample processing module was loaded onto biophotonic sensor surface. The sensor has multiple microring resonators functionalized with primers that are complementary to methylated DNA molecule (target). Unlike conventional polymerase chain reaction (PCR) which requires thermal cycling, amplification occurs at a constant temperature, and it allows for amplification and detection simultaneously on the single chip surface. It also allows for label –free detection of target DNA in real-time. By taking advantage of complementary metal-oxide semiconductor (CMOS) technology, silicon microring resonator sensor was fabricated with high reproducibility and specificity and its utility has been demonstrated in the detection of biomarkers for clinical application, such as diagnosis of infectious and genetic diseases.

Being coupled with sample processing module, detection module performed analysis of methylation status of *RAR β* gene in human breast cancer cell line. The whole analysis process including sample pretreatment was completed within 80 min, which is

considerably faster than conventional Methylation-specific PCR techniques (3-24 h). Additionally, by the novel LOC based technique, 1% mutant cell was detectable from the mixture containing methylated and unmethylated DNA which was unable to achieve by other detection techniques (conventional PCR, real-time PCR). As DNA methylation in *RAR β* gene has been known to be closely related to a few human cancers, monitoring its DNA methylation pattern is necessary to deliver successful clinical treatment.

This novel method was able to perform rapid sample processing followed by detection of DNA methylation. It has shown considerable improvement in both detection ability (1% mutant) and reduced analysis time. I expect this versatile analytical device to serve as a DNA methylation analytical system and also be part of integrated systems with existing PCR technique and next generation sequencing technologies.

3.2. Working principle and device design

3.2.1. Working principle of LOC based DNA methylation analysis platform

The working principle of on-chip bisulfite modification based DNA methylation analysis is illustrated in Fig. 10-12. In order to discriminate methylated allele from unmethylated allele, bisulfite modification must be carried out as a pretreatment process. Sample preprocessing module selectively converts unmethylated cytosine to uracil on-chip as shown in Fig. 10 and the converted DNA was driven into detection module for methylation pattern analysis (SMR chip)(Fig. 11). First, genomic DNA (gDNA) isolated from MCF-7 was bisulfite treated passing through the reaction chamber. During the reaction, all the unmethylated cytosines in the original sequence were replaced by uracil sulphonates (U*), while methylated cytosines are protected. (Fig. 10_i) Once DNA exits microchamber region, it merges with chaotropic agent within micromixer. Then the gDNA mixture was driven into the main channel, being captured onto glass surface in the presence of chaotropic agent via electrostatic force. (Fig. 10_ii) To complete the bisulfite-mediated conversion to uracil, NaOH solution was injected into the main

channel for desulfonation. Inefficient sample transfer between each processing steps, which is involved in most of the column based bisulfite conversion protocols, was avoided by using the microfluidic platform. To operate continuous fluid flow, the reaction chamber was designed to reduce fluid velocity by using multi-layered fabrication.

Sample pre-processing module

Figure 10 Working principle of sample pre -processing module. Human gDNA in bisulfite solution was driven into the module for on-chip bisulfite conversion. Bisulfite conversion was carried out in microchamber at 70 °C. On-chip Bisulfite conversion gives rise to sequence difference by converting unmethylated cytosine to uracil while methylated cytosine is unaffected. After conversion, subsequent desulfonation and purification processes were performed within microchannel before elution. Bisulfite converted DNA sample was collected through an outlet in TE buffer and used for analysis on detection module.⁹²

After bisulfite conversion, simultaneous amplification and detection of bisulfite converted DNA was performed in real-time on detection module. DNA methylation is detected by measuring resonant wavelength shifts. The working principle of detection module is illustrated in Fig. 11-12. Detection module consists of silicon microring sensor chip. (Fig. 11) A target DNA strand can be detected when its sequence is complementary to primer immobilized on the chip surface. When the sequence is matched, hybridization of target template to immobilized primer occurs, and it allows for amplification. Detection module employs RPA technique as an amplification method. The protocol is modified to apply to the microfluidic platform. Each SMR sensor chip surface was functionalized with either methyl- or unmethyl-specific forward primers. When the original sequence contains methylated cytosine, the bisulfite converted templates are amplified and bound to methyl-specific primer, which leads to large shifts in resonant wavelength. (Fig. 12) No amplification occurs on the chip with unmethyl-specific primer. For unmethylated DNA, only a background wavelength shift is observed when the sample is loaded onto the methyl-specific chip, which indicates primer sequence immobilized on the surface chip is not complementary to converted unmethylated DNA. This implies DNA sample was initially unmethylated. Unmethylated DNA induces large wavelength shifts on the chip with unmethyl specific primer. Based on resonant wavelength shifts it is possible to determine whether original DNA sequence had methylated cytosine or not within target region before bisulfite conversion. Resonant wavelength shift can be collected in real-time, which makes the LOC based DNA methylation analysis technique attractive for rapid detection. By taking advantage of sample preprocessing module and detection module, the LOC system was able to analyze DNA methylation status within 60-80 min, substantially reducing hands-on process and sample loss during the reaction.

Detection module

Figure 11 Detection module based on silicon microring sensor chip. The inset shows microring structures functionalized with either Methyl or Unmethyl forward primer. After bisulfite conversion in sample preprocessing module converted DNA was loaded onto sensor chip in RPA solution containing Recombinase, reverse primer and Polymerase.

Figure 12 The detection of DNA methylation on detection module (silicon microring sensor)

3.2.2. Design of microfluidic sample pre-processing module for on-chip bisulfite conversion

A 2D schematic of the microfluidic sample pre-processing module for on-chip bisulfite conversion is shown in Fig. 13. The microfluidic lab-on-a-chip system was designed to perform 1) DNA analysis process including sample preparation, modification, and detection on single biochip in a continuous manner without any active components, 2) incubation of liquid sample at 70°C for ~20 min for complete bisulfite conversion reaction to occur, 3) passive mixing of the sample with a binding buffer to capture converted DNA onto channel surface, 4) binding of converted DNA onto channel surface via electrostatic force, 5) desalination and desulfonation during final washing step, 6) elution of modified DNA for downstream detection and 7) detection of the resultant amplicons by means of isothermal solid phase amplification integrated with silicon microring resonator.

Figure 13 Schematic of sample pre-processing module design (Top view). Inset figure shows three-dimensional structure of split and recombine (SAR) micromixer.⁹²

The microfluidic system was designed to perform sample processing step including bisulfite modification and subsequent purification on a single biochip. The device consists of a reaction chamber for DNA bisulfite modification process, three-dimensional micromixer for passive mixing of binding buffer with pre-modified DNA, and microchannels for adsorption of modified DNA onto channel surfaces and subsequent purification and desulfonation. Along the flow path, fluid manipulation was carried out in a continuous flow, thereby bypassing the difficulties in fabrication, integration, and operation of active components such as micropumps and microvalves. Especially, 3D micromixer structure was fabricated by simple laser ablation process using polymer, which was capable of performing efficient mixing without needing to

incorporate mechanical transducer. The mixing mechanism behind the 3D micromixer is primarily a combination of chaotic advection and lamination. Two streams introduced into microfluidic channel merge in micromixer and go through the multi-lamination process, which refers to repetitive split and recombine procedure, as they proceed along the series of the 3D serpentine structure of micromixer. Continuous lamination increases the area of the interface between two fluids where diffusion can occur. Three-dimensional serpentine structure not only induced lamination but also generated chaotic advection simultaneously. In other words, transverse flow components oriented perpendicular to the direction of the mainstream can be generated, and they, in turn, can help increase interfacial area. Additionally, an abrupt increase in the cross-sectional area also accelerates mixing by expansion effect. Basic 3D split-and-recombine (SAR) structure was modified to introduce expansion effect within the mixer. When two fluid flows enter into expansion region, a sudden change in fluid velocity and direction occurs, which enhances mixing of two fluids due to instability caused by expansion effect.

2. Micromixer

Figure 14 Photograph of the fabricated device. Bisulfite conversion chamber is visualized with red dye on the left-hand side. Mixing between two fluids is visualized within micromixer region. Two streams from inlet I and inlet II are mixed thoroughly in 4 mixing units of three-dimensional micromixer as they proceed towards DNA Capture microchannel, shown as purple.

3.2.3. Numerical simulation of mixing of two fluid flows in 3D mixer

Due to small dimensions of the microfluidic device, fluid flows in microchannel are predominantly laminar flow. To realize efficient mixing of bisulfite converted DNA with chaotropic binding buffer within the short path length without active elements, passive micromixer is necessary between reaction chamber and microchannel. In the experiment, bisulfite converted DNA was introduced into micromixer whereby it was mixed with binding buffer prior to DNA adsorption on microchannel. In order to achieve efficient mixing between two buffers within short effective mixer length, I investigated the mixing performance of 3D serpentine micromixer and compared it with several types of mixers by simulation.

To assess the mixing efficiency of designed 3D serpentine micromixer and numerically determine the minimum mixing path length, computational fluid dynamics (CFD) simulations were investigated under steady-state condition. I also compared the simulation result of other established micromixers. CFD software COMSOL Multiphysics was used for all numerical studies. I investigated mixing performance of the 3D micromixer within four mixing units. Two streams were driven into mixing region through dedicated inlets and driven out through the single outlet. One stream was introduced through upper left inlet boundary and tracer traveled with this stream. The other stream was introduced through lower left inlet boundary without the tracer. Dimensions of channels are set to be the same as the fabricated prototype and the total length of the domain in the simulation was ~20 mm. The diffusivity of a solute (species) in water was set to $1 \times 10^{-10} \text{ m}^2/\text{s}$. For discretization of fluid flow, first order elements were used to calculate pressure and second order elements were selected to calculate velocity. The total pressure drop across mixer was evaluated, and the mixing result was visualized. The total number of domain elements, boundary elements, and edge

elements were 32040, 24344 and 4817 respectively. The number of degrees of freedom was 973014.

In order to calculate the mixing results, Comsol solves Navier-Stokes equation as well as the Convective-Diffusion equation. The incompressible Navier-Stokes momentum equation describes the viscous fluid motion and is known as

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

, where u is the velocity, ρ is the fluid density, p is the pressure, η is the dynamic viscosity and f is the body force. NS equation is solved with boundary conditions:

1. no-slip condition at the all solid walls
2. Specified fluid velocity at inlet boundary
3. Pressure specified at the outlet boundary, in this case, set to have zero pressure at the outlet.

In simulation, when body force is zero, the equation is solved in non-dimensional form, so equation (1) can be converted into

$$\frac{\partial u'}{\partial t'} + u' \cdot \nabla' u' = -\nabla' p' + \frac{\eta}{L\rho u} \nabla'^2 u' \quad (3)$$

where U and L are a characteristic velocity and a characteristic length.

The pressure drop from the inlet to the recent boundary was calculated by p' from the equation. The value obtained from Comsol is dimensionless pressure drop so it can be converted into dimensional form by multiplying the number by pressure standard p_s .

$$\Delta p = p_s * \Delta p' \quad (4)$$

$$p_s = \rho * (U_s)^2 \quad (5)$$

And also, Comsol calculates a pressure drop at a boundary as

$$P' = \frac{\int p(x,y) dx dy}{\int dx dy} \quad (6)$$

To observe the mixing effect, mass transport of dissolved species was calculated by solving convective-diffusion equation. For incompressible fluid flow, without sink or source, with a constant diffusion coefficient, a simplified form of convective-diffusion equation is commonly known as

$$\frac{\partial c}{\partial t} = D \cdot \nabla^2 c - \vec{v} \cdot (\nabla c) \quad (7)$$

where c is the concentration of species, D is the diffusion coefficient and $\vec{v}$ is the average velocity of the flow. CD equation is solved with boundary conditions defined as:

1. Solid walls are not permeable, that is, zero flux on all the walls.
2. Convective flux on the outlet boundary
3. The initial concentration at the inlet boundary, in this case, concentration is 1 at one inlet boundary and 0 at the other inlet boundary.

In simulation, convective-diffusion equation is also solved in non-dimensional form by introducing dimensionless variables, which results in

$$Pe \cdot \frac{\partial c'}{\partial t'} + Pe \cdot v' \cdot \nabla' c' = \nabla'^2 c' \quad (8)$$

Peclet number Pe is defined as

$$Pe = \frac{v_s \cdot x_s}{D} \quad (9)$$

where v_s is average velocity at the inlet and x_s is the characteristic length.

As a result, the total pressure drop across mixer was 47.086Pa. And the mixing result at a flow velocity of 3.2 mm/s is shown in Fig. 15.

Figure 15 The mixing result at initial flow velocity 3.2 mm/s at the inlet boundary in three-dimensional micromixer

Compared to the mixing results of 2D modified tesla structure by Hong *et al.*⁹³ (Fig. 16) across the same distance from the inlet, the result from 3D serpentine micromixer used in microfluidic device shows higher mixing efficiency.

Figure 16 The mixing result at a flow velocity of 3.2 mm/s in modified tesla micromixers.

However, 2D cross-sectional plots (Fig. 17) from the region near entrance shows that lamination of fluids was not perfectly vertical (e, f, g) which must be improved either by modifying the design parameter or flow velocity of the input buffer.

Figure 17 2D cross-sectional plots of lamination in the first mixing unit.

Quantitative analysis was performed to compare the mixing performance of two mixer designs. The mixing efficiency for each mixer design was quantified and investigated by numerical simulation as described above. The average Reynolds number $\overline{Re}$ is calculated by using the formula

$$\overline{Re} = \frac{\rho V L}{\mu} \quad (10)$$

where ρ is the fluid density, V is the average flow velocity, L is the characteristic length, which is the hydraulic diameter of the microchannel and μ is the dynamic viscosity of the mixed fluid, respectively.

The concentration of the input tracer at the two inlets is 1 (upper) and 0 (lower) respectively and 0.5 represents the complete mixing. The input flow rates at two inlets were equal. To compare the mixing performance, the mixing efficiency ζ is defined as

$$\zeta = 1 - \sqrt{\frac{\sigma^2}{\sigma_{max}^2}} \quad (11)$$

where σ is the variance of the mass fraction on a cross sectional plane perpendicular to the direction of fluid flow. σ_{max} is the maximum variance before mixing at the inlet. ζ varies between 0 and 1, where 0 indicates that no mixing occurs and 1 indicates complete mixing. Therefore, higher ζ represents better mixing performance. The variance σ is calculated using the formula below

$$\sigma = \sqrt{\frac{1}{N} \sum_{i=0}^N (c_i - c_{mix})^2} \quad (12)$$

where N is the total number of sampling, c_i is the local mass fraction at the pixel i on cross sectional plane and c_{mix} is the mass fraction after complete mixing.

Fig. 18 shows mixing efficiency with the varying Reynolds numbers at the outlets of 3D mixer and Tesla mixer for comparison of mixing performance within the same mixing length. The mixing efficiency of the 3D mixer exhibits the maximum mixing efficiency at a very low input flow velocity ($\overline{Re}=0.1$). This is mainly due to sufficient residence time for diffusion to occur at a low flow velocity. As $\overline{Re}$ increases up to $\overline{Re} = 5$, mixing efficiency decreases, which is presumably due to the reduced residence time for diffusion as compared to $\overline{Re} = 0.1$ flow condition. Interestingly, as $\overline{Re}$ increases beyond 5 up to 20, the mixing efficiency greatly increases. Within this flow rate range, the flow velocity is high enough to generate the inertial force, which enhance convective effect that improves mixing. At $\overline{Re} = 20$, the mixing efficiency is comparable to the result obtained at $\overline{Re} = 0.1$, while it enables faster mixing at higher flow velocity. Therefore, the input flow velocity was set such the velocity satisfied the $10 < \overline{Re} < 20$. When the $\overline{Re}$ exceeds 20, Mixing efficiency decreases consistently, indicating insufficient residence before the fluid streams exit the mixer region.

On the other hand, the numerical simulation result of mixing efficiency for the tesla mixer is in agreement with the numerical simulation result of tesla mixer reported previously.⁹⁴ Mixing efficiency drop occurs in $0.1 < \overline{Re} < 1$ indicating reduced residence time for diffusion. Then mixing efficiency increases as $\overline{Re}$ increases up to 100. Tesla mixer exhibits better mixing performance than 3D mixer within $40 < \overline{Re} < 100$. This indicates convective mixing effect dominates at high flow velocity enhancing the mixing efficiency. Since the sample processing was carried out in continuous stream on microfluidic platform, sufficient residence time is required to complete the bisulfite conversion on the chip. Therefore, the desired Re is below 20 where 3D mixer outperforms tesla mixer in this $\overline{Re}$ range.

Figure 18 Numerical simulation results of 3D mixer (Red) used in the sample processing module and tesla mixer (Blue) for comparison of mixing performance with varying Re from 0.1 to 100.

3.2.4. Design of silicon microring sensor

As detection module, I utilized silicon microring resonator for detection of methylated DNA. Structures of silicon microring resonator and waveguides used for optical detection of converted DNA products were described in previous work by our group.⁷⁸ A layout of silicon microring sensor array is shown in Fig.19.

Figure 19 Layout of silicon microring sensor array. The sensor array consists of four racetrack shaped microrings.⁷⁸

Briefly, a sensor array has four individual racetrack shaped microrings coupled with one straight waveguide for signal input, and output waveguide of each ring is located on the opposite side for signal output. Among four rings, three rings were used for sensing platform of methylated DNA amplicons through opened sensing windows where access of target molecule to ring surface is allowed. The other ring buried underneath the SiO₂ cladding layer was used as a reference sensor for measuring signal drift caused by temperature variation. The diameter of rings is 10 μm, and the distance between microrings is set to 750 μm. In order to prevent resonant spectral overlapping, coupling path length was designed to range from 2 to 2.042 μm. Dimensions of linear I/O bus waveguides are 500 nm x 220 nm. The spacing between rings and linear waveguide at coupling region is 220 nm.

As shown in Fig. 19, microring resonator consists of a ring-shaped waveguide coupled to two straight waveguides. A fraction of the incident light is transmitted to the drop port when coupling occur. When the waves traveling through ring shaped waveguide build up a round trip phase shift that equals an integer multiple of 2π , the waves interfere constructively and the cavity is in resonance. Determination of design parameters and the parametric optimization can be performed by using commercially available photonic device simulation software. Lumerical FDTD solution was used to design and simulate a silicon microring resonator. By using the FDTD solution software, geometric parameters can be determined to achieve desired characteristic parameter such as free spectral range (FSR) and quality factor (Q-factor).

The important parameters in designing ring resonator are the effective circumference of the ring L , the propagation constant β of the circulating mode, self-coupling coefficient r and cross-coupling coefficient k . The effective index varies with the wavelength of incident light and the wavelength range between the two adjacent resonances is called FSR and is given by

$$\text{FSR} = \frac{\lambda^2}{n_g L} \quad (13)$$

where n_g is the group index. For the high index waveguide made of an SOI, the bending loss is low due to strong confinement within the waveguide. Given the desired FSR and FWHM of the drop, Q factor is estimated by using the formula:

$$Q = \frac{\lambda}{2\delta\lambda} = \frac{n_g L \pi}{\lambda} \frac{|r|}{1-|r|^2} \quad (14)$$

where bending loss and other sources of loss are ignored for simple approximation. Typically, to establish biosensor using silicon microring resonator as a detection module, Q factor is to be >20000 and FSR is to be 17 nm. Since the device was fabricated using

commercially available SOI wafer with 220 nm thick top silicon layer, the height of the waveguide is set as 220 nm.

In order to find the ring circumference to achieve desired FSR, the group index in wavelength range from 1500 nm to 1600 nm is calculated. The group index n_g is approximately 4.3 at 1550 nm. Using this group index, the desired total ring length can be calculated in wavelength unit as

$$L = \frac{\lambda^2}{n_g FSR} \approx 32.9 \mu m \quad (15)$$

Next, from equation (10), the amplitude of r can be derived as

$$|r| = \sqrt{\left(\frac{n_g L \pi}{2 Q \lambda}\right)^2 + 1} - \frac{n_g L \pi}{2 Q \lambda} \quad (16)$$

Given the group index $n_g \approx 4.3$, total ring length $L \approx 32.9 \mu m$ at 1550 nm, *coupling coefficients* r and k are determined. Coupling length is derived from the coupling power:

$$k^2 = \sin^2 \frac{\pi \Delta n L}{\lambda} \quad (17)$$

where Δn is effective index difference between the even-mode and odd-mode. Using the gap 220 nm between the linear waveguide and ring, Δn at 1550 nm is calculated and the coupling length can be determined. Desired coupling length is $2 \mu m$ to achieve sufficient coupling for the structure in our device. As a result, the radius of the ring can be determined given the desired coupling length calculated above and the total ring length. Consequently, the ring radius is set as $5 \mu m$.

3.3. Materials and Methods

3.3.1. Fabrication of microfluidic device for on-chip bisulfite modification of human genomic DNA

The microfluidic device was designed using AutoCAD software and fabricated using laser cutting process (Universal Laser Systems; Scottsdale, AZ, USA) that enables easy and rapid prototyping and low-cost manufacturing. (Fig. 20) The chip is made of Poly (methyl methacrylate) thin film with melting temperature higher than the incubation temperature needed. Geometries of microfluidic flow paths, as well as inlets and outlet, were patterned onto 114 μm thick double-sided tape sheets (3M 9475LE; St. Paul, MN, USA) using laser machine system. For complete DNA conversion process, the fluid flow rate must be low enough when the stream is passing through DNA bisulfite conversion chamber. To realize varied flow rates within microfluidic networks 500 μm thick PMMA layer was inserted between two layers of double sided tape. Patterned microchannels can be accessed by through holes which act as inlets and outlet on the top layer. Prior to assembly, commercially available glass slide (75mm x 26mm) was cleaned by immersing in piranha bath for 30 min and rinsing with deionized water. To enclose microfluidic networks, protective layers on both sides of double-sided tape were then removed and the PMMA layer was sandwiched between the piranha-cleaned glass slide (bottom layer) and top layer by applying pressure gently. Connectors were manufactured with 2.5 mm thick PMMA sheet and used to connect the microfluidic channels and tubings by holding them in their through-holes. Connectors were attached on top layers using adhesive layer patterned at the bottom and Tygon tubings (AAC02548; Cole-Parmer; Vernon Hills, IL, USA) were placed into through-holes. To prevent a leak, they were sealed by applying epoxy around the edges.

Figure 20 Fabrication of sample pre-processing module. The sample pre-processing module was built with multiple layers of PMMA sheets by laser cutting process. The bottom layer consists of a blank glass slide (piranha cleaned) for DNA binding.

To perform stable and efficient bisulfite conversion on-chip, feedback temperature control is essential. (Fig. 21) It was able to monitor and maintain reaction temperature by introducing temperature control module. The module consists of a Peltier thermoelectric cooler, copper layer and thermocouple. A 1mm thick copper block was mounted on the Peltier thermoelectric cooler (ThermaTEC Series; Laird Technologies; Earth City, MO, USA), and the thermocouple (HYP-1; Omega Engineering; Stamford, CT, USA) was embedded and glued to it by using thermally conductive paste (OMEGATHERM 201; Omega Engineering) for good thermal contact. Dimensions of the copper layer were chosen to cover the area of the microchamber. A thermoelectric cooler controller (Series 800; Alpha Omega Instruments Corp.; Lincoln, RI, USA) was connected to the module to monitor temperature in real time manner.

Figure 21 Sample pre-processing module is mounted on Peltier thermoelectric cooler for feedback temperature control.

3.3.2. Fabrication of silicon microring resonator chip for methylation specific amplification and detection

The fabrication process of silicon microring sensor chip used as a detection platform was illustrated in previous work by our group.⁷⁸ Briefly, the sensor chip was fabricated with Silicon-On-Insulator(SOI) wafer that consists of 220 nm thick silicon layer on top of 2 μm thick buried oxide (BOX) layer. Microring resonators and Input/output bus waveguides were created through patterning by 248 nm deep UV lithography and subsequent reactive ion etching (RIE) process. The substrate was immersed in an etchant bath till silicon layer was completely removed. After RIE process a 1.5 μm thick cladding layer was deposited on the substrate with high-density plasma (HDP) CVD. Then sensing window was etched to expose sensing microrings by RIE dry etching process.

3.3.3. On-Chip DNA methylation analysis

Using the LOC technique, analysis of DNA methylation status was performed. Methylation status of DNA isolated from human cancer cell-line, MCF-7, was analyzed. Workflow of DNA methylation analysis using LOC system is illustrated in Fig. 22. First DNA was isolated from human cancer cell-line (MCF-7). Isolated DNA was driven into sample pre-processing module for bisulfite conversion and subsequent purification process. Bisulfite converted DNA was then analyzed on silicon microring sensor (SMR) chip for methylation studies. Workflow of each process will be described in detail in this chapter.

Figure 22 Workflow of DNA methylation analysis using Lab-on-a-Chip system

3.3.4. Sample preprocessing module

To start DNA methylation analysis, DNA must be isolated from cells. An epithelial cell line derived from human mammary gland cells, MCF-7, was used to obtain genomic DNA. Cell line was cultured in high-glucose Dulbecco's modified Eagle medium (DMEM, Life Technologies) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humid incubator with 5% ambient CO₂ as described by Shin *et al.*⁹⁵ Once confluent, cells were taken out from the flask using 0.25% Trypsin-EDTA (Biowest) solution. Human genomic DNA was extracted from the cell line using QIAmp DNA mini kit (Qiagen;Hilden, Germany).

Next, DNA isolated from cancer cells was converted in microfluidic sample preprocessing module. The microfluidic device was used for on-chip DNA bisulfite

modification. Bisulfite conversion occurs in highly concentrated bisulfite solution. Bisulfite solution was prepared as following order:

Bisulfite solution was prepared by dissolving 0.832 g of sodium bisulfite (NaHSO_3) salt in 1ml deionized water (dH_2O). Then 0.3 ml of 3N sodium hydroxide (NaOH) was added to the solution and dissolved carefully at 50°C for 10 min in a dry heating bath. Since conversion efficiency varies depending on pH level, pH of the final solution was monitored and adjusted to ~ 5.4 . In a separate tube, 10 mg of hydroquinone was added in 2.5 ml of dH_2O . Then 150 μl of bisulfite solution and 30 μl of hydroquinone solution were mixed by thorough pulsed vortexing. Sodium bisulfite and hydroquinone were purchased from Sigma-Aldrich. Prior to bisulfite conversion, gDNA isolated from cancer cells was denatured in 20 μl of 0.3N NaOH at 37°C for 10 min in a thermocycler. Since only cytosines in single-stranded DNA are susceptible to bisulfite treatment, complete denaturation of the DNA undergoing analysis is essential. After denaturation, DNA sample was added to 180 μl mixture of bisulfite and hydroquinone solution. Prior to sample loading into sample preprocessing module, the device was washed with dH_2O to pre-wet the inner microfluidic walls, thereby preventing evaporation of water from sample solution during incubation and washing any residual solvent. The mixture of genomic DNA and premixed bisulfite solution was driven into bisulfite conversion chamber region through Inlet 1 at a flow rate of 10 $\mu\text{l}/\text{min}$ using syringe pump. The temperature of the mixture was elevated to 70°C when passing through the chamber region by heat transfer from a Peltier heater module attached to the back side of sample preprocessing module. Once the sample left the conversion chamber, it was mixed with 7.0 M guanidine hydrochloride (GuHCl) (Inlet 2) in three- dimensional mixers at a flow rate of 6.4 mm/s . By electrostatic interaction, bisulfite converted DNA molecules were adsorbed onto microchannel surface in the presence of chaotropic salt.

After bisulfite conversion, subsequent desulfonation and purification were performed. For complete bisulfite conversion of DNA, removal of residual salt and reagent is critical. In order to remove residual bisulfite salt wash buffer (80% ethanol; 10 mM Tris-HCl, pH 8.0) was introduced through Inlet 3 for 5 min at 10 μ l/min. After washing, desulfonation buffer (0.3M NaOH in 90% EtOH) was injected in the same manner and incubated for 10 min at room temperature. Finally, 70% EtOH was introduced for final washing for 5 min, and the microfluidic surface was dried. The bisulfite converted DNA was eluted by adding TE buffer (pH 8.0). The eluted DNA was investigated on detection module (SMR chip) of LOC system.

On the other hand, Bisulfite modification by the conventional method was performed using CpGenome DNA modification kit (Millipore-Merck) to compare with the proposed assay. The sample was incubated in bisulfite solution overnight (~16 h) according to the protocol provided by the supplier.

3.3.5. DNA methylation analysis by conventional End-point PCR and real-time PCR

Conventional end-point PCR and real-time PCR were also carried out to validate a novel technique of DNA methylation status analysis. All primer sets used for conventional PCR of RAR β gene are described in Table 2. For conventional PCR, 2 μ L of either bisulfite modified or unmodified DNA were amplified in a final volume of 25 μ L PCR mixture containing 1x PCR buffer, 2.5 mM MgCl₂, 0.25 mM deoxynucleotide triphosphates (dNTPs), 25 pmol of forward and reverse primer, and 1 unit of Taq DNA polymerase. All reagents were obtained from Taq PCR core kit (Qiagen). Amplification was performed in a thermocycler (initial denaturation step at 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min). Each PCR product was loaded onto 2 % agarose gel stained with

ethidium bromide (EtBr) (Sigma-Aldrich), separated by gel electrophoresis and visualized under UV irradiation.

For real-time PCR, amplification was carried out using LightCycler, according to the protocol provided by Roche Diagnostics. Briefly, 5 μ L of bisulfite-modified DNA was amplified in a final volume of 20 μ L PCR mixture containing 4 μ L of DNA Master mix, 25 pmol of forward and reverse primer. The reaction initiated with a pre-incubation step at 95 °C for 10 min followed by 50 cycle amplification step (95 °C for 10 s, 60 °C for 30 s, and 72 °C for 10 s), and finally cooling step at 40 °C for 30 s.

Table 2 Primer sequences for DNA amplification by detection module or conventional PCR (MS-PCR/Real-time PCR)

Method	Primer set	Sequence
Detection module	RARMF	5'-NH ₂ -(CH ₂) ₆ -AAAAAATTGAAGGTTAGTAGTTCGGGTAGGGTTATC-3'
	RARMR	5'-NH ₂ -(CH ₂) ₆ -AAAAATCCGAATCCTACCCCGACGGTGCCAGACAAA-3'
	RARUF	5'-NH ₂ -(CH ₂) ₆ -AAAAAATTGAAGGTTAGTAGTTGGGTAGGGTTATT-3'
	RARUR	5'-NH ₂ -(CH ₂) ₆ -AAAAATCCAAATCCTACCCCAACAATACCCAAACAAA-3'
MS-PCR/ Real-time PCR	Methyl-Forward	5'-GGTTAGTAGTTCGGGTAGGGTTATC-3'
	Methyl-Reverse	5'-CCGAATCCTACCCCGACG-3'
	Unmethyl-Forward	5'-TTAGTAGTTGGGTAGGGTTATT-3'
	Unmethyl-Reverse	5'-CCAAATCCTACCCCAACA-3'
	Wild-type Forward	5'-ATTTGAAGGTTAGCAGCCCG-3'
	Wild-type Reverse	5'-GCATCCCAGTCCTCAACAG-3'

3.3.6. Preparation of detection module

Silicon microring resonator chips functionalized with methylation specific primers were used as detection module. Reagents used in functionalization process of the sensor surface, including glutaraldehyde (GAD), 3-aminopropyltriethoxysilane (APTES), sodium cyanoborohydride (5.0 M in 1 M NaOH) were supplied by Sigma-Aldrich (St. Louis, MO). Primers used for methylation specific on-chip amplification/detection of

RAR β gene were custom-ordered by Sigma-Aldrich. To immobilize forward primer onto a solid surface, primers were 5'-modified with an amine group, and C6 alkyl group was used to link the amine group and oligonucleotide. All reagents used for on-chip amplification were obtained from TwistAmp Basic kit (TwistDx, Cambridge, UK).

In order to analyze DNA methylation status of gDNA isolated from cancer cells using silicon microring sensors as detection module, the sensor surface was functionalized with forward primer. Two sets of sensor chips with immobilized primers either methylated-specific (M primer) or unmethylated-specific (U primer) were prepared for comparison. Functionalization of the sensor surface was carried out in the following order:

1. Oxygen plasma treatment: Functionalization started with oxygen plasma treatment of chips. Sensor surface was plasma cleaned for 1 min.

2. Silanization of the substrate: After plasma treatment, the substrate was silanized with 2% APTES in 95% ethanol at room temperature for 2 h. After incubation, sensor chips were immersed in 95% ethanol and rinsed thoroughly for 5 min x 3times on a shaker and dried with nitrogen gas flow.

3. Curing chips: Then chips were cured in a convection oven at 120 °C for 15 min. Set the temperature prior to this step and let it stabilize.

4. The addition of glutaraldehyde (GAD): After curing, a solution of 2.5% GAD in dH₂O containing 5mM sodium cyanoborohydride was added onto chip surface, incubated for 1 h, and rinsed with dH₂O.

5. The immobilization of primers: To immobilize forward primer onto sensor chip surface, 100 μ l of 2 mM RAR β gene forward primer solution containing 5mM sodium cyanoborohydride was added to the sensing area, where microrings are located, and incubated for 16 h at room temperature. After incubation, chips were rinsed with MES

buffer and dH₂O to remove any unbound primer and residual buffer, then dried with nitrogen gas.

6. Finally, PMMA well (6mm x 1.5mm x 1mm) was attached to the chip to enclose the sensing area. This will facilitate sample loading and application of mineral oil on top.

3.3.7. DNA methylation analysis on detection module

An external near-infrared tunable laser was used as a light source of the optical measuring system. Input wavelength is tunable between 1530 nm to 1580 nm for spectrum scan.⁷⁸ Light traveled through an inline polarization controller to generate independent wave plates to change the polarization state of the transmitted light in single mode fiber (SMF). Then light entered into the linear input waveguide through a vertical grating coupler placed at the end of the waveguide. A grating coupler (12 μ m width, etching depth = 70 nm, pitch period Λ = 630 nm, filling factor = 0.5 and N = 24) is coupled to a linear waveguide adiabatic tapered. Light is coupled into output linear waveguide when exiting from three sensor rings and the output optical fiber receives optical signals passing through a vertical grating coupler. The output SMF was connected to a power detector. EXFO IQS-12004B DWDM passive component test system was used to measure transmission spectra of the sensor.

To investigate the functionality of the microfluidic device for on-chip bisulfite conversion and its specificity, methylation specific amplification of bisulfite treated DNA products from the microfluidic device was performed using the detection module. Methylation-specific isothermal solid-phase amplification/detection technique is based on recombinase polymerase amplification (RPA) method, an isothermal alternative to conventional PCR, developed by TwistDx Ltd (Cambridge, UK). The detailed working principle of detection module will be discussed in result & discussion section. The

protocol provided by the supplier was modified for the on-chip application. Detection module was operated in the following order:

1. Preparation of RPA reagents and primer mix: In a sterile, nuclease-free PCR tube on ice, 47 μL of RPA cocktail solution was prepared by adding 29.4 μL of rehydration buffer, 15 μL of distilled water, 2.4 μL of RAR β reverse primer (10 μM) and mixed via pipetting.

2. The mixture was added to freeze-dried enzyme pellet. Mix the reagents carefully in the tube.

3. Then 2.4 μL of 280 mM magnesium acetate (MgAc) was added and mixed with reagents.

4. To begin detection, 10 μL of the final solution was taken out and added into tube with 2 μL of bisulfite treated DNA (eluted from sample processing module) and immediately applied to the sensing area of detection module, where sensor rings were located, and the whole detection module was placed on top of Peltier heater module to start amplification reaction.

5. Prior to measurement, 10 μL of mineral oil was added to template-primer solution to cover the sensing window for prevention of evaporation during the amplification process. The temperature was set to 37 $^{\circ}\text{C}$ and monitored by real-time feedback control.

6. The resonance spectrum data was collected within the wavelength range of 1530-1580 nm every 5 min at least for 30 min using optical setup. For data analysis, collected raw data file was imported to OriginPro data analysis software, and resonance spectra were generated and analyzed.

3.4. Results & Discussion

3.4.1. Optimization of bisulfite treatment

Conventional bisulfite treatment requires hours of exposure to bisulfite solution. Extremely long incubation of DNA template in bisulfite solution is likely to cause degradation of DNA sample during the reaction. And also, there is a chance that conversion errors such as incomplete reaction or over-conversion can occur, which will lead to over- or under-estimate of methylation pattern in the analysis. Therefore, it is desirable to decrease reaction time to minimize the sample loss due to degradation. I found the optimal reaction time that yields the maximum amplification efficiency and applied it to the sample preprocessing module technology.

In order to reduce incubation time required for bisulfite conversion, I investigated the effect of molarity of bisulfite salt and incubation temperature. A few method of bisulfite conversion with high molarity and temperature have been reported, and Genereux *et al.*⁹⁶ investigated conversion error and time depending on molarity and temperature. I used modified protocol of high molarity/temperature to accelerate the reaction and perform bisulfite conversion on the sample preprocessing module under the modified condition.

To investigate the effect of molarity/temperature on conversion, I prepared both low and high molarity bisulfite solution separately. For low molarity/temperature conditions (Low MT), I used 4.9M bisulfite and 55 °C, under which typical bisulfite conversion occurs. For high molarity/temperature conditions (High MT), I used 8M bisulfite and 70 °C as suggested by Shiraishi and Hayatsu⁹⁷. The conversion efficiency over time was examined varying the reaction time ranging 5 min to 4 h to find out the minimum required reaction time for complete conversion. After bisulfite conversion reaction followed by desulfonation and purification process, bisulfite converted DNA was eluted from the sample pre-processing module and analyzed by methylation specific-PCR (MS-PCR). Methylation-specific primer and wild type primer sets were used for

amplification. Wild-type primer set was used to investigate any incomplete conversion. The amplification results using wild type primer set served as control. (Fig. 23) The amplicons were visualized under UV via gel electrophoresis. Gel electrophoresis is a conventional method to detect biomolecules (DNA, RNA and protein) of different sizes following the amplification by PCR. Briefly, amplicons of different sizes can be separated traveling through the gel in electric field and the traveling distance varies with the size of the molecule. As shown in Fig. 23A, the molecular weight size marker is located in the first lane. The traveling distance is approximately inversely proportional to the logarithm of the size of the biomolecule. That is, bands in different lanes at the same distance from the sample loading well indicate the bands contain DNA with approximately the same size. And the band intensity is quantitatively analyzed by comparing against the size marker loaded on the same gel. Under High MT condition, RAR β gene was successfully amplified by Methyl-specific primer (M primer) using template treated for 20 min bisulfite conversion while showing a low intensity of amplification by Wild-type primer (W primer), which indicates high conversion efficiency was achieved under high MT condition. (Fig. 23A) On the other hand, under Low MT condition, high intensity of amplification by W primer was observed using template treated 40 min, which indicates considerable incomplete conversion for a duration less than 40 min under Low MT condition. And after 240 min of incubation, severe loss of DNA samples detected was observed under both low MT and high MT condition. (Fig. 23B) Conversion efficiency using the sample pre-processing module as a function of the reaction time of bisulfite conversion was illustrated in Fig. 23C. This indicates that long incubation in bisulfite solution can cause degradation of DNA. Hence, it was inferred that higher conversion efficiency could be achieved in a microfluidic environment using the sample pre-processing module under high MT

condition. From genetic analysis results, high molarity/temperature condition for rapid on-chip bisulfite conversion was adopted.

There are two major reasons behind the reduction of reaction time using the new protocol. First, as reported by M. Shiraishi *et al.*⁹⁷, the conversion of unmethylated cytosine to uracil can be largely improved by increasing the concentration of bisulfite solution and temperature. Conventional bisulfite reaction is done at 55°C in 4~5M bisulfite solution. In order to achieve high-speed bisulfite conversion, different temperature/molarity condition in microfluidic environment with modified protocol was investigated. As a result, the microfluidic approach was able to reduce bisulfite reaction time from 16 h (conventional method) to 20 min~40 min.

The use of microfluidic as a sample processing platform may contribute to reduction of conversion time by using the smaller size in microscale environment. Presumably the modified protocol can also be applied to “conventional” method, however, subsequent purification steps are still problematic using conventional protocol. Efficient purification by continuous flow in sample processing module also contributed to reduction of time in sample preparation step.

Figure 23 Conventional PCR results for optimization of temperature and molarity condition. (A) Human gDNA (1 μ g) isolated from MCF-7 cells was used as a template. Gel electrophoresis of MS-PCR products amplified using template treated on sample pre-processing module under Low MT or High MT condition. (M= Methyl-specific primer, W=Wild-type primer and L= 50 bp DNA ladder) (B) After 240 min of incubation, serious loss of DNA samples detected was observed under both low MT and high MT condition. (C) Conversion efficiency using the sample pre-processing module as a function of the reaction time of bisulfite conversion. Incubation time varied from 5 min to 4 h. Conversion efficiency was analyzed by obtaining the normalized intensity of products from MS-PCR.

3.4.2. Characterization of microfluidic sample preprocessing module by Methyl-specific PCR

To validate the utility of sample pre-processing module as an on-chip assay for bisulfite conversion of the whole genome, I also compared the conversion efficiency of proposed microfluidic approach with that of the conventional bisulfite conversion method (in-tube). It is illustrated in Fig. 24A that the methylated RAR β gene was successfully amplified with M primer after 50 min of bisulfite conversion reaction using the sample processing module, while no amplicon was observed from sample amplified by W primer. In addition, it shows that bisulfite conversion reaction time including conversion, desulfonation and purification process was significantly reduced to less than an hour in microfluidic environment under high MT condition, while the equivalent amplification of RAR β can be achieved by using the current module. Moreover, I demonstrated detection of products with as little as 100 ng of input DNA converted in the sample preprocessing module within 50 min as shown in Fig. 24B. The gel electrophoresis results show that the proposed method has equivalent or higher conversion rate than the conventional method using input DNA as little as 100 ng. Therefore, the sample preprocessing module alone could serve as a useful tool for on-chip bisulfite conversion and reduce processing time yet provide higher conversion efficiency under optimized condition

Figure 24 Characterization of sample pre-processing module for on-chip bisulfite conversion by Methyl-specific PCR. (A) Comparison of conversion efficiency. Genomic DNA from MCF-7 (1 µg) was used as an input. The normalized intensities of amplicons from MS-PCR by M primer (Blue) and W primer (Red) set were compared. The inset shows gel electrophoresis results. (B) Quantitative analysis of DNA methylation using a varying amount of input DNA after conversion by on-

chip and conventional techniques. (1 μ g, 500 ng, and 100 ng from left). Average normalized intensity was obtained from gel electrophoresis results.

3.4.3. DNA methylation analysis using detection module

For validation of LOC system for analysis of DNA methylation status, DNA methylation detection was performed using the detection module and compared the result with analysis using conventional method, MS-PCR and real-time PCR. (Fig. 25) DNA isolated from MCF-7 cells (**Methylated template**) was first bisulfite converted using the microfluidic sample preprocessing module and analyzed by using the detection module, MS-PCR and real-time PCR. (Fig. 25A) Human gDNA that had not been treated with bisulfite conversion (**Untreated Template**) was also used as a control to test accuracy and specificity of the bisulfite treatment by proposed method. Each chip was functionalized with either methylation-specific primer (**M primer**) or unmethylation-specific primer (**U primer**). The immediate jump was observed in resonant wavelength shift after 5 min from the chip with M primer in the presence of bisulfite-modified DNA, indicating the methylation of the target in RAR β . The increase in wavelength shift was observed up to 20 min (665 pm). (Fig. 25B) From chip with U primer, by contrast, a slight increment in resonant wavelength shift was observed (373 pm) in the presence of bisulfite-modified DNA (Methylated template), which is likely due to non-specific binding between bisulfite modified methylated target and unmethylation-specific primer. In the case of untreated gDNA, a similar level of increase in resonant wavelength shift as **methylated target-U primer** was shown. Hence, I inferred that the great increase in resonant wavelength shift from Methylated target- M primer is true positive induced by sequence alteration of unmethylated cytosine with high accuracy and specificity.

By MS-PCR, bisulfite treated gDNA from MCF-7 is successfully amplified by M primer, while untreated gDNA from MCF-7 was strongly amplified by wild-type primer (W primer). (Fig. 25C) No amplification was observed by U primer and W primer in the presence of bisulfite treated gDNA, which indicates the bisulfite modification in the microfluidic environment was fairly complete, in agreement with the result by LOC based detection module. Low C_T value from amplification by M primer in the presence of bisulfite converted gDNA in real-time PCR also indicated successful conversion of gDNA by the LOC system.

Moreover, to overcome long incubation time during primer immobilization process, I examined the long-term stability and reusability of the LOC system. (Fig. 26) First, RAR β gene forward primers were immobilized on functionalized surface of chips in batches as aforementioned. After immobilization process, the chips were vacuum-sealed in foil pouches and stored at 4°C for 0, 7 and 14 days, respectively. Then the chips were used for detection of DNA methylation of input genomic DNA (1 μ g) by measuring resonant wavelength shift after 30 min amplification. Detection process and experimental condition were the same as described above. As a result, I found no significant loss of stability of primers immobilized over a long period.

Figure 25 Analysis of on-chip bisulfite modified DNA by detection module, MS-PCR (Methylation specific PCR) and real-time PCR. (A) Workflow of bisulfite modified DNA analysis using detection module. (B) DNA methylation analysis of bisulfite treated DNA by the detection module. Resonant wavelength shift was measured during amplification on detection module functionalized with either M or U primer. (C) Analysis of bisulfite treated DNA by MS-PCR. Bisulfite treated gDNA from MCF-7 by using proposed method(I) compared to untreated (No bisulfite conversion) gDNA from MCF-7(II); M=methylated, U=unmethylated, W=Wild-type specific primer set, respectively. (D) DNA methylation analysis of bisulfite converted DNA (BS converted) by real-time PCR. Amplification with methylated specific primer (M+BS converted), unmethylated-specific primer (U+BS converted) of RAR β , and NTC (No template).

Figure 26 Repeatability of LOC based DNA methylation detection system (sample pre-processing & detection module). (A) Long-term stability of grafting primers on silicon microring resonator (SMR) for storage possibility of the detection module. (B) Reusability of the detection module. All experiments were performed using the same SMR chip for detection of the bisulfite converted gDNA. The used chip was washed with NaOH to remove all bound molecules on the surface. (C) Reusability of sample pre-processing module. All experiments were performed using the same sample pre-processing module for bisulfite conversion of gDNA from MCF-7. The used chip was washed with dH₂O to get rid of all bound molecules on the glass substrate. The DNA-modified was analyzed by MS-PCR using the methyl-specific (M) primer.

3.4.4. Quantitative analysis of DNA methylation status

Typically, clinical specimens of breast cancer have a high degree of heterogeneity carrying diverse subtypes of breast cancer cells intermixed with different percentage of healthy cells. Furthermore, breast cancer subtypes respond differently to treatment.⁹⁸ Therefore, a rapid detection system that is able to identify target mutation with high specificity is required to improve the effectiveness of clinical treatment. Accordingly, the sensitivity of the new DNA methylation detection scheme was evaluated by serial dilution assay of breast cancer cells with Jurkat cells (Wild-type RAR β gene). (Fig 27) Genomic DNA (1 μ g) extracted from each sample that contains 1 %, 10 %, 50 %, 100 % of MCF-7 cells and 100 % of Jurkat cells (No mutant included) were used. Each DNA sample was bisulfite treated and detected using the new LOC technique. (Fig. 27A) As a result, the average resonant wavelength shifts measured after 30 min by using each sample were 251.93 pm $\pm$ 1.83, 334.75 pm $\pm$ 20.7, 473.21 pm $\pm$ 16.06, 724.28 pm $\pm$ 74.68, and 868.89 pm $\pm$ 14.15, respectively. (Fig. 27B) Mutant allele was successfully detected by proposed method from MCF-7/Jurkat sample with a small fraction of MCF-7, as little as 1 %, which was not detected by real-time PCR. (Fig. 28) The superiority of the proposed method in terms of minimum detection limit (<1 % Mutant) over real-time PCR (>10 % Mutant) is presumably due to the inherent limitation of real-time PCR, as in detection of fluorescence from non-specific fluorescent dye intercalating in any double-stranded DNA. Non-specific DNA amplification (Primer dimer formation) observed might induce a high level of background as low C_T value from no template control indicates (~26.7), which hampered detection of a mutant allele from the sample with a low fraction of cancer cells. This may cause misinterpretation in profiling DNA methylation pattern. In addition, the resonant wavelength shifts showed a strong dependency on the fraction of MCF-7 in the mixture. A dependency of resonant

wavelength shifts on the concentration of target mutant cells suggests the potential for application as an efficient method of quantifying the heterogeneity of cancer cells.

Figure 27 Quantitative study of DNA methylation. Human genomic DNA (1 μ g) was extracted from serial dilutions of MCF-7 including RAR β mutation with Jurkat cells (RAR β wild-type). The ratios of MCF-7 cells were 0%, 1%, 10%, 50% and 100%, respectively. Human gDNA (1 μ g) extracted from the mixture was bisulfite converted in sample pre-processing module and DNA methylation was quantitated by the detection module. Error bars shows standard error of the mean. (A) Resonant wavelength shifts during amplification on detection module up to 30 min. Dotted line (100% *) represents resonant wavelength shift using bisulfite converted DNA of the sample with 100% MCF-7 and unmethyl-specific primer. (NTC) (B) Resonant wavelength shifts as a function of the ratio of MCF-7 cells. The presence of DNA methylation was successfully detected from a sample containing MCF-7 as little as 1 %. The shaded section represents mutation detected by LOC technique. The dashed line shows detection limit determined by resonant wavelength shift of bisulfite converted DNA from a sample with 0 % MCF-7 cell and unmethyl-specific primer).

Figure 28 DNA methylation analysis of bisulfite converted DNA by real-time PCR. Human genomic DNA (1 μ g) was extracted from serial dilutions of MCF-7 including RAR β mutation with Jurkat cells (RAR β wild-type). Extracted DNA was bisulfite converted under the same condition as on-chip detection. The mutation was detected from a sample containing MCF-7 no less than 10 %. The dashed line represents cut-off value for detection of bisulfite converted DNA determined by threshold cycle of a sample with 0 % MCF-7 cell. (NTC= No template)

3.5. Conclusion

In this chapter, I present a Lab on a Chip system that can serve as a useful tool in a wide range of epigenetic testing based on DNA methylation biomarker for both research and clinical purpose. To the best of my knowledge, this is the first LOC system for DNA methylation detection from cancer cells that is based on on-chip bisulfite conversion. I demonstrated successful bisulfite conversion and subsequent real-time, label-free detection of DNA methylation in RAR β gene within 90 min using the novel LOC technique. On-chip bisulfite conversion by sample preprocessing module was first validated by end-point PCR and real-time PCR using bisulfite converted DNA sample. Then I determined the detection limit of 1% mutant using mutant/wild-type mixture. A

miniaturized and automated assay offers easy handling of reagents and samples bypassing use of bulk instrumentation.

Furthermore, I demonstrated that the LOC system is a suitable choice of method for analyzing the DNA methylation from human gDNA with a rapid and simple process yet high sensitivity as compared to other PCR-based methylation analysis techniques that are in general use recently. This modular approach will accelerate individual optimization and reconfiguration of the bisulfite pretreatment and detection module. Even though isothermal amplification based on RPA has been chosen as a detection method in this study, microfluidic bisulfite treatment module is compatible with the majority of bisulfite based detection techniques. Current sample pre-processing module is applicable to a variety of analysis methods, such as MS-HRM assay, which are preceded by bisulfite conversion. Therefore, potential applications to alternative sensing techniques are under investigation. This novel method needs further testing with large clinical samples. Nevertheless, this LOC system holds great potential for DNA methylation analysis and incorporation to current bisulfite-based sequencing techniques as a cancer diagnostic tool for both research and clinical use.

In summary, there are three major noteworthy features in the proposed LOC based DNA methylation analysis technique. To the best of my knowledge this is the first microfluidic system on which bisulfite conversion are practiced. By leveraging its microscale environment, it allows for rapid sample processing. In addition, integration of bisulfite conversion module with silicon microring is a new approach to reduce total analysis time. Using the sample treated in sample processing unit, silicon microring was able to perform DNA methylation analysis with minimized human intervention. Lastly, real-time and label-free detection is also important feature that arises from silicon microring resonator technology combined with isothermal amplification method.

A Disposable Microfluidic Platform for RNA Isolation

In this chapter, I present a novel method of RNA isolation utilizing disposable Lab-on-a-chip system that provides high-quality RNA from a variety of biological samples. Despite a huge advance in Lab-on-a-Chip (LOC) technology, miniaturized diagnostic platform based on RNA has been falling behind due to lack of on-chip RNA isolation techniques. This is mainly because of challenges in handling RNA samples as RNA is more susceptible to degradation than DNA. RNA analysis also plays an important role in understanding the expression of genes associated with diseases as well as detection of pathogenic diseases. To meet the increasing need for RNA analysis in Point-of-care (POC) testing, development of reliable LOC based RNA isolation method is to be preceded. By taking advantages of pH-dependent reversible DMA binding to RNA, RNA isolation was performed on polymer microfluidic LOC system without the need for chaotropic salts or alcohol within 30 min. Gene expression analysis was successfully carried out using RNA isolated from different cell lines (HEK293, Caco-2, MCF-7 and T24) by following real-time RT-PCR. In addition, I validated the utility of assay in viral RNA isolation by performing Dengue virus serotype identification. High recovery rate, rapid processing with minimized contamination demonstrated here, therefore, shows that this novel method can be an ideal candidate for potential RNA isolation technique in gene expression research and diagnosis of infectious diseases caused by RNA viruses in POC testing.

4.1. Introduction

Development of miniaturized diagnostic systems is becoming increasingly popular in molecular diagnostics taking advantages of Lab-on-a-Chip (LOC) techniques.⁹⁹ One of

the most active applications of miniaturized diagnostic systems is nucleic acid testing (NAT) due to its high sensitivity and specificity, which is critical for efficient diseases monitoring and successful identification of appropriate treatment with shorter window period especially for acute infectious diseases such as Dengue fever. The use of nucleic acid based LOC platforms offers many benefits over conventional laboratory-based techniques. For examples, Point-of-care testing using LOC based NAT can provide rapid detection of pathogens (Bacteria, viruses) for diagnosis of infectious diseases in low-resource environments.^{100,101} POC testing enables early detection without the need for bulky instrument involved in typical centralized laboratory-based testing. Hence, it can be a powerful tool for the effective control of infectious diseases by addressing access barriers to health care services experienced by patients specifically in developing countries. Indeed, a huge effort has been taken to develop miniaturized NAT platform for identification of pathogenic infection such as HIV to replace current screening system in developing countries where inadequate health care facilities hinder early diagnosis for efficient treatment.¹⁰² And also, LOC based NAT can be a low-cost and efficient tool for Point-of-Care (POC) genetic analysis of diseases such as cancers in resource-limited settings.¹⁰³

In fact, a number of nucleic acid-based analysis platforms have been developed, however, the majority of them are based on DNA. This is mainly due to the nature of RNA such as low structural stability of RNA as compared to DNA which results in short half-life.¹⁰⁴ And also, RNA is more vulnerable to degradation during cell lysis process caused by ubiquitous RNases present both in the cellular lysate itself and environment, which makes RNA analysis challenging and special precautions are required to prevent sample contamination when dealing with RNA samples.²⁸ Despite challenges associated with sample handling in RNA analysis, there has been increasing the need for LOC

platforms for RNA analysis since RNA analysis plays crucial roles in the identification of genetic diseases like cancer, quantitative studies of gene expression and pathogen detection. While quite a few LOC techniques involved in RNA studies have been reported, majority of techniques are focused on detection that needs off-chip RNA isolation.¹⁰⁵⁻¹¹¹ RNA isolation in a miniaturized platform has remained relatively unexplored. Lack of reliable LOC based RNA isolation techniques has limited the development of integrated analytical systems for RNA analysis.¹¹² Therefore, development of reliable LOC RNA isolation technique that facilitates the RNA handling is necessary to realize practical RNA analysis platform for Point-of-care application.

Many RNA isolation methods have been practiced in molecular diagnostics, such as phenol-chloroform based extraction⁴, Oligo-dT based affinity chromatography^{26,27} and silica based purification²⁸. These conventional extraction methods like phenol-chloroform based extraction and silica based purification have been widely used due to its high yield and rapid stabilization of RNA. But the use of these conventional methods is restricted to laboratory-based diagnosis level because laborious hands-on processes, as well as centrifugation steps, are extensively involved during purification procedure for most of conventional RNA extraction methods. And also, in the case of viral RNA extraction, low concentration of viral RNA extracted in large volume makes pathogen detection more challenging due to sample dilution during conventional sample processing. Moreover, contaminants carryover like phenol and chaotropic reagents may cause inhibition of enzymatic reactions during PCR or other downstream analysis procedures, which makes it difficult to fully realize automated on-chip assay. In this respect, microfluidic-based LOC techniques can solve the current issues associated with conventional isolation methods and address the needs for RNA analysis in POC testing.

In order to overcome the challenges involved in RNA isolation, there have been a few attempts to bring RNA extraction onto microfluidic chip assay. Typically, total RNA is extracted from biosamples and captured onto solid phase matrix under specific buffer condition. After removal of impurities, elution buffer flows through the matrix releasing captured RNA molecule by changing the binding affinity. One of the most common methods is silica based solid phase extraction (SPE). In the presence of chaotropic salt, RNA is bound to a silicon substrate as the lysates containing RNA is introduced into the microfluidic channel while other residual contaminants including proteins are removed during the washing step. This method has been employed in a number of publications^{29,113-115} as the purification mechanism is similar to that of DNA isolation. However, typical silica-based isolation methods utilize alcohol to enhance nucleic acid binding like as silica column based purification, which is likely to be included in collected eluent despite thorough washing. Alternatively, mRNA isolation has been practiced on microfluidic device taking advantage of oligo(dT) affinity purification technique that selectively binds to poly (A) tail of mRNA.^{31,32} This protocol is originated from oligo(dT) conjugated resin column for mRNA enrichment and modified protocol has been implemented onto a microchip. This method can provide reproducible, and high mRNA extraction efficiency but the utilization of this technique is limited to analyzing gene expression of eukaryotic cells. To circumvent the problem associated with the use of chaotropic agents, microfluidic-based RNA isolation in aqueous solution was devised.^{116,117} For example, Hagan K. *et al.*³⁰ demonstrated RNA isolation from cancer cells using pH dependent Chitosan-RNA binding in a miniaturized device. Silica microbead surfaces were coated with chitosan for reversible RNA binding whereby the bound RNA molecules can be released simply by adjusting the pH level of buffer above pKa of the amino group during elution step. It provided higher RNA recovery rate (71%)

as compared to silica binding method while avoiding the use of chaotropic salts or alcohol during the extraction process. However, it involved long processing time for preparation of chitosan coated silica and integration into microchip prior to extraction.

Most of these LOC-based RNA isolation techniques rely on expensive fabrication process and involve complicated preparation procedures using microparticles^{31,118-123} or porous structures^{115,116,124} to enhance the isolation efficiency. As an alternative, our group recently demonstrated a novel non-chaotropic DNA extraction technique based on dimethyl adipimidate (DMA) on a microfluidic system.^{125,126} DMA was able to capture DNA onto either amine modified silicon or polymer microfluidic channel surfaces forming amidine bond at pH 8-10 and release DNA molecules above pH 10. Neither chaotropic salt nor alcohol is required during the process which is beneficial in downstream analysis procedure. Moreover, this method eliminated the need for complicated fabrication process by using cost effective rapid prototyping. It has proven its utility in DNA extraction from eukaryotic and prokaryotic cells as well as body fluid.

In this chapter, I present my achievement which is focused on 1) RNA isolation utilizing Lab-on-a-chip system that enables rapid sample preparation for subsequent gene expression analysis and pathogen detection, 2) Demonstration of gene expression analysis using the isolated RNA from the LOC system and 3) Identification of Dengue virus serotypes using viral RNA isolated using LOC system.

The assay exploits reversible DMA binding to capture and release RNA by adjusting buffer pH level in a microfluidic device. The microfluidic device was fabricated with a hydrophilic polyester film that allows for cost effective manufacturing and rapid prototyping. The RNA isolated from assay has been proven to be suitable for reverse transcription, PCR detection with high purity and integrity and the results are comparable to that of commercial kit yet with a simple procedure. I validated the utility

of assay in gene expression level analysis by purifying RNA from various human cancer cell lines. And also, identification of Dengue virus serotypes was performed successfully using the purified RNA in the assay, which suggests that the assay has great potential to serve as a diagnostic tool for pathogen detection for POC application.

4.2. Working principle

The working principle of a microfluidic system for RNA purification is illustrated in Fig. 29. Off-chip cell lysis was carried out using detergent-based lysis buffer and the lysate was injected onto the chip. The mixture contains DMA as a non-chaotropic binding agent. DMA, known as a protein crosslinker, has imidoester groups at both ends, which is reactive towards amine group. Imidoester group in DMA reacts with an amine group in nucleic acid and an amine group on the chip surface, forming amidine bond. Before loading, the lysate was treated with DNase and Proteinase K to get rid of contaminating genomic DNA and protein as they will compete with the binding reaction between RNA and DMA, thereby reducing RNA recovery rate. As cell lysate is loaded onto the microfluidic device, DMA in sample mixture captures RNA molecule onto amine modified chip surface. Binding reaction between DMA and amine group is pH dependent. For efficient binding, the pH level of the sample was monitored and maintain ~pH 8. After sufficient incubation for binding, PBS was loaded onto the chip to get rid of contaminants and cell debris while retaining RNA-DMA complex on the surface. In order to retrieve purified total RNA from the chip surface, 10 mM sodium bicarbonate (pH 10.6) was used as an elution buffer. Since DMA-RNA reaction is reversible at the high pH level, RNA was released from the surface and collected in the elution buffer.

1. Surface treatment

3. On-chip RNA isolation

2. Cell lysis

Figure 29 Schematic of Lab-On-a-Chip system for rapid and simple RNA isolation. Chemical lysis was carried out, and the lysate was injected onto the chip with DMA as a binding agent. Before loading, the lysate was treated with DNase and Proteinase K to remove gDNA and protein. As cell lysate is loaded onto the microfluidic device, DMA/RNA conjugates were captured onto amine-modified polymer chip surface. As the binding reaction is pH dependent, the pH level of the sample was monitored and maintain ~pH 8. After incubation for RNA binding, contaminants and cell debris were removed during washing step while retaining RNA-DMA complex on the surface. After washing, 10 mM sodium bicarbonate (pH 10.6) was used as an elution buffer. Above pH 10, RNA was released from the surface and collected in the elution buffer. Total RNA can be utilized for subsequent downstream analyses.

4.3. Materials and Methods

4.3.1. Device fabrication

The prototype of on-chip RNA extraction device was designed using CorelDRAW software and constructed from polyester sheets by laser cutting process (Universal Laser Systems, Scottsdale, AZ, USA) for rapid prototyping and low-cost manufacturing as described previously.⁵¹ The device consists of 6×6 micro-reservoirs array for RNA binding connected to fluidic channel network with single inlet and an outlet for sample injection. Microfluidic structures were patterned on a double-sided adhesive layer (3M 9475LE, USA). And for top and bottom layers, clear hydrophilic polyester film (3M, USA) was used. Through holes were drilled on the top layer for inlet and outlet ports. Prior to assembly, the surfaces of both top and bottom layers were activated in oxygen plasma treatment system (Femto, Diener Electronic, 80W, 5-10 min, 2 sccm). After plasma treatment, protective layers attached to double-sided adhesive layer were removed immediately, and the layer was sandwiched between top and bottom layer. Then inlet and outlet connectors were aligned and connected to through holes on the top layer and Tygon tubings (AAC02548; Cole-Parmer, VernonHills, IL, USA) were inserted into holes. Epoxy glue was then applied to prevent leak around the port. After assembly, microfluidic reservoirs were functionalized in 2% (3-Aminopropyl)triethoxysilane (APTES) in RNase-free water for 1 hr at 65°C then washed with 1ml ultrapure DEPC-treated water.

4.3.2. RNA isolation from cancer cells on chip

Total RNA isolation from cells was performed using the LOC system. (Fig. 30)

Figure 30 RNA isolation process using the disposable microfluidic platform. Cells were lysed and homogenized off-chip using chemical lysis buffer. Then the lysate was mixed with proteinase K and DNase, as well as DMA, followed by sample loading on a microfluidic platform. The lysate was incubated for 10-20 min to complete gDNA & protein removal and binding reaction between DMA-RNA complex and microfluidic channel surface. After incubation, thorough purification is required to remove residual cell debris and impurities before elution. RNA was eluted by increasing buffer pH level thereby reversible reaction occur to release RNA from the chip surface. RNA isolated from LOC system was analyzed for downstream application.

Detergent based cell lysis buffer provided by One Biomed was used for RNA extraction from cancer cells. Cells were pelleted by centrifugation at 1200 rpm for 5 min, and the culture medium was removed. Then cell pellets were resuspended in 50 μ l PBS by vigorous pipetting for homogenization. In order to isolate RNA from cells, 100 μ l of lysis buffer was mixed with cells in tubes. Then 100 mg/mL DMA dissolved in DEPC water was added to cell/lysis buffer mixture as a binding agent. To eliminate genomic DNA and protein, the lysate was treated with 20 μ l of RNase-free DNase set (Qiagen) and Proteinase K (Qiagen) at room temperature. And the mixture was loaded onto the microfluidic chip using 1 mL sterile syringe and infusion pump (KD Scientific) at a constant speed then incubated for 10 min at room temperature to enable DMA-RNA complex to bind to the amine-modified surface. After incubation, microfluidic chip surface was washed thoroughly with PBS at flow rate 100 μ l/min to remove residual contaminants and cellular debris. Finally, RNA was released in elution buffer by increasing pH level (pH>10). The final concentration and purity of RNA were determined by Pelkin Elmer Multimode plate reader.

4.3.3. Viral RNA isolation on chip

To demonstrate the utility of microfluidic RNA extraction chip in virus detection, three different serotypes of Dengue virus were used. DENV-1 strain 159, DENV-2 strain New Guinea C and DENV-4 strain 242270 were derived by incubating virus in Vero cells in culture. For viral RNA extraction on the chip, 14 μ l of cell culture supernatant containing heat inactivated virus was diluted with 36 μ l PBS to 2.8×10^2 pfu/ μ l. Lysis buffer containing 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS and 10% Triton x-100 was added to the sample and mixed thoroughly by pipetting. DMA in DEPC treated water was mixed with lysate. Then the sample was injected onto microfluidic RNA extraction chip using 1mL sterile syringe. After 10 min incubation, chip surface

was washed with PBS at a flow rate 100 μ l/min. Viral RNA-DMA complex captured on the chip was eluted in 100 μ l sodium bicarbonate at flow rate 33.3 μ l/min. For comparison, RNA isolation was also carried out by an on-column method using commercially available kit. All reagents were obtained from QIAamp viral mini kit (Qiagen). In accordance with protocol provided by the supplier, 14 μ l of cell culture supernatant containing heat inactivated virus was diluted in PBS up to 140 μ l. Viral RNA was eluted in 100 μ l Buffer AVE from the column after purification.

For cell preparation, human embryonic kidney (HEK) 293 cells and two human cancer cell lines, T24 (Human urinary bladder carcinoma) and MCF-7 (Human breast adenocarcinoma) were cultured in Minimum Essential Medium (MEM, Biowest) supplemented with 10% fetal bovine serum (FBS, Biowest) in a 37°C humid incubator with 5% ambient CO₂. For Caco-2 (Human colorectal adenocarcinoma cells), Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) was used as culture medium. Once confluent, cells were taken out from the flask using 0.25% Trypsin-EDTA (Biowest) solution.

For gene expression analysis and quantitation of isolated RNA, either one-step or two-step real-time reverse transcription (RT) PCR were performed as a detection method. For cancer cell RNA isolation, two-step real-time RT-PCR was carried out. Reverse transcription was conducted using Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer's protocol. A mixture of Anchored-oligo(dT)₁₈ primer and random hexamer primer was used for cDNA synthesis. Subsequently, real-time RT-PCR was carried out using LightCycler FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's protocol. In brief, PCR Mix contained 5 μ l cDNA template with 250 nM forward and reverse primer in 20 μ l. One-step real-time RT-PCR was performed using LightCycler RNA Master SYBR Green I (Roche)

following viral RNA isolation. RT-PCR Mix contained 5 µl RNA template with 200 nM forward and reverse primer, 3.25 mM Mn(OAc)₂ and 7.5 µl LightCycler RNA Master mix in 15 µl. The amplification condition consists of 20 min reverse transcription of template RNA at 61°C, 30 s initial denaturation at 95°C, 45 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 18 s. All primer sets used for amplification are described in Table 3.

Table 3. Primer sequences for RT-PCR

Sample types	Primer set	Sequence
Cancer cell lines	18S rRNA Forward	5'-GCTTAATTTGACTCAACACGGGA-3'
	18S rRNA Reverse	5'-AGCTATCAATCTGTCAATCCTGTC-3'
	EGFR Forward	5'-AAACTGCACCTCCATCAGTG-3'
	EGFR Reverse	5'-ATTCGTTGGACAGCCTTCAAG-3'
Dengue Viruses	DEN-1-Forward	5'-GAC ACC ACA CCC TTT GGA CAA-3'
	DEN-1-Reverse	5'-CAC CTG GCT GTC ACC TCC AT-3'
	DEN-2-Forward	5'-CAT GGC CCT KGT GGC G-3'
	DEN-2-Reverse	5'-CCC CAT CTY TTC AGT ATC CCT G-3'
	DEN-4-Forward	5'-TGA AGA GAT TCT CAA CCG GAC-3'
	DEN-4-Reverse	5'-AAT CCC TGC TGT TGG TGG G-3'

4.4. Results & Discussion

4.4.1. Numerical simulation

The fabricated microfluidic RNA extraction chip consists of contraction-expansion array (CEA) to ensure rapid mixing at high volume flow rate during sample loading and washing processes. Fluid flowing through sudden contraction-expansion region

experience multi vortices at high Re , which is beneficial to effective mixing during the operation. In this configuration, two types of secondary flows can be generated within the cavity array. At high Re , a horizontal vortex in expansion area is created due to flow detachment, along with cross sectional counter-rotating vortex that induced at the entrance of contraction channel area due to pressure gradient. The multi vortices in the designed CEA channel were numerically investigated via CFD simulations by using COMSOL Multiphysics. Fig. 31A includes two units of contraction-expansion array configuration. Abrupt contraction (Contraction ratio=5.6) produced fluid acceleration in contraction area. The streamlines at the entrance of expansion cavity are illustrated in Fig. 31B. Horizontal vortex in expansion cavity appears when Re is high enough ($Re > 12$) to cause fluid detachment and the vortex grows as Re increases. A secondary flow in a cross sectional plane was also observed at the same Re near the contraction cavity entrance. (Fig. 31C) These multi-vortices in horizontal and cross sectional plane may facilitate fluid mixing at high Re thereby enhances efficient and rapid sample processing ensuring high-throughput operation. Multi-vortexing effect on RNA-DMA surface binding efficiency is under investigation in CEA geometries for future development of integrated microfluidic RNA extraction system with multiple fluid streams.

Figure 31 Numerical simulation of multi-vortices in the microfluidic RNA extraction chip. (A) Fluid flow field calculation by computational fluid dynamics simulation. Contraction-expansion array (CEA) geometries were used in the microfluidic RNA extraction chip. (B) Simulation results in a horizontal plane (x-y plane) at different Reynolds numbers ($Re = 7.4, 15$, and 37) illustrate the vortex at the entrance of the expansion area at a high flow rate due to flow detachment. (C) Velocity field calculation (y and z components) in a cross-sectional plane (y-z plane) shows four counter-rotating vortices near the entrance of the contraction area.

4.3.2. Polymer surface silanization

Mechanism of RNA binding on microfluidic walls is based on the reaction between Dimethyl adipimide (DMA) and RNA. To capture DMA-RNA complex onto the microfluidic channels, the surface must be functionalized with amino silane. For silanization procedure, (3-Aminopropyl)triethoxysilane (APTES) was used thereby creating an amine-reactive group on the surface. In order to realize rapid prototyping, polyester film was used as a material for the microfluidic device. For reliable and reproducible RNA binding on the polymer surface, I investigated conditions under which stable silanization of polymer surface is achievable. Since thermoplastics has low glass transition temperature, silanization of polymer microfluidic surface was carried out at low temperature (from RT to 65°C) with APTES in water unlike silanization process for conventional materials such as glass and silicon (at 120°C in ethanol).

Hydrophilicity of the film surface is known to increase significantly when the surface is coated with APTES. To determine the stability of silane on polymer surface after surface modification, I investigate hydrophilicity of the surface after each treatment step by measuring water contact angle. The shape of distilled water drop on the surface after each step was captured and the contact angle was analyzed. (Fig. 32A) To begin contact angles was first measured on the intact surface before treatment. (69.33 ± 0.47) Then polymer surface was treated with oxygen plasma and a significant decrease in contact angle was observed. (25.00 ± 0.45) Oxygen plasma treated polymer surface was then placed in 5% aqueous APTES solution for 1 hr. Polymers treated at room temperature yielded $64.93^\circ \pm 1.39$, which indicate incomplete silanization with APTES according to the previous studies¹²⁶. On the other hand, water contact angle on polymer silanized at 65°C was $37.3^\circ \pm 1.15$. This indicates incubation of polymer surface during silanization process at higher temperature can accelerate silanization and increase the wettability of the surface. (Fig. 32B)

A

1. Intact surface

2. After plasma

3-1. After APTES(RT)

3-2. After APTES(65°C)

B

Figure 32 Water contact angle measurement on the polymer surface. The contact angles were measured on the intact surface and after oxygen plasma treatment, APTES treatment respectively. APTES treatment was performed either at room temperature (3-1) or 65 °C for 1h.

4.3.3. Optimization of disposable microfluidic RNA isolation chip.

Optimization of RNA isolation procedure on microfluidic RNA purification chip was performed. (Fig. 33-34) In order to assess the ability of DMA to enhance binding of RNA onto an amine-modified polymer surface, 1 μ g of total RNA extracted from HEK-293 cells was injected into microfluidic device and eluents were collected to determine the absorbance in a spectrophotometer. RNA yield and purity were estimated from absorbance data at 260 nm and 280 nm accordingly. In the presence of DMA, the average RNA yield was $87.1\% \pm 3.61$, while it was $73.8\% \pm 6.53$ in the case without DMA. (Fig. 33A) To evaluate the suitability of RNA sample collected from microfluidic chips for down-stream applications, purity of samples was assessed. RNA sample purity was ~ 1.9 in the presence of DMA, which is within the acceptable range of purified RNA (Fig. 33B). A noticeable improvement in purity using DMA indicates that DMA enhances the DNA binding onto the amine-modified surface while other contaminants are removed efficiently by washing with PBS.

Figure 33 Optimization of disposable microfluidic RNA purification chip. (A) Comparison of total RNA purification efficiency on APTES treated the surface with and without DMA. In order to investigate the RNA recovery rate of the assay, 1 μ g of total RNA extracted from HEK-293 cells was injected with DMA as a binding agent(Black) or without DMA(Grey). RNA recovery rate was determined by absorbance at 260 nm using a spectrophotometer. (B) Purity of each sample was determined by ratio A_{260}/A_{280}

Next, the effect of DNase and Proteinase K treatment of lysate was investigated. Since DNA and protein also can react with a functional group of DMA, residual genomic DNA and protein in cell lysate may reduce RNA binding affinity towards DMA. And also, gDNA can be coamplified with cDNA in subsequent PCR, which may affect the expression level analysis results. Typically real-time RT-PCR is a method of quantitation of transcript in gene expression studies.¹²⁷ There are two methods (Absolute and relative quantitation) of analyzing gene expression by using real-time RT-PCR. Absolute quantitation of transcript can be performed by comparing threshold cycle (C_T) values to a standard curve. On the other hand, relative quantitation can be done by normalization of C_T values to the C_T values of a reference transcript. In real-time PCR, C_T values are inverse to the amount of input DNA or RNA so that it can be a measure of the number of copies in the sample. Higher C_T values indicate low amounts of targeted DNA or RNA. Fold difference is equal to $2^{-(\Delta C_T)}$. Therefore, real-time PCR is commonly used method for evaluation of the performance of RNA isolation techniques. Detection mechanism is based on fluorescence signal generated during the amplification. During PCR, the number of double stranded DNA increases and fluorescent dye (SYBR Green I dye) intercalate generating fluorescent signal. The fluorescent signal from SYBR Green I dye is measured at 530 nm at the end of each amplification cycle. In order to optimize the DNase treatment, different amount of DNase I (Qiagen) (5, 10 and 20 μ l) was added to HEK-293 cell lysates during off-chip lysis step and RNA was extracted from the assay for gene expression analysis.

EGFR expression level relative to a housekeeping gene (18S rRNA) was determined by real-time quantitative RT-PCR and the results between samples were compared (Fig. 34). As a result, I found C_T values of both housekeeping gene and EGFR increased as

input amount of DNase I rose from 5 to 20 μ l. This variation is presumably due to residual contaminating gDNA after incomplete gDNA digestion when the input DNase I concentration is too low. However, the variation in C_T value of housekeeping gene (ΔC_T 1.7) is higher than that of EGFR gene (ΔC_T 1.2) which leads to variation in EGFR expression level. This indicates removal of gDNA by DNase treatment from lysate can help in the interpretation of gene expression data. In the same manner, I studied the effect of proteinase K on expression level. The input amount of DNase I was 20 μ l in this case. EGFR expression level also varies depending on input volume of Proteinase K. As illustrated in Fig 34, the expression level decreased as input Proteinase K increased. And, I found C_T value of both genes decreased when using a higher volume of Proteinase K, indicating that higher number of RNA was adsorbed to the surface after Proteinase K treatment. Consequently, I found the enhancement in RNA yield using DMA as a solid phase in RNA purification and optimized DNase and Proteinase K treatment conditions

Figure 34 Effects of DNase and Proteinase K treatment on relative gene expression level. 5 μ l, 10 μ l and 20 μ l of DNase and Proteinase K were added into HEK-293 cell lysates, respectively and RNA was extracted from the assay for expression level analysis. EGFR expression levels relative to a housekeeping gene (18S rRNA) were determined by real-time quantitative RT-PCR.

4.3.4. Characterization of disposable microfluidic RNA isolation chip

Under optimized cell lysis conditions, RNA isolation was performed on microfluidic assay by using human embryonic kidney (HEK) 293 cells and compared to the on-column method. RNA was extracted from 1×10^6 cells and used as a template for real-time quantitative RT-PCR. C_T values of the housekeeping gene (18S rRNA) of each sample were compared to estimate the amount of eluted RNA. As a result, RNA extracted from microfluidic assay yielded 4~5 cycles higher C_T compared to C_T obtained using the on-column method, which indicates lower amplification efficiency. (Fig. 35A) To address inhibition during amplification, I investigated the effect of washing volume before RNA elution on amplification efficiency. As a result, a noticeable decrease in C_T values of 18S rRNA was observed as washing volume increases. The average C_T value of 18S rRNA decreased from 10.7 to 8.0 by increasing the volume of PBS injected from 1 to 2 ml during washing step, indicating that sufficient surface washing is needed before elution to remove PCR inhibitors that can be possibly carried over and affect the performance of real-time PCR which leads to false negative results. By optimizing washing for efficient removal of inhibitors, C_T obtained from the sample prepared by using the microfluidic method was equivalent to C_T of the on-column method.

To evaluate the efficiency of the assay in total RNA extraction, total RNA was isolated from 10-fold serial dilutions from 1×10^6 to 1×10^2 cells. RNA was collected in 100 μ l elution buffer, and 5 μ l of each sample was used as a template for real-time quantitative RT-PCR. As a result, 18S ribosomal RNA transcript was detected in down to 100 cells by real-time RT-PCR using the novel method (Fig. 35B). As a result, the utility of assay in downstream RNA analysis was demonstrated despite the difference in C_T value of 18S rRNA.

Figure 35 Characterization of disposable microfluidic RNA isolation chip. (A) Effects of input PBS volume during washing step on gene expression level. Total RNA was isolated from 10^6 HEK 293 Cells. Before elution 1ml (LOC) and 2 ml (LOC*) of PBS was used as washing buffer for on-chip RNA isolation. C_T values of reference gene (18S rRNA) were investigated. C_T values of RNA extracted using on-column method is included for comparison. (B) Quantitative analysis of extracted RNA. Total RNA was purified from serial dilutions of HEK-293 cells (10^6 to 10^2 cells) using microfluidic RNA isolation chip and analyzed by real-time quantitative RT-PCR.

4.3.5. RNA isolation from various cancer cell lines and gene expression analysis

RNA isolation is primarily carried out as a preparative process in gene expression profiling. Since the good quality and integrity of RNA is essential for successful subsequent analysis procedures such as reverse transcription and real-time PCR, I validated the utility of assay in gene expression analysis by isolating RNA from four different human cell lines with the microfluidic method. RNA was isolated from HEK 293 cells and various human cancer cell lines, T24 (Human urinary bladder carcinoma), MCF-7 (Human breast adenocarcinoma) and Caco-2 (Human colorectal adenocarcinoma) cells (3×10^5 cells each) using both microfluidic RNA extraction assay and silica based on-column method (Qiagen) for comparison. Using 5 μ l of isolated RNA from each assay, two-step RT-PCR was conducted to estimate gene expression level in each cell line. For relative quantitation of EGFR, I used threshold cycle data of EGFR and 18S rRNA. I compared the amplification curves of the housekeeping gene (18S rRNA) to estimate the amount of eluted RNA. (Fig. 36) The amount of target (EGFR) was normalized to reference gene (18S rRNA).¹²⁸ Transcript level of EGFR relative to 18S rRNA for samples purified by the novel assay was consistent across samples. (Fig. 37) RNA using the assay consistently showed higher expression level, which is mainly due to higher C_T value of 18S rRNA while C_T of EGFR remains equal. This suggests isolation performance of the assay is equivalent for target transcript, which is low abundance transcript in the total cellular RNA pool. There was a little variation in absolute C_T value of reference gene, however, RNA from the new assay has shown equivalent expression level to that of the on-column method.

A

B

Figure 36 RNA purification from various cancer cell lines and gene expression level analysis. RNA was isolated from MCF-7, T24 and Caco-2 cells (3×10^5 cells each) using the microfluidic RNA isolation chip and on-column method. (A) Real-time quantitative RT-PCR of eluted RNA from different cancer cell lines. 5 μ l of eluted RNA from each assay was used as a template. Amplification curves for a housekeeping gene (18S rRNA) are shown (B) Comparison of threshold cycles between microfluidic RNA isolation chip and on-column method. Using the amplification data from A, threshold cycles are compared

A **B**

Figure 37 RNA isolation from various human cell lines and gene expression level analysis. (A) RNA was isolated from HEK 293 (1×10^6 cells each) using the microfluidic RNA extraction chip and on-column method. To analyze gene expression level, real-time quantitative RT-PCR of eluted RNA from different human cell lines was carried out by using 5 μ l of eluted RNA from each assay as template and threshold cycle data of target gene(EGFR) were compared. (B) RNA was isolated from HEK 293, MCF-7, T24 and Caco-2 cells (3×10^5 cells each) using the microfluidic RNA extraction chip and on-column method. From the threshold cycle data of 18S rRNA and EGFR, relative gene expression levels were determined.

4.3.6. Detection of Dengue virus using microfluidic RNA isolation chip

Low abundance of viral RNA is one of the challenges involved in viral RNA isolation due to sample dilution during purification process when using conventional method. By using LOC based RNA isolation technique, I tested the ability to purify viral RNA from Dengue virus. To apply novel RNA extraction method to viral RNA detection, I compared the viral RNA isolation efficiency of lysis buffers. From DENV-1 sample, viral RNA was eluted, and the amount of purified viral RNA in each sample was estimated by real-time RT-PCR. (Fig. 38) For viral RNA extraction, the lysis buffer used for total RNA isolation from human cancer cells (LB1) showed low recovery rate both on column and chip. The other detergent-based lysis buffer which contains Triton X-100 (LB2) showed comparable recovery rate with chaotropic lysis buffer on the column. I quantitated viral RNA eluted by on-chip method and silica based on-column method and compared threshold cycle data obtained by one-step real-time RT-PCR. As a result, an on-chip method using Triton x-100 based lysis buffer resulted in lower threshold cycle as compared to an on-chip method with the other detergent-based lysis buffer, which was ~2.9 cycles higher than that of the on-column method. Further experiments on Dengue viral RNA extraction was performed under optimized condition using Triton X-100 based lysis buffer.

Figure 38 Comparison of viral RNA isolation efficiency. In order to compare virus lysis efficiency, Dengue-1 virus supernatant was mixed with chaotropic or non-chaotropic (LB1 and LB2) lysis buffer and loaded onto silica spin column. (Left) Eluted RNA from the spin column was used as a template for real-time RT-PCR for quantitative analysis. Threshold cycle of LB2 is comparable to that of chaotropic reagent from a commercially available kit (chaotropic method). Using non-chaotropic lysis buffer (LB1, LB2) viral RNA extraction from Dengue-1 virus was performed on the chip and eluted RNA was quantified by real-time RT-PCR. (Right) As a result, improvement in amplification was observed using LB2 on chip (LOC2) as compared to LB1 on chip (LOC1).

Dengue infections are caused by Dengue virus transmitted by mosquitoes. The dengue virus genome is single stranded RNA.¹²⁹ There are four distinct serotypes of the virus, DENV-1, DENV-2, DENV-3 and DENV-4 that cause dengue fever. Under optimized buffer conditions, the utility of microfluidic RNA extraction chip in virus detection was investigated by using three different dengue virus serotypes (DENV-1, DENV-2 and DENV-4) in cell culture supernatants. Virus supernatant was mixed with lysis buffer containing DMA and processed on microfluidic RNA isolation chip. For comparison, on-column viral RNA isolation was conducted in parallel. The eluted samples from both assays were analyzed by real-time RT-PCR. (Fig. 39) Amplification was carried out with 5 µl of RNA template. As a result, all three serotypes were successfully amplified in both eluates. RNA integrity was also qualitatively assessed by end-point RT-PCR and the following electrophoresis. Comparable band intensity after amplification was observed from all serotypes by both methods without noticeable RNA degradation. This suggests the potential for application as an efficient purification method for identifying Dengue virus serotypes.

Figure 39 Detection of Dengue virus from eluted viral RNA using microfluidic RNA extraction chip. Viral RNA extraction from three different dengue virus serotypes. The utility of microfluidic RNA extraction chip in virus detection was investigated by using three different dengue virus serotypes (DENV-1, DENV-2 and DENV-4). Virus supernatant was mixed with lysis buffer containing DMA and processed on microfluidic RNA isolation chip. (A) The eluted viral RNA samples were analyzed by real-time RT-PCR. (B) For comparison, conventional on-column isolation was conducted. RNA integrity was also qualitatively assessed by conventional RT-PCR and the following electrophoresis.

4.4. Conclusion

I have validated a novel LOC approach to RNA isolation employing DMA as a binding agent which allows for streamlined process reducing the risk of contamination associated with conventional methods, thereby achieving high-quality RNA preparation. The entire purification procedure can be performed within 30 min. The assay has shown high RNA recovery rate (~87%) in the presence of DMA. The optimization was carried out to enhance recovery rate and purity by removing contaminants that can compete with the binding reaction between RNA and DMA. The RNA quality and integrity was investigated by means of real-time RT-PCR and end point RT-PCR and the result was equivalent to that of commercially available kit for target transcript. I successfully demonstrated analysis of EGFR gene expression in different human cell lines (HEK 293, Caco-2, MCF-7 and T24) and Dengue viral RNA (three types) detection by performing real-time RT-PCR with samples prepared on the assay. The RNA yielded consistent results with no significant degradation. The LOC based RNA isolation assay provides many advantages over conventional RNA preparation methods. First of all, unlike typical silica-based assay, the purification process was carried out without the need for chaotropic agents or alcohol, thereby reducing the risk of sample contamination that may cause PCR inhibition. Second, the procedure has been streamlined by using microfluidic platform that minimizes manual process and inefficient sample transfer. Moreover, the microfluidic platform offers RNase-free environments, which makes the new assay more attractive as an alternative to conventional purification techniques, as it can solve the challenges regarding RNA degradation caused by RNase that hampers accurate quantitation and evaluation of gene expression level.

LOC based RNA isolation has emerged as a great interest in recent publications, however, the majority of techniques still involves expensive fabrication processes and laborious preparation procedures. By using RNA-DMA binding on amine-modified

polymer microfluidic platform, I was able to achieve cost-effective fabrication and rapid prototyping. Furthermore, the assay is simple to use with little human intervention which makes it suitable to build micro total analysis system being integrated with LOC detection techniques. The integrated system will be able to serve as a diagnostic tool in POC RNA testing for monitoring pathogen infection and genetic diseases for near-patient diagnosis.

In summary, there are three major noteworthy features in the proposed LOC based RNA isolation technique. To the best of my knowledge, the LOC approach proposed here is the first RNA isolation method that employs DMA as a binding reagent. Secondly, very few studies have been reported to realize RNA isolation from real biosamples (cell lines, Dengue) without using chaotropic salt. Lastly, the LOC system exhibits high RNA isolation efficiency and high throughput processing taking advantage of sudden expansion-contraction structures.

Lab On A Chip System For Particle Separation

In this chapter, I present a microfluidic lab-on-a-chip system that can perform size based nanoparticle separation by dead-end filtration with ultrasonic irradiation. Despite the increasing demand for nanoscale biomolecule analysis for point-of-care application, nanoparticle separation still remains a challenge in many application, due to huge sample loss during separation, low throughput, large scale input materials required and sophisticated technologies involved, which hinders the development of an integrated analytical system for POC application. As the separation efficiency may affect the subsequent sample processing and analysis, robust and reliable size-based separation technique is necessary. By using simple and rapid polymer prototyping, I was able to build a lab on a chip system that consists of a microfluidic network with embedded membrane filters with different pore size cutoff and an ultrasonic transmitter for acoustic agitation, which is to enhance the separation performance. Using the novel method, I successfully demonstrated fractionation of 15 nm AuNP from polydisperse nanoparticle solution in the presence of ultrasonic wave (28-40 kHz) generated by the transducer incorporated with the microfluidic system during the separation. Ultrasonic irradiation helps in preventing cake formation and reversing the fouling process by acoustic agitation. Consequently, a significant increase in flow rate during separation process as well as improvement in recovery of target size nanoparticle was observed. This novel microfluidic platform can serve as a powerful tool for both sample preparation process and analytical process in POC application.

5.1. Introduction

In this chapter, I present a microfluidic lab-on-a-chip system with embedded ultrasonic transmitter that can perform size based nanoparticle separation by dead-end filtration.

Using the novel method, I was able to fractionate 15 nm AuNP from polydisperse (15/30 nm) nanoparticle solution in the presence of ultrasonic wave (28-40 kHz) transmitted by the transducer integrated with the microfluidic system during the separation to prevent the formation of cake layer and detach foulants adhering to membrane surface by agitation. Typically, the processing time was ~10 min by enhancing the flow rate in the presence of ultrasonic treatment during the process. In addition, it was able to achieve 70% of recovery rate in Au NP separation, showing significant improvement in recovery rate compared to the case without ultrasonic treatment. The novel microfluidic lab on a chip holds great potential as a useful tool that provides many advantages over other microfluidic size-based technique such as 1) simple fabrication by employing commercially available track-etched membrane filter with sharp pore size cutoff to bypass complicated fabrication process for building sieving microstructure, 2) easy sample handling by means of automatic process, 3) high recovery rate (70% in case of 60 nm Au NP) of target size NP, 4) No additional chemical pretreatment required by using membrane filtration 5) Fast and straightforward processing, 6) Little dilution of sample

5.2. Working principle of ultrasonic assisted nanoparticle separation

The working principle of ultrasonic assisted nanoparticle separation on a microfluidic device is illustrated in Fig. 40. The separation mechanism is straightforward using dead-end filtration. The microfluidic system for size based nanoparticle separation consists of a microfluidic device with track-etched polycarbonate membrane filters with sharp pore size cutoff incorporated with an ultrasonic transducer. To isolate monodisperse nanoparticle from the mixture, membrane filters with a pore size larger and smaller than the particle size. As shown in Fig. 40, target (Green) particles penetrate the membrane with larger cutoff value while larger particles settle onto the membrane. To capture the

target particles, a membrane with smaller cutoff was introduced enabling purification before collection of target particles. Impurities and particles smaller than the target flow through the second membrane filter and proceed to waste reservoir.

Figure 40 Schematic of a microfluidic platform for nanoparticle separation. Track-etched polycarbonate membranes with different pore size were embedded to capture target molecules. Particles larger than cut-off of the first membrane will remain on the membrane (waste), while target molecules penetrate the first molecule with large pores and capture in microchamber underneath the second membrane with the lower cut-off. Captured molecules are collected in elution buffer by applying negative pressure to microfluidic networks connected to a syringe pump. In order to enhance recovery rate by detaching particles on fouled membrane surface, ultrasonic transducers were incorporated with the microfluidic platform.

As one of the critical limitations in dead-end filtration is sample loss and decrease in permeability during the separation process due to particle adsorption on the membrane surface and formation of cake layer near the surface, ultrasonic wave was exploited to

detach fouled particles on the surface and enhancing recovery of target particles. (Fig. 41) For efficient ultrasound irradiation, the transducer was located near the membranes to minimize the attenuation as the wave propagates through the material. And the chamber was designed to fit the active area of the transducer to maximize the coverage of ultrasound irradiation. To prevent leakage at the interface of PDMS layer and the membranes, negative pressure was applied instead of injection of samples through the inlet.

Figure 41 Effect of ultrasonic treatment during NP separation

5.3. Materials and Methods

5.3.1. Chip design and fabrication

The prototype of microfluidic nanoparticle separation device was designed using AutoCAD software and constructed by a standard soft lithography process. For filtration of nanoparticles, the device consists of two circular reservoirs connected by a microfluidic network as illustrated in Fig. 42. The microfluidic network patterned onto the bottom layer, which connects the two reservoirs. The side channels connected to the reservoir for sample collection are patterned onto the middle layer. And the top layer serves as a top cover to form a closed microfluidic network while the left reservoir remains open for sample loading. The right reservoir has a microfluidic barrier

structures to guide the fluid flow uniformly to cover the entire surface of membrane so that it enables the even distribution of the particles in filtrate on the membrane surface.

The left reservoir serves as a sample loading well where a mixture of nanoparticles was loaded. The membrane filters with different pore sizes including 15 nm and 30 nm were integrated with three PDMS layers in case of 20 nm NP separation process. First, the structures of each PDMS layer were patterned onto transparency mask and supplied by Fineline Imaging (Colorado Springs, CO) at 32000 dpi resolution. To fabricate a master mold, SU-8 2025 was spun onto a silicon substrate using spin coater at 2000 rpm for 30 sec to achieve a uniform thickness of 40 μm . Then the master went through soft bake process to evaporate solvent at 65 °C for 2 min and 95°C for 5 min followed by UV exposure ($200 \text{ mJ}/\text{cm}^2$) for 30 sec. For Post exposure bake (PEB) the master was cured at 65 min for 1 min and 95 min 3 min. Then the pattern was developed by immersing the master in the developer solution for 5 min and rinsing with Deionized water and isopropyl alcohol. Excess water must be removed in a spin dryer. After patterning process, the SU-8 mold was modified with trimethylchlorosilane (Sigma-Aldrich, USA) under vacuum. Next, the PDMS layers were fabricated by standard soft lithographic techniques. Briefly, PDMS was mixed with a curing agent at a ratio of 10:1 and poured onto the SU-8 master mold to form a replica of the device. Degassing was performed to remove entrapped bubbles from the layer. Then the layer was baked in a convection oven at 65 °C for an hour for curing and peeled off from the master mold. Two reservoir regions were punched with biopsy punches and through holes at inlet and outlet were punched for fluidic access. Membrane filters were cut using laser cutting process into the desired size. Pore sizes of membrane filters varied depending on the target nanoparticle size. To integrate membrane filter on PDMS bottom layer, uncured elastomer/curing agent mixture was applied along the edge of the reservoir. Then the

membrane was aligned, placed at the center of the reservoir and pressed gently with round tip tweezer so that the uncured mixture was absorbed into filter layer. The bottom layer and membrane filter was cured in an oven at 65 °C for at least 30 min. Three PDMS layers were oxygen plasma treated to activate the surface, aligned and brought into contact with each other to form a microfluidic platform. Tygon tubings were inserted into through holes at inlets and outlet regions. After integration and assembly, no significant leak was observed during operation. SU-8 master mold can be reused after washing with IPA and deionized water. The microfluidic device was coupled with ultrasonic transducers by using silicone at the interface of the transducer and PDMS layer to minimize the energy loss and attenuation during operation.

Figure 42 Fabrication of microfluidic nanoparticle separation device. (A) Each PDMS layers were patterned by standard soft lithographic technique. Membrane filters were embedded in the interface between the bottom layer and middle layer using uncured PDMS prepolymer. Left reservoir served as a sample loading well. (B) A microfluidic nanoparticle separation device with sample loaded in sample reservoir.

5.3.2. Ultrasonic assisted nanoparticle separation on microfluidic device

Prior to separation, microfluidic network was rinsed with 0.1 % Poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (Pluronic F108)(Sigma-Aldrich) solution to remove residual impurities and treat the fluidic PDMS surface thereby preventing adsorption of nanoparticle on membrane surface or fluidic channel wall and formation of fouling on the membrane surface. To demonstrate ultrasonic assisted nanoparticle separation on microfluidic device gold nanoparticles (Nanocs, NY, USA) with a varied diameter (15, 30, 50, 80, 100, 200, 400, 600 nm) were used. In the case of 60 nm/ 100 nm mixture, 10 μ l of the stock solution of 60 and 100 nm (1.9×10^9 / mL and 3.8×10^9 / mL respectively) Au nanoparticles in 0.1mM PBS were added into 1 $\times$ PBS, and the final volume was brought up to 100 μ l. The mixture was loaded into sample reservoir of assembled microfluidic device with membrane filter for separation. (Fig. 43) The mixture was driven into microfluidic device by the negative pressure generated by syringe pump operating on “withdraw” mode. Flow velocity was set to 0.1 mL/h. The microfluidic device was irradiated with ultrasonic waves generated by ultrasonic transducer during the separation. Typically, 40 kHz square wave was generated by a function generator and 50 times amplified through high voltage amplifier (WMA-300, Falco systems) and used to excite the ultrasonic transducer. During the separation process, other inlets and outlets were closed than the waste port. Prior to collection of target particles, 0.1 % Pluronic F108 solution was injected at 0.1 mL/h for 10 min as a washing buffer. To retrieve 60 nm gold nanoparticle (target) from the chamber region between two membrane filters, backwashing with the ultrasonic wave was performed. 60 nm Au NP were collected in either 0.1 % F108 solution or PBS for downstream analysis. The retentate settled on the first membrane was collected by pipetting up and down several times for measurement.

Figure 43 Microfluidic device for on-chip nanoparticle separation consists of three PDMS layers. Membrane filters with different cutoff size (15 nm, 30 nm) were sandwiched between the bottom layer and middle layer in the case of 20/ 50 nm nanoparticle separation. Particles larger than cut-off of the first membrane remain in sample loading reservoir, and the rest can penetrate the membrane. Particles and buffer solution can go through the second membrane with smaller cut-off (15nm), while the target size particle (20nm) stay in chamber region between those two membrane filters.

5.3.3. Characterization of Populations of Nanoparticles

UV-Vis spectroscopy was used to characterize the populations of nanoparticles before and after filtration process by quantifying the absorption intensity of each solution. The wavelengths of peak absorbance depend on sizes of gold nanoparticles. Typically, peak absorbance occurs within 564 – 574 nm and 538 – 544 nm for 100 nm and 60 nm Au NP respectively. Populations of nanoparticles were characterized by measuring the wavelengths of peak absorbance and intensity on a multimode plate reader (Pelkin Elmer). Samples (Filtrates, retentates) collected from the microfluidic device after separation were analyzed within 400 – 700 nm wavelength range and the results were

compared with that of stock solution. Based on concentration and volume loaded, the recovery rate and sample loss were calculated.

To validate the filtration efficiency fluorescence image was obtained using fluorescent microscope. For visualization of NP adsorbed onto membrane surface, 350 and 450 nm fluorescently labeled polystyrene nanoparticles were filtered on microfluidic device incorporated with 400 nm pore membrane filter and the membrane was taken out of the device and investigated under a microscope. The 450 nm particles settled onto membrane were considered sample loss and the number of particles were counted automatically using Image J software.

In addition, particle size distribution was also determined by dynamic light scattering (DLS) using isolated filtrate and retentate prepared in 0.1 % F108 solution. The distribution data were acquired and compared with distribution data of each stock solution. All experiments were carried out at least 3 times.

5.4. Results & Discussion

5.4.1. Characterization of microfluidic size based NP separation with ultrasonic irradiation

The efficiency of nanoparticle filtration using the microfluidic system with ultrasonic irradiation was evaluated using monodisperse 60 nm gold nanoparticles and single track etched polycarbonate membrane filter with 80 nm pore size cutoff. (Fig. 44) Twenty microliters of gold nanoparticle stock solution were diluted with 80 μ L 0.1 % F108 solution and placed into loading reservoir. To investigate the efficiency of ultrasonic treatment, filtration was performed with ultrasonic irradiation and the UV-Vis spectroscopy results were compared with the results of filtration without irradiation. Recovery rates and loss were calculated through normalization by absorbance profile of feed solution. In the case of filtration without ultrasonic irradiation, significant sample loss was observed after the filtration likely due to adsorption of particles onto membrane

surface during the process. Peak absorbance of filtrate indicates 21 % of input was recovered after filtration, yielding very low recovery rate. From the peak absorbance of retentate, I assumed 17.9 % of input could not penetrate the membrane, and the particles were retrieved by manual pipetting. On the other hand, with ultrasonic irradiation, significant enhancement in recovery rate was observed. The absorbance of retentate solution was negligible indicating few 60 nm particles were recovered in the solution. It was able to achieve 70.6% of recovery rate introducing ultrasonic treatment during the separation on a microfluidic device, which is 3.9 times higher than the case without ultrasonic treatment. It is crucial to maximize the recovery rate while no particles were detectable from the retentate solution to obtain monodisperse target size nanoparticles from the mixture.

Figure 44 60 nm Au NP filtration using membrane filter with 80 nm pore size cutoff embedded in the microfluidic size based particle separation device. (A) The absorbance was measured using the feed, filtrate, retentate sample collected after filtration without ultrasonic irradiation. (w/o US) (B) The absorbance results were compared to the case with ultrasonic irradiation (w/ US). (C) In the presence of ultrasonic irradiation, 70.6 % of input 60 nm particles were successfully collected while 21% of input particles were collected without ultrasonic treatment.

5.4.2. Effect of surface treatment

In the initial attempt to determine filtration efficiency of the microfluidic device, it was found that the filtration efficiency is greatly dependent on the membrane surface condition. Once the volume loaded into sample reservoir passed through the membrane, and the membrane surface started to dry, Au NP settled on the membrane surface were compacted and the foulants formed a cake layer which makes it difficult to re-suspend particles from the membrane surface for downstream analysis regardless of vigorous pipetting. To maximize the filtration efficiency and recovery rate, the membrane filter surface must be kept wet during and after the filtration. To demonstrate this phenomenon, monodisperse fluorescently labeled polystyrene (PS) NPs were used and visualized under fluorescent microscope. The hydraulic diameter of NP was 450 nm and the pore size cutoff of the membrane filter used for filtration was 400 nm. Fig. 45 shows the comparison of removal rate between the case with ultrasonic treatment and without the treatment on either wet or dry surface. Fig 45-1 shows the 450 nm particles on 400 nm filter surface when 100 μ l of sample passed through the membrane. Under wet surface condition (Fig. 45-2 and 3), the effectiveness of ultrasonic cleaning was examined. As a result, most of the input particles were successfully removed from the filter surface by both back flushing or pipetting in both cases. The loss after filtration without ultrasonic treatment was 5% while no detectable quantities of nanoparticles were found from the surface after ultrasonic cleaning. This indicates that under the wet surface condition, nanoparticles larger than the pore size of the membrane filter tend to stay in suspension or the cake layer formed during the filtration is easier to be removed from the membrane surface during cleaning process combined with ultrasonic irradiation. In contrast to a wet surface, severe fouling of membrane filter was observed once the membrane surface is dry indicating the irreversible formation of cake layer with foulants on the surface under dry surface condition. (Fig. 45-4 and 5) Ultrasonic

irradiation exhibited an improvement in removal rate, however, significant losses were detected from both cases. As a result, 65% of input particles were successfully removed from the surface without ultrasonic cleaning, while 70 % of input were removed by ultrasonic cleaning. Hence, I assumed that 30 % of nanoparticles larger than pore size were presumably adsorbed on membrane surface irreversibly under dry surface condition. Therefore, the membrane filter surface needs to remain wet during and after filtration before recovering target particles, and ultrasonic cleaning enhances the removal of particles from the surface.

Figure 45 Comparison of particle removal rate under different surface condition (wet/dry). Under the dry surface condition, particles irreversibly formed cake layer near membrane filter surface yielding 65 -70 % recovery rate. On the other hand, most of the input particles are collected when membrane filter remains wet during the filtration.

To examine the utility of microfluidic device in size based nanoparticle sorting, filtration of 100 / 400 nm NP mixture was performed and the particle size distribution was investigated by dynamic light scattering (DLS) technique. (Fig. 46) A mixture of two different sized polystyrene particles was driven into the microfluidic device and pressurized to initiate size based filtration. For 100 / 400 nm separation, 80 nm and 200 nm track etched membrane filter was selected for separation. The filtrate was collected from the reservoir by back flushing with ultrasonic treatment. Fig. 46 shows particle size distribution profiles of 100 nm and 400 nm particle stock solution, filtrate and mixture. Size distribution of polydisperse mixture was examined before filtration. As a result, the filtrate showed size distribution profile similar to original 100 nm stock solution without a detectable shift in peak wavelength and spectrum broadening. I compared the size distribution profile measured by light scattering technique to the size estimated by absorbance spectra. As a result, peak absorbance of the filtrate was ~70% of stock solution with little variation in peak wavelength indicating successful size discrimination and high recovery rate.

Figure 46 Fractionation of 100nm polystyrene NP from polydisperse mixture solution by using ultrasonic assisted microfluidic particle sorting. Dynamic light scattering (DLS) spectra were obtained from filtrate (Green) and a mixture containing 1:1 polydisperse 100nm and 400 nm particles (Black). Light scattering spectra of a stock solution of 100nm and 400 nm were obtained for comparison. (Red and blue respectively) Size distribution of filtrate shows that size of particles collected in the filtrate is close to 100 nm stock solution.

5.4.3. Effect of orientation of ultrasonic treatment

The effect of orientation of ultrasonic transmitter on the performance of the device in particle fractionation and recovery rate was investigated using 60 nm Au NP. Particle fractionation was carried out with track-etched membrane filters; the mean pore sizes cutoff of which were 80 nm. Ultrasonic transmitter was incorporated into the device at the feed side or permeate side to examine the effect of orientation of ultrasonic transmitter on the fractionation capability. The 40 kHz square US waves were irradiated on membrane from the permeate side or feed side. Fig. 47A shows the layouts of the microfluidic particle filtration system with embedded ultrasonic transmitter. Output voltage through amplifier was 40 Vp-p. To investigate ultrasonic assisted filtration performance, 50 μ l of 60 nm particle stock solution was diluted with 50 μ l 0.1 % F108 surfactant solution. To initiate filtration, 100 μ l of sample was driven into the device. To maximize the recovery, 100 μ l of 0.1% F108 was reloaded when the first sample passed through so that the residual particles were collected in collection reservoir. After the filtration, collected samples were examined using UV-Vis spectrophotometer and absorbance spectra scan was performed within 350 - 700 nm wavelength range. As a result, the higher recovery rate of 60 nm Au NP was obtained from the sample filtered using feed side ultrasonic treatment at the input concentration studied. (57%) (Fig. 47B) In the case of ultrasound irradiation at the permeate side of the membrane, it yielded 48% of original concentration using the same input sample.

Figure 47 Effect of the orientation of ultrasonic treatment on recovery rate. Ultrasonic wave with 40 kHz, 40Vp-p output voltage generated by ultrasonic transmitter was focused onto membrane filters either at the permeate side or feed side of the membrane. (A) Layouts of the microfluidic systems for particle filtration for ultrasonic treatment from the permeate side (left) and the feed side (right) of the membrane filters. The distance between the transducer and membrane remains 300 μm for the experiment. (B) Absorbance spectra of 60 nm Au NP collected in the filtrate solution by ultrasonic treatment from the permeate side (left) and feed side (right) of the membrane filters were compared with the absorbance using an original stock solution of 60 nm Au NP.

In addition, volumetric flow rate was monitored every 30 sec up to 20 min using each transmitter configuration under the same pressure and input concentration condition. (Fig. 48) As a result, a similar trend was also seen in volumetric flow rate results. The higher volumetric flow rate was achieved when ultrasound was irradiated onto membrane surface from the feed side of the membrane. A significant decrease in flow rate occurred after 5 min filtration when ultrasound was irradiated from the permeate

side of the membrane, indicating critical fouling of the membrane. Despite the decrease in flow rate in case of ultrasound irradiation from the feed side, the flow rate was stabilized after 10 min initial separation process. On the other hand, flow rate consistently decreased in the case of ultrasonic irradiation from the permeate side, showing the similar trend as the case of no ultrasonic treatment. The flow rate was double with ultrasonic irradiation compared to no ultrasonic treatment when the ultrasonic propagated from the feed side. According to the flow rate results, I assumed that the orientation of the ultrasonic transmitter affected the effectiveness of the ultrasonic treatment in preventing the formation of cake layer and fouling of membrane as well as recovery of the target particles.

Figure 48 Volumetric flow rate of sample filtered using microfluidic particle separation system as a function of filtration time. Samples containing 60 nm Au NP were processed using the microfluidic system with ultrasonic irradiation from the feed side (green), permeate side (blue) or without ultrasonic treatment. As a result, a decrease in flow rate was observed in three cases. However, the highest flow rate was achieved with ultrasonic irradiation from the feed side, and the steady flow rate was observed after 600 sec of the filtration process. For the case of the ultrasound treatment from the permeate side, it showed the same level of fouling as the case of no ultrasound treatment, which indicating that the ultrasonic irradiation is not effective when the ultrasonic waves propagate from the permeate side of the membrane filter

5.5. Conclusion

I have demonstrated a novel microfluidic approach to size-selective nanoparticle separation by taking advantage of Lab-on-a-Chip technique that can carry out ultrasonic assisted dead-end filtration. This novel method allows for single chip size based nanoparticle separation providing high resolution at nanometer scale by employing membrane filters with different pore size cutoff. The efficiency of nanoparticle filtration using the microfluidic system with ultrasonic irradiation was investigated by separating monodisperse 60 nm gold nanoparticles in solution. As a result, I successfully performed size based separation of 60 nm gold nanoparticles from 60 /100 nm poly-disperse with recovery rate as high as 70 %, substantially reducing the sample loss during filtration by using ultrasonic cleaning effect generated by ultrasonic transmitter module embedded in the LOC system. It was shown that acoustic agitation reverses the formation of cake layer near the membrane surface, which leads to enhancement in flow rate as well as recovery rate of target size particle trapped between two membrane filters. In addition, I also investigated several key factors that affect the size based separation and filtration such as membrane surface conditions and orientation of ultrasonic treatment to determine the optimum conditions. Since the effectiveness of ultrasonic assisted filtration is dependent on multiple factors, further studies are necessary to elucidate the correlation among these factors.

As current technologies for biological analysis rely on size based separation that usually requires large input material, it limits the analysis techniques to biological samples which are submicron scale, or samples for which it is easier to accumulate large numbers such as blood cells. And these methods typically employ highly sophisticated technologies (ultracentrifugation, field-flow fractionation) for external field application to perform separation, which makes it difficult to realize a Point-of-Care analysis of nanoscale biomolecules. Lack of robust and reliable size based separation technique for

nanoscale biosamples with smaller scale input leads to huge demand for the development of a miniaturized lab-on-a-chip platform that can perform particle separation on a single chip. Hence, the novel microfluidic-based NP size based separation technique has great potential to serve as a powerful tool for biomolecule separation for both preparative and analytical purposes. This versatile microfluidic platform would help in building a miniaturized analytical system that can be widely applied in many areas such as point of care molecular testing for disease diagnosis. One of potential application in research field is ribosome profiling, which involves many separation steps to fractionate monosome (~30 nm) from polysome population. Current ribosome profiling relies on ultracentrifugation and size exclusive chromatography, which result in huge sample loss and long processing time. My future work will involve further validation of utility of the proposed device and biocompatibility in sample preparation for ribosome profiling by performing monosome separation on the LOC system.

Conclusions

In recent years, a substantial technological advance has been made in manufacturing, semiconductor engineering and biomedical technologies, which makes it possible for microfluidic systems to miniaturize and integrate multiple functions that had been routinely performed by hands in chemical and biological science. One of the most active applications of microfluidic is POC testing, which requires handy and compact diagnostic platform for near patient testing and easy access to the outcome. By using a little amount of biosample on microfluidic LOC platform, rapid diagnostic testing can be performed with streamlined processes without the need for sophisticated equipment. Despite many advantages microfluidic LOC system can provide, the use of LOC techniques are still limited to some particular application. To fill this gap in the market, I present LOC technologies that can address challenges current technologies are facing.

I have shown a microfluidic-based LOC technique for DNA methylation detection that can be used in POC cancer diagnosis in chapter 3. The novel LOC DNA methylation detection technique consists of sample pre-processing module and detection module. Employing an on-chip bisulfite conversion, it allows easy sample preparative process, reducing processing time and labor involved. To the best of my knowledge, this is the first LOC technique for DNA methylation analysis based on bisulfite conversion. Using bisulfite converted DNA from sample processing module, detection module based on silicon microring sensor functionalized with oligonucleotides was able to detect DNA methylation from as little as 1% cancer cell, which is unachievable using traditional techniques such as real-time PCR or end-point PCR. Combined with sample processing module, detection module can be a useful tool for POC cancer diagnostic platform,

which can rapidly deliver outcomes for the efficient decision-making process. This addresses the challenges involved with current DNA methylation techniques which adopt off-chip sample processing, which is of little practical application for POC diagnostic testing.

Unlike DNA, RNA is prone to degradation during the sample processing procedure caused by RNase activity. To deal with these challenges associated with RNA sample handling, I presented a novel method for RNA isolation from various biosamples employing disposable microfluidic platform in chapter 4. Taking advantage of a reversible reaction between DMA and RNA molecules, I demonstrated RNA isolation from various human cell-lines as well as viruses on the chemically modified polymer microfluidic surface. This novel method offers many benefits over current RNA isolation techniques by using a non-chaotropic agent for RNA binding. In this study, I compared the performance of the novel RNA isolation method, such as yield, quality and compatibility with downstream analysis, with current gold standard silica column based technique. As a result, it provided high quality and high yield RNA from both cell lines, yet with streamlined processing procedure using the microfluidic platform. The utility of microfluidic LOC system in RNA isolation has been proven by performing gene expression analysis and infectious disease detection using RNA isolated on the microfluidic platform.

In chapter 5, I demonstrated nanoparticle separation for biological application on microfluidic LOC system. Acoustic agitation generated by ultrasonic transducer embedded in microfluidic device enhances nanoparticle filtration by preventing the formation of cake layer near the membrane surface which leads to an increase in flow rate and recovery rate of target size particle. I have proven its ability to discriminate nanoparticles based on their size using different size gold nanoparticles in solution. Due

to inherent problems of dead-end filtration many filter-free techniques were developed as alternatives. By using ultrasound during filtration, it provides a straightforward size-selective nanoparticle separation method without the need for external field application. This versatile nanoparticle separation technique can be used in many biological and chemical research and POC diagnostic testing integrated with detection technologies.

The basic principle of LOC system for size based NP separation suggested in this thesis is very straightforward using the dead-end membrane for filtration. However, it has shown a great improvement in separation performance employing the ultrasonic agitation. Even though many studies have been reported for ultrasonic assisted filtration, most of them were practiced in macroscale with sophisticated ultrasonic transducer system. The LOC system suggested here uses rapid and simple integration of ultrasonic transducer into microfluidic system and enables nanoscale particles. To the best of my knowledge, this LOC approach is the first nanoparticle separation method based on combination of dead-end membrane filtration and ultrasonic treatment in microscale environment. This system was built with simple rapid prototyping process unlike previous works for dead-end filtration for NP separation.

The LOC techniques presented in the previous chapters have shown great promise as powerful tools that can provide sample preparation and process in resource-limited setting or near patient testing. These techniques can be used independently as sample processing module or connected with downstream analytical assays to form fully integrated diagnostic platform. By adopting microfluidic formats, these techniques are potentially compatible with many LOC technologies.

Future Work

As discussed above, I have successfully demonstrated the functionalities of the novel microfluidic LOC based approaches that can be potentially used in POC diagnostic testing. A feasibility of these new concepts taking advantage of microfluidic technologies has been verified by performing sample processing and subsequent testing using biological samples such as human cancer cell-lines. The research work described above were to evaluate the proof-of-concept and the results shown in previous chapters show the functionalities of the techniques in device level. As these techniques are still at early stage of development, they need system level verification of feasibility for real-world application.

7.1. Multiplexing of LOC system for DNA methylation analysis

I developed a prototype LOC microsystem designed for on-chip methylation specific PCR. A key sensing component of the LOC system is a silicon biophotonic sensor with the ability to provide highly sensitive, label-free DNA methylation detection. The scalability and inherent multiplexing capability of silicon biophotonic sensor make it an attractive platform for simultaneously delivering information on the distribution of DNA methylation in multiple genes from the same DNA template.¹³⁰ The microfluidic LOC system described in chapter 2 was designed, constructed, and investigated for single gene detection. Next step for further development of this prototype into the analytical system for practical use is multiplexing. I will be working on the expansion of current technique to a multiplexed format for analysis of multiple genes, which is based on arrays of silicon microring resonators. Each ring will be functionalized with different primer allowing parallel methylation analysis of multiple genes.

7.2. Integration of LOC system for DNA methylation analysis

In chapter 2, I presented sample processing module for on-chip bisulfite conversion and detection module based on silicon biophotonic sensor. For a real-world application, two components need to be brought together to build integrated analytical system. I envision an integrated LOC system that incorporates the optimized microfluidic bisulfite conversion module and the multiplexed silicon microring resonator arrays. For integration of microfluidic module with the biophotonic sensor, the introduction of the microvalve, micromixer and channels must be exercised to deliver bisulfite converted DNA sample mixed with PCR reagents necessary for amplification. The integrated LOC system will be able to practice simultaneous bisulfite modification reaction, purification, amplification and detection. The LOC system will move towards truly low-cost, single-use disposable microTAS system operating with a system equipped with an optical measuring platform, thermal control module, and pumping system.

7.3. RNA analysis on disposable microfluidic platform

In chapter 3, I presented a novel method for RNA isolation using the disposable microfluidic platform. Subsequently, reverse transcription PCR (RT-PCR) is commonly used for further analysis of isolated RNA for gene expression profiling. The results shown in chapter 3 were obtained by performing off-chip RT-PCR. To improve real world applicability of the LOC system, the realization of on-chip RT-PCR is desirable. Before releasing purified RNA from the microfluidic channel surface, reagents necessary for RT-PCR will be introduced and the RNA adsorbed onto chip surface will undergo RT-PCR. Combined with the microfluidic analytical system the LOC can serve as a fully integrated diagnostic platform for gene expression studies for the POC use.

7.4. Size based biomolecule separation on microfluidic LOC system with ultrasonic treatment

I have demonstrated size based nanoparticle separation using the novel method adopting microfluidic LOC system with ultrasonic irradiation for enhancement of particle separation. I used different size gold and polymer NP for a demonstration of particle separation. Therefore, next step to further investigate the feasibility of this LOC system is to exercise size selective separation using biosample. Monosome separation will be carried out using the LOC system as a preparative sample processing in ribosome profiling to show the feasibility of the system for both research and clinical use.

Bibliography

- 1 B. W. Stewart and C. P. Wild, 2014.
- 2 A. P. Feinberg and B. Tycko, *Nat Rev Cancer*, 2004, **4**, 143–153.
- 3 P. A. Jones and S. B. Baylin, *Cell*, 2007, **128**, 683–692.
- 4 P. Chomczynski and N. Sacchi, *Nat Protoc*, 2006, **1**, 581–585.
- 5 H. Aviv and P. Leder, *PNAS*, 1972, **69**, 1408–1412.
- 6 B. Kowalczyk, I. Lagzi and B. A. Grzybowski, *Current Opinion in Colloid & Interface Science*, 2011, **16**, 135–148.
- 7 A. Lenshof and T. Laurell, *Chem. Soc. Rev.*, 2010, **39**, 1203–16.
- 8 P. Sajeesh and A. K. Sen, *Microfluid Nanofluid*, 2014.
- 9 D. Huh, J. H. Bahng, Y. Ling, H.-H. Wei, O. D. Kripfgans, J. B. Fowlkes, J. B. Grothberg and S. Takayama, *Anal. Chem.*, 2007, **79**, 1369–1376.
- 10 S. S. Kuntaegowdanahalli, A. A. S. Bhagat, G. Kumar and I. Papautsky, *Lab on a Chip*, 2009, **9**, 2973–8.
- 11 J. Shi, H. Huang, Z. Stratton, Y. Huang and T. J. Huang, *Lab on a Chip*, 2009, **9**, 3354–7.
- 12 X. Lu and X. Xuan, *Anal. Chem.*, 2015, **87**, 6389–6396.
- 13 A. Stark, D. J. Shin, T. Pisanic, K. Hsieh and T.-H. Wang, *Biomed Microdevices*, 2016, **18**, 1022–7.
- 14 A. De, W. Sparreboom, A. van den Berg and E. T. Carlen, *Biomicrofluidics*, 2014, **8**, 054119–12.
- 15 F. J. Steemers and K. L. Gunderson, *Biotechnology Journal*, 2007, **2**, 41–49.
- 16 P. Wang, H. Wu, Z. Dai and X. Zou, *Chem. Commun.*, 2012, **48**, 10754–3.
- 17 W. C. Maki, N. N. Mishra, E. G. Cameron, B. Filanoski, S. K. Rastogi and G. K. Maki, *Biosensors and Bioelectronics*, 2008, **23**, 780–787.
- 18 A. A. I. Sina, L. G. Carrascosa, R. Palanisamy, S. Rauf, M. J. A. Shiddiky and M. Trau, *Anal. Chem.*, 2014, **86**, 10179–10185.
- 19 J. G. Herman, J. R. Graff, S. Myöhänen, B. D. Nelkin and S. B. Baylin, *PNAS*, 1996, **93**, 9821–9826.
- 20 C. A. Eads, K. D. Danenberg, K. Kawakami, L. B. Saltz, C. Blake, D. Shibata, P. V. Danenberg and P. W. Laird, *Nucleic Acids Research*, 2000, **28**, e32–00.
- 21 Z. Xiong and P. W. Laird, *Nucleic Acids Research*, 1997.
- 22 H. Yin, B. Sun, Y. Zhou, M. Wang, Z. Xu, Z. Fu and S. Ai, *Biosensors and Bioelectronic*, 2014, **51**, 103–108.
- 23 W. C. Maki, N. N. Mishra, E. G. Cameron, B. Filanoski, S. K. Rastogi and G. K. Maki, *Biosensors and Bioelectronics*, 2008, **23**, 780–787.
- 24 J. Shim, G. I. Humphreys, B. M. Venkatesan, J. M. Munz, X. Zou, C. Sathe, K. Schulten, F. Kosari, A. M. Nardulli, G. Vasmatzis and R. Bashir, *Sci. Rep.*, 2013, **3**, 1–8.
- 25 J. Wang, Z. Zhu and H. Ma, *Anal. Chem.*, 2013, **85**, 2096–2101.
- 26 R. E. Pemberton, P. Liberti and C. Baglioni, *Analytical Biochemistry*, 1975, **66**, 18–28.
- 27 J. A. Bantle, I. H. Maxwell and W. E. Hahn, *Analytical Biochemistry*, 1976, **72**, 413–427.
- 28 S. C. Tan and B. C. Yiap, *Journal of Biomedicine and Biotechnology*, 2009, **2009**, 574398–10.
- 29 Y.-K. Cho, T.-H. Kim and J.-G. Lee, *J. Micromech. Microeng.*, 2010, **20**,

- 065010–8.
- 30 K. A. Hagan, W. L. Meier, J. P. Ferrance and J. P. Landers, *Anal. Chem.*, 2009, **81**, 5249–5256.
 - 31 G. Jiang and D. J. Harrison, *Analyst*, 2000, **125**, 2176–2179.
 - 32 B. C. Satterfield, S. Stern, M. R. Caplan, K. W. Hukari and J. A. A. West, *Anal. Chem.*, 2007, **79**, 6230–6235.
 - 33 J. D. Robertson, L. Rizzello, M. Avila-Olias, J. Gaitzsch, C. Contini, M. S. Magoń, S. A. Renshaw and G. Battaglia, *Sci. Rep.*, 2016, 1–9.
 - 34 P. Sajeesh and A. K. Sen, *Microfluid Nanofluid*, 2013, **17**, 1–52.
 - 35 O. Akbulut, C. R. Mace, R. V. Martinez, A. A. Kumar, Z. Nie, M. R. Patton and G. M. Whitesides, *Nano letters*, 2012, **12**, 4060–4064.
 - 36 P. Arosio, T. Müller, L. Mahadevan and T. P. J. Knowles, *Nano letters*, 2014, **14**, 2365–2371.
 - 37 I. Arnaud, J.-P. Abid, C. Roussel and H. H. Girault, *Chem. Commun.*, 2005, 787–2.
 - 38 M. Hanauer, S. Pierrat, I. Zins, A. Lotz and C. Sönnichsen, *Nano letters*, 2007.
 - 39 A. H. Latham, R. S. Freitas, P. Schiffer and M. E. Williams, *Anal. Chem.*, 2005, **77**, 5055–5062.
 - 40 T. R. Gaborski, J. L. Snyder, C. C. Striemer, D. Z. Fang, M. Hoffman, P. M. Fauchet and J. L. McGrath, *ACS Nano*, 2010, **4**, 6973–6981.
 - 41 W. Zhao, L. Lin and I.-M. Hsing, *Langmuir*, 2010, **26**, 7405–7409.
 - 42 J. de Jong, R. G. H. Lammertink and M. Wessling, *Lab on a Chip*, 2006, **6**, 1125–15.
 - 43 S. D. Noblitt, J. R. Kraly, J. M. VanBuren and S. V. Hering, *Analytical ...*, 2007.
 - 44 H. Wei, B.-H. Chueh, H. Wu, E. W. Hall, C.-W. Li, R. Schirhagl, J.-M. Lin and R. N. Zare, *Lab on a Chip*, 2011, **11**, 238–245.
 - 45 J. Linkhorst, T. Beckmann, D. Go, A. J. C. Kuehne and M. Wessling, *Sci. Rep.*, 2016, 1–8.
 - 46 G. Belfort, R. H. Davis and A. L. Zydney, *Journal of Membrane Science*, 1994, **96**, 1–58.
 - 47 T. N. Shendruk, R. Tahvildari, N. M. Catafard, L. Andrzejewski, C. Gigault, A. Todd, L. Gagne-Dumais, G. W. Slater and M. Godin, *Anal. Chem.*, 2013, **85**, 5981–5988.
 - 48 N. T. Ingolia, G. A. Brar, S. Rouskin and A. M. McGeachy, *Nat Protoc*, 2012.
 - 49 H. M. Kyllönen, P. Pirkonen and M. Nyström, *Desalination*, 2005, **181**, 319–335.
 - 50 X. Shi, G. Tal, N. P. Hankins and V. Gitis, *Journal of Water Process Engineering*, 2014, **1**, 121–138.
 - 51 J. Yoon, M. K. Park, T. Y. Lee, Y.-J. Yoon and Y. Shin, *Lab on a Chip*, 2015, **15**, 3530–3539.
 - 52 A. Alrifaiy, O. A. Lindahl and K. Ramser, *Polymers*, 2012, **4**, 1349–1398.
 - 53 J.-I. Hahm and C. M. Lieber, *Nano letters*, 2004, **4**, 51–54.
 - 54 M. T. Martínez, Y.-C. Tseng, N. Ormategui, I. Loinaz, R. Eritja and J. Bokor, *Nano letters*, 2009, **9**, 530–536.
 - 55 Y. Shin, A. P. Perera and M. K. Park, *Sensors and Actuators B: Chemical*, 2013, **178**, 200–206.
 - 56 A. L. Washburn, M. S. Luchansky, A. L. Bowman and R. C. Bailey, *Anal. Chem.*, 2010, **82**, 69–72.
 - 57 F. Lisdat and D. Schäfer, *Anal Bioanal Chem*, 2008, **391**, 1555–1567.

- 58 Y. Wang, D. Zheng, Q. Tan, M. X. Wang and L.-Q. Gu, 2011, 1–7.
- 59 T. Matsuoka, B. Choul Kim, C. Moraes, M. Han and S. Takayama, *Biomicrofluidics*, 2013, **7**, 041301–13.
- 60 A. Kundaje et al., *Nature*, 2015, **518**, 317–330.
- 61 M. Esteller, *Carcinogenesis*, 2006, **27**, 1121–1125.
- 62 H. Heyn and M. Esteller, *Nature Reviews Genetics*, 2012, **13**, 679–692.
- 63 P. W. Laird, *Nat Rev Cancer*, 2003, **3**, 253–266.
- 64 A. P. Bird, *Nature*, 1985, **321**, 209–213.
- 65 X. Guo, *J. Biophoton.*, 2012, **5**, 483–501.
- 66 S. Zeng, D. Baillargeat, H.-P. Ho and K.-T. Yong, *Chem. Soc. Rev.*, 2014, **43**, 3426.
- 67 B. Sepulveda, J. S. Del Rio, M. Moreno, F. J. Blanco, K. Mayora, C. Domínguez and L. M. Lechuga, *Journal of Optics A: Pure and Applied Optics*, 2006, **8**, S561.
- 68 F. Prieto, B. Sepulveda, A. Calle, A. Llobera, C. Domínguez, A. Abad, A. Montoya and L. M. Lechuga, *Nanotechnology*, 2003, **14**, 907.
- 69 J. Vörös, J. J. Ramsden, G. Csucs, I. Szendrő, S. M. De Paul, M. Textor and N. D. Spencer, *Biomaterials*, 2002, **23**, 3699–3710.
- 70 N. Zaytseva, W. Miller, V. Goral, J. Hepburn and Y. Fang, *Appl. Phys. Lett.*, 2011, **98**, 163703.
- 71 Y. Fang, A. M. Ferrie, N. H. Fontaine, J. Mauro and J. Balakrishnan, *Biophysical journal*, 2006, **91**, 1925–1940.
- 72 M. S. McClellan, L. L. Domier and R. C. Bailey, *Biosensors and Bioelectronic*, 2012, **31**, 388–392.
- 73 A. J. Qavi and R. C. Bailey, *Angew. Chem. Int. Ed.*, 2010, **49**, 4608–4611.
- 74 O. Scheler, J. T. Kindt, A. J. Qavi, L. Kaplinski, B. Glynn, T. Barry, A. Kurg and R. C. Bailey, *Biosensors and Bioelectronic*, 2012, **36**, 56–61.
- 75 A. J. Qavi, A. L. Washburn, J.-Y. Byeon and R. C. Bailey, *Anal Bioanal Chem*, 2009, **394**, 121–135.
- 76 J. T. Kindt and R. C. Bailey, *Current opinion in chemical biology*, 2013, **17**, 10.1016/j.cbpa.2013.06.014.
- 77 K. W. Kim, Y. Shin, A. P. Perera, Q. Liu, J. S. Kee, K. Han, Y.-J. Yoon and M. K. Park, *Biosensors and Bioelectronic*, 2013, **45**, 152–157.
- 78 M. K. Park, J. S. Kee, J. Y. Quah, V. Netto, J. Song, Q. Fang, E. M. La Fosse and G.-Q. Lo, *Sensors and Actuators B: Chemical*, 2013, **176**, 552–559.
- 79 K. De Vos, I. Bartolozzi, E. Schacht, P. Bienstman and R. Baets, *Opt. Express, OE*, 2007, **15**, 7610–7615.
- 80 M. Ronen, D. Avrahami and D. Gerber, *Lab on a Chip*, 2014, **14**, 2354–2362.
- 81 S. Fang Lim, A. Karpusenko, J. J. Sakon, J. A. Hook, T. A. Lamar and R. Riehn, *Biomicrofluidics*, 2011, **5**, 034106–8.
- 82 C.-H. Wang, H.-C. Lai, T.-M. Liou, K.-F. Hsu, C.-Y. Chou and G.-B. Lee, *Microfluid Nanofluid*, 2013, **15**, 575–585.
- 83 M. Kantlehner, R. Kirchner, P. Hartmann, J. W. Ellwart, M. Alunni-Fabbroni and A. Schumacher, *Nucleic Acids Research*, 2011, **39**, e44–e44.
- 84 T. Y. Lee, Y. Shin and M. K. Park, *Lab on a Chip*, 2014, **14**, 4220–4229.
- 85 P. Hou, M. Ji, C. Ge, J. Shen, S. Li, N. He and Z. Lu, *Nucleic Acids Research*, 2003, **31**, e92–92.
- 86 J. Shim, G. I. Humphreys, B. M. Venkatesan, J. M. Munz, X. Zou, C. Sathe, K. Schulten, F. Kosari, A. M. Nardulli, G. Vasmatazis and R. Bashir, *Sci. Rep.*, 2013, **3**, 1–8.

- 87 K. Goto, D. Kato, N. Sekioka, A. Ueda, S. Hirono and O. Niwa, *Analytical Biochemistry*, 2010, **405**, 59–66.
- 88 P. W. Laird, *Nature Reviews Genetics*, 2010, **11**, 191–203.
- 89 H. K. Komori, S. A. LaMere, A. Torkamani, G. T. Hart, S. Kotsopoulos, J. Warner, M. L. Samuels, J. Olson, S. R. Head, P. Ordoukhanian, P. L. Lee, D. R. Link and D. R. Salomon, *Genome Research*, 2011, **21**, 1738–1745.
- 90 Y. Zhang, V. Bailey, C. M. Puleo, H. Easwaran, E. Griffiths, J. G. Herman, S. B. Baylin and T.-H. Wang, *Lab on a Chip*, 2009, **9**, 1059–1064.
- 91 M. Kalofonou and C. Tournazou, *Sensors and Actuators B: Chemical*, 2013, **178**, 572–580.
- 92 J. Yoon, M. K. Park, T. Y. Lee, Y.-J. Yoon and Y. Shin, *Lab on a Chip*, 2015, **15**, 3530–3539.
- 93 C.-C. Hong, J.-W. Choi and C. H. Ahn, *Lab on a Chip*, 2004, **4**, 109.
- 94 A.-S. Yang, F.-C. Chuang, C.-K. Chen, M.-H. Lee, S.-W. Chen, T.-L. Su and Y.-C. Yang, *Chemical Engineering Journal*, 2015, **263**, 444–451.
- 95 Y. Shin, A. P. Perera, C. C. Wong and M. K. Park, *Lab on a Chip*, 2013, **14**, 359–368.
- 96 D. P. Genereux, W. C. Johnson, A. F. Burden, R. Stöger and C. D. Laird, *Nucleic Acids Research*, 2008, **36**, e150–e150.
- 97 M. Shiraishi and H. Hayatsu, *DNA Res*, 2004, **11**, 409–415.
- 98 R. Rouzier, C. M. Perou, W. F. Symmans, N. Ibrahim, M. Cristofanilli, K. Anderson, K. R. Hess, J. Stec, M. Ayers, P. Wagner, P. Morandi, C. Fan, I. Rabiul, J. S. Ross, G. N. Hortobagyi and L. Pusztai, *Clinical Cancer Research*, 2005, **11**, 5678–5685.
- 99 P. Yager, T. Edwards, E. Fu, K. Helton, K. Nelson, M. R. Tam and B. H. Weigl, *Nature*, 2006, **442**, 412–418.
- 100 A. Niemz, T. M. Ferguson and D. S. Boyle, *Trends in Biotechnology*, 2011, **29**, 240–250.
- 101 A. M. Foudeh, T. Fatanat Didar, T. Veres and M. Tabrizian, *Lab on a Chip*, 2012, **12**, 3249–18.
- 102 C. D. Chin, T. Laksanasopin, Y. K. Cheung, D. Steinmiller, V. Linder, H. Parsa, J. Wang, H. Moore, R. Rouse, G. Umvilighozo, E. Karita, L. Mwambarangwe, S. L. Braunstein, J. van de Wijgert, R. Sahabo, J. E. Justman, W. El-Sadr and S. K. Sia, *Nature Medicine*, 2011, **17**, 1015–1019.
- 103 C.-M. Chang, W.-H. Chang, C.-H. Wang, J.-H. Wang, J. D. Mai and G.-B. Lee, *Lab on a Chip*, 2013, **13**, 1225–19.
- 104 J. Wen, L. A. Legendre, J. M. Bienvenue and J. P. Landers, *Anal. Chem.*, 2008, **80**, 6472–6479.
- 105 K. Yamanaka, M. Saito, K. Kondoh, M. M. Hossain, R. Koketsu, T. Sasaki, N. Nagatani, K. Ikuta and E. Tamiya, *Analyst*, 2011, **136**, 2064–5.
- 106 M. Ikeda, N. Yamaguchi, K. Tani and M. Nasu, *Journal of Microbiological Methods*, 2006, **67**, 241–247.
- 107 C. S. Liao, *Nucleic Acids Research*, 2005, **33**, e156–e156.
- 108 N. V. Zaytseva, V. N. Goral, R. A. Montagna and A. J. Baeumner, *Lab on a Chip*, 2005, **5**, 805–7.
- 109 Y. Li, C. Zhang and D. Xing, *Microfluid Nanofluid*, 2010, **10**, 367–380.
- 110 Y. Sun, R. Dhumpa, D. D. Bang, J. Høgberg, K. Handberg and A. Wolff, *Lab on a Chip*, 2011, **11**, 1457–7.
- 111 N. V. Zaytseva, R. A. Montagna and A. J. Baeumner, *Anal. Chem.*, 2005, **77**, 7520–7527.

- 112 J. Kim, M. Johnson, P. Hill and B. K. Gale, *Integr. Biol.*, 2009, **1**, 574–13.
- 113 Q. Cao, M. Mahalanabis, J. Chang, B. Carey, C. Hsieh, A. Stanley, C. A. Odell, P. Mitchell, J. Feldman, N. R. Pollock and C. M. Klapperich, *PLoS ONE*, 2012, **7**, e33176–11.
- 114 K. J. Shaw, E. M. Hughes, C. E. Dyer, J. Greenman and S. J. Haswell, *Laboratory Investigation*, 2013, **93**, 961–966.
- 115 A. Bhattacharyya and C. M. Klapperich, *Sensors and Actuators B: Chemical*, 2008, **129**, 693–698.
- 116 B. E. Root, A. K. Agarwal, D. M. Kelso and A. E. Barron, *Anal. Chem.*, 2011, **83**, 982–988.
- 117 K. A. Hagan, C. R. Reedy, M. L. Uchimoto, D. Basu, D. A. Engel and J. P. Landers, *Lab on a Chip*, 2011, **11**, 957–961.
- 118 W.-C. Lee, K.-Y. Lien, G.-B. Lee and H.-Y. Lei, *Diagnostic Microbiology and Infectious Disease*, 2008, **60**, 51–58.
- 119 C.-J. Liu, K.-Y. Lien, C.-Y. Weng, J.-W. Shin, T.-Y. Chang and G.-B. Lee, *Biomed Microdevices*, 2008, **11**, 339–350.
- 120 2006, 1–6.
- 121 C.-H. Wang, K.-Y. Lien, T.-Y. Wang, T.-Y. Chen and G.-B. Lee, *Biosensors and Bioelectronic*, 2011, **26**, 2045–2052.
- 122 M. J. Jebrail, A. Sinha, S. Vellucci, R. F. Renzi, C. Ambriz, C. Gondhalekar, J. S. Schoeniger, K. D. Patel and S. S. Branda, *Anal. Chem.*, 2014, **86**, 3856–3862.
- 123 J. Wen, C. Guillo, J. P. Ferrance and J. P. Landers, *Anal. Chem.*, 2007, **79**, 6135–6142.
- 124 D. Chen, M. Mauk, X. Qiu, C. Liu, J. Kim, S. Ramprasad, S. Ongagna, W. R. Abrams, D. Malamud, P. L. A. M. Corstjens and H. H. Bau, *Biomed Microdevices*, 2010, **12**, 705–719.
- 125 Y. Shin, A. P. Perera, C. C. Wong and M. K. Park, *Lab on a Chip*, 2014, **14**, 359–368.
- 126 Y. Shin, S. Y. Lim, T. Y. Lee and M. K. Park, *Sci. Rep.*, 2015, 1–11.
- 127 M. W. Pfaffl, *Nucleic Acids Research*, 2001.
- 128 D. Ling and P. M. Salvaterra, *PLoS ONE*, 2011, **6**, e17762–8.
- 129 E. A. Henchal and J. R. Putnak, *Clin. Microbiol. Rev.*, 1990, **3**, 376–396.
- 130 J.-Y. Byeon and R. C. Bailey, *Analyst*, 2011, **136**, 3430–3433.