Development of novel microfluidic systems for the establishment of highly sensitive protein-based optical biosensors and self-powered POC DNA analysis system

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**NANYANG
TECHNOLOGICAL
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**Development of novel microfluidic systems for the
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and self-powered POC DNA analysis system**

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Summary

Over last few decades, human being have been suffered from various infectious diseases. Lab-on-chip (LOC) technologies have played great role in revolutionizing the way *in vitro* medical diagnostics are done and provide easy to use, cost-effective miniaturized systems with faster analysis time, which can be used for near-patient or Point-Of-Care (POC) tests. The POC technology enables effective prevention and early detection of diseases and it helps to drastically deplete the mortality. Therefore, in this dissertation, I focused on the development of POC protein and nucleic acid biomarkers-based infectious disease diagnostic systems.

Proteins and nucleic acids (DNA and RNA) are mostly used as biomarkers to identify the specific infectious diseases. The single specific biomarker in body fluids is, however, not sufficient to be used in clinical setting for the detection due to their low-concentration (few pg/ml). Unlike nucleic acid biomarkers, the amplification of protein biomarkers would be impracticable itself. Therefore, I have developed the Rotationally Focused Flow (RFF) method to enhance the sensitivity of protein biomarker based biosensor.

The RFF was made by adding a less dense fluid, ethanol, to focus the target fluid near the sensor surface in a simple T-shape microchannel, which results in reducing the distance between target molecules and immobilized probe molecules. The focusing rate of target fluid can be controlled by changing volumetric fraction ratio between two fluids. I verified the formation of the RFF in the T-shaped microchannels using both computational fluid dynamics simulations and flow experiments with fluorescent beads. The sensitivity enhancement using RFF method was investigated with label-free silicon microring resonator sensor using the biotin-streptavidin interaction as a model system. Remarkably, the sensitivity of microring sensor with the RFF method was increased by 8-order of magnitudes compared to the single

flow method where only target fluid was injected to the microchannel. I conducted a series of experiments and utilized these data to verify the sensitivity enhancement in the RFF method and to rule out other potential contribution factors including denaturation of target molecules. The investigation clearly showed that the RFF method is a simple and effective way to enhance the sensitivity of label-free optical sensor and can be widely applied to any biosensor without requiring additional instruments.

Microfluidic lab-on-chip (LOC) system is the most suitable platform for the satisfaction of POC diagnostic system requirements. Fluidic pumps and valves are among the key active components for LOC system, however, they often require on-line electrical power or battery and make the whole system bulky and complex, therefore limit its application in POC testing. In order to solve the problem, I have developed two types of self-powered actuators with corresponded valves.

First self-powered actuator & valve is a disposable switchgear controllable vacuum actuator which is made of two disposable syringes, poly (methyl methacrylate) switchgear and O-ring. In the vacuum actuator, an opened syringe and a blocked syringe are bound together and act as a working syringe and an actuating syringe, respectively. The negative pressure in the opened syringe is generated by restoring force of the compressed air inside a blocked syringe and utilized as the vacuum source. A Venus symbol shape of switchgear provides multi-function including reagent reservoir, push-button for the vacuum actuator, and on-off valve. Using the vacuum actuator, I have developed a self-powered switch-controlled nucleic acid extraction system (SSNES) for the establishment of disposable and equipment-free sample preparation device of POC diagnostic system. The SSNES consists of three sets of vacuum actuators, switchgears and microfluidic components. The SSNES can be widely used as self-powered and disposable system for DNA extraction and the syringe based

vacuum actuator would be easily utilized for diverse applications with various microchannels as a self-powered fluidic pump.

Second self-powered actuator & valve is that a hydration reaction based self-powered actuator. The working principle of the actuator is that hydrogen gas is generated by hydration reaction of powder with water and expanded to push in reagents through reaction zone toward the outlet of microchannel. The gas flow can be controlled with attaching a cover tape to or detaching a cover tape from the holes (inlets and outlets) of microchannels. In addition, I also have developed and integrated exothermic reaction based non-instrumented RPA device which performs the amplification of DNA with self-powered heating. Based on the developments, I present the development of a non-instrumented DNA analysis system (NIDAS) that enable to carry out total analysis of DNA biomarkers from sample to answer within 1 hour.

Chapter 1:

1. Introduction

1.1 Motivation

Over last few decades, human being have been suffered from various infectious diseases. Moreover, since 1970, about 40 kinds of new infectious diseases have been discovered and continued till now. According to the report from World Health Organization (WHO), over 20 % of all death has been caused by infectious diseases in worldwide. Especially, in developing countries, over 95 % of deaths from infectious diseases are caused by poor surroundings for proper diagnostics and treatments¹. Lab-on-chip (LOC) technologies have played great role in revolutionizing the way *in vitro* medical diagnostics are done and transforming bulky and expensive laboratory instruments and labor-intensive tests to easy to use, cost-effective miniaturized systems with faster analysis time, which can be used for near-patient or Point-Of-Care (POC) tests¹⁻³. The POC technology enables effective prevention and early detection of diseases and it helps to drastically deplete the mortality^{4,5}. In addition, The POC diagnostic system provides patients their health status in relatively faster, cheaper and easier way than existing medical diagnosis. Therefore, many research groups have given enormous efforts to develop POC diagnostic systems to reduce the mortality of infectious diseases. In the first stage of POC diagnostic system development, some requirements such as: i) Affordable, ii) Sensitive, iii) Specific, iv) User-friendly, v) Rapid and robust, vi) Equipment-free and vii) Deliverable to end-users (ASSURED) should be considered^{6,7}. These requirements can provide a most suitable diagnostic platform for use in resource-limited settings.

The specific identification of various infectious diseases is urgent for the accurate diagnosis. Proteins and nucleic acids (DNA and RNA) are mostly used as biomarkers to identify the specific infectious diseases. Although many of both typed biomarkers have been examined as promising candidate biomarkers of various infectious diseases, few selected protein or nucleic acid biomarkers have benefits to indicate few specific infectious diseases (e. g. heat-shock protein (hsp) antigen for tuberculous meningitis (TBM) disease)⁸. Therefore, the development of protein analysis system and nucleic acid analysis system are important for the diagnosis of infectious diseases and have been actively studied in parallel.

Various protein and nucleic acid biomarkers exist in body fluids such as saliva, blood and urine. Total 200 biomarkers of various diseases have been discovered in a urine sample and they include specific biomarkers for top 5 leading death from infectious diseases (lower respiratory infections, HIV, diarrheal diseases, tuberculosis and Malaria)⁹⁻¹¹. Despite blood and saliva samples also contain the specific biomarkers, urinary biomarkers have been more actively utilized for the development of POC diagnostic system due to advantages that the sufficient volume of urine sample can be obtained in noninvasive, pain-free manners and the sample is non-infectious and non-biohazardous in disposal¹². In addition, a separation step or a separation technique is not required for the analysis of urine biomarker since urine is relatively clean compared to blood with abundant red blood cells. Therefore, structural design of urinary biomarker based POC diagnostic system is relatively simple than other body fluids based system and in this project, I choose urine sample as a test sample for the detection of urinary biomarkers of infectious diseases.

The single specific biomarker in body fluids is not sufficient to be used in clinical setting for the detection due to their low-concentration. In the concentration range of infection biomarkers in body fluids can be as low as few pg/ml and in some cases, the analysis system requires sub pg/ml level sensitivity to distinguish patients with target disease

from healthy control¹³. Therefore, in order to detect the low-concentration biomarkers, effective enhancement methods of the biomarker concentration (amplification) or the analysis system sensitivity is essential. As is well known, the concentration of specific nucleic acid biomarker can be easily amplified to the detectable levels with thermal cycling or isothermal processes. Unlike nucleic acid biomarkers, the amplification of protein biomarkers would be impracticable itself. Therefore, it is desirable to develop sensitive read-out device and a microfluidic system to enhance the sensitivity of the read-out device.

Recently, biosensors have been widely used as immuno-sensors (i.e. read-out device for protein biomarkers) for the detection of target biomolecules in urine samples because they offer several advantages such as rapid and continuous measurement, high sensitivity and specificity and less usage of reagents¹⁴. Biosensors can be categorized according to transduction technologies such as optical¹⁵, resonance¹⁶, thermal¹⁷, magnetic¹⁸ and piezoelectric¹⁹ biosensors and each type of biosensors has different characteristics for various applications. Among the optical biosensors, especially label-free sensors have gained significant attractions because the target molecule can be detected as its natural form without any alteration or labelling and such sensors are inexpensive, easy to handle and allow quantitative and real-time detection²⁰. However, the sensitivity and detection limit of label-free optical sensor may not meet the requirement for the detection of extremely low concentration protein biomarkers (sub pg/ml) to be useful in clinical setting and the specificity of the sensor can suffer in low concentration range¹³. One of methods to enhance the sensitivity and the detection limit is modification of optical biosensor surface with carbon nanomaterials such as carbon nanotubes and graphene oxides^{21, 22}. The modification is effective to achieve a few pg/ml level of detection, however equipment and material costs are expensive and high technology with time consuming process is necessary for the modification. Therefore, for the realization of the use of label-free optical sensors in practical

applications, it is critical to develop a highly sensitive (the detection limit of sub pg/ml) sensor system with broad dynamic range in order to detect clinically relevant protein biomarkers.

As one of the efforts to improve the sensitivity and the detection limit of biosensors, researchers have developed different methods to enhance binding kinetics between analytes and immobilized ligands on the surface of biosensors by reducing the boundary layer thickness. Typically, convective transportation dominantly occurs in a microfluidic device due to its high Péclet number (Pe) over 10^3 based on extremely narrow width of microchannel and low diffusivity of analyte solutions^{23, 24}. In this case, binding kinetics can be effectively augmented by increasing initial reactive flux, which is directly enhanced by the reduction of boundary layer thickness²⁵⁻²⁷. Another approach to enhance binding kinetics is the electrokinetic manipulation of biomolecules using dielectrophoresis (DEP) technique and pre-concentrator based on Nafion nanochannels²⁸⁻³¹. However, simply reducing the height may induce excessively high pressure drop ($\Delta P \propto \Delta h^{-2}$)^{9,10} that could cause breakage of the bonding between microfluidic channels³² and the electrokinetic manipulation require electrodes on the sensor chip and additional electronic devices for the generation of DEP force and osmotic force, which limits universal application to different types of biosensors. Therefore, it is desirable to develop a straightforward and simple microfluidic method that can be directly integrated with any sensor platforms and induce enhanced performances for target applications.

Microfluidic lab-on-chip (LOC) system is the most suitable platform for the satisfaction of ASSURED requirements. Basically, bio-analytical devices integrated with microfluidic component can be used for multi-step processes, which used to be separately performed at large scale, in a miniaturized system with fast analysis time, high sensitivity and specificity^{33, 34}. Especially, polymeric material based microfluidic components have many

advantages including low cost fabrication, duplicability and disposability. For few decades now, classical POC diagnostic devices, “dipstick”, has been actively used for tests of pregnancy, influenza, cardiac markers and other infectious diseases³⁵⁻³⁷. These inventions successfully provide cost-effective and easy to use method. This type of a POC diagnostic system, however, has limitations to be used for multi-step and complex fluid handling required for molecular diagnostics³⁷. The workflow for molecular diagnostics is typically divided into few steps including sample preparation, target amplification (optional step for nucleic acids) and signal read-out. Especially, sample preparation and target amplification steps in the molecular diagnostics need the implementation of several active components (pumps, valves, reactors and heating elements) onto the microfluidic component due to its multi-step protocol (e.g. separation, lysis, purification and amplification). Fluidic pumps, valves and heating elements are among the key active components for LOC system, however, they often require on-line electrical power or battery and make the whole system bulky and complex, therefore limit its application in POC testing. A number of pump and valve components for microfluidic control have been actively studied for the development of equipment-free and user-friendly POC diagnostic systems. Microfluidic devices that utilize capillary force¹⁹⁻²⁰ such as Microfluidic Paper-based Analytical Devices (μ PADs)³⁸⁻⁴¹ as well as gravity-driven pumps^{22, 23} have been widely used as actuation sources to transport samples in POC diagnostic systems. Recently, for the self-powered microfluidic device, the method to pre-load vacuum inside the microfluidic device via degas process has been developed and used⁴²⁻⁴⁴. Several research groups have developed hand-operation pumps as a powerless pump on POC devices⁴⁵⁻⁵⁰. The pumps are operated with direct pressure by pushing elastic polymers implemented on inlets of POC devices. A disposable syringe has been popular choice as a power source³⁰⁻³². It can act as vacuum source or pressure source. Chin *et al.*, recently developed a POC assay that utilized a tubing pre-loaded with multistep reagents and

a syringe as vacuum source³². They successfully demonstrated that such a device can perform immunoassay for the diagnosis of infectious diseases in POC setting. While the pumps have advantages in low-cost and simple process of fabrication, they have limitations for the precise flow rate control required reaction steps of multi-step protocol for sample processing in molecular diagnostics. More importantly, the different flow rate can be required for different process step in a device, which cannot be provided by these pump devices. Together with the development of self-powered pumps, various types of self-powered valves have been developed for microfluidic devices⁵¹⁻⁵⁶. Microvalves are urgent as an active component in bio-analytical process to control fluid flows by opening or closing fluidic passageways. The valve operation is strongly related with actuation mechanism and its application. The target amplification is frequently carried out by an enzymatic reaction based method such as polymerase chain reaction (PCR). Heating elements, however, are essential due to the thermal cycling process of the PCR. Unlike the conventional PCR, isothermal amplification methods enable to amplify DNA at one constant temperature. Recently, several isothermal methods such as loop-mediated isothermal amplification (LAMP), recombinant polymerase amplification (RPA), rolling circle amplification (RCA) and helicase dependent amplification (HDA) have been frequently studied and used for the DNA-based POC diagnostic applications⁵⁷⁻⁶². Together with the development of isothermal amplification methods, researcher have given an enormous efforts to establish non-instrumented nucleic acid amplification device with various isothermal amplification methods^{63, 64}. Non-instrumented RPA device which is the most suitable isothermal amplification method for POC applications due to low required temperature and time, however, is not developed yet. Therefore, it is necessary to develop straightforward and simple self-powered controllable pump with a suitable microvalve and non-instrumented RPA device to establish equipment-free POC diagnostic system.

1.2 Objectives

The final objectives of the project are that the development of: i) a microfluidic system to enhance the sensitivity of protein biomarker based biosensors for the protein-based POC diagnosis of infectious diseases and ii) a fully disposable system which consists of a controllable self-powered actuator, DNA extraction channel component, non-instrumented RPA device with colorimetric detection method for the DNA-based POC diagnosis of infectious diseases.

1.3 Scope

The dissertation is based on real work done by the author in NTU and IME.

The following sequence of tasks for the successful execution during his Ph.D. period;

Chapter one introduces a motivation of the project for the issue of current works and outlines objectives and scope of the dissertation.

Chapter two provides reviews about current methods of; sensitivity enhancement of protein-based biosensors by improving binding kinetics, POC systems which used self-powered actuators & valves and non-instrumented DNA amplification.

Chapter three suggests rotationally focused flow (RFF)-based microfluidic system for the enhancement of label-free optical biosensor and indicates simulation results of rotationally

focused flow, fabrication process of the microfluidic system, flow experiment procedures using fluorescent beads and quantitative measurement results of sensitivity enhancement.

Chapter four proposes a self-powered switch-controlled nucleic acid extraction system (SSNES) for the sample preparation step of a POC diagnostic process. This chapter includes modelling process of a self-powered disposable syringe-based actuator, functional test results of switchgear component, design & fabrication of the SSNES, operating procedure of the SSNES and downstream analysis results.

Chapter five suggests a non-instrumented DNA analysis system (NIDAS) for DNA-based POC diagnostic process. This chapter includes selection of hydration reactive powder mixture for self-powered actuator, test results of the actuating performance, experimental demonstration of performance of exothermic reaction based recombinant polymerase amplification (RPA) device, design & fabrication process of the NIDAS and experimental preparation & procedures of the NIDAS.

Chapter six concludes the dissertation and represents future works

Chapter 2:

2. Literature Reviews

2.1 Sensitivity enhancement of protein-based biosensors by improving binding kinetics in microfluidic devices

The sensitivity of sensor is most important factor to diagnosis diseases. Unfortunately, sensitivity of most sensors may not meet the requirement for the detection of extremely low concentration target molecule (sub pg/ml) to be useful in clinical setting and the specificity of the sensor can suffer in low concentration range. Therefore, it is critical to develop a microfluidic system which can enhance the sensitivity of sensors to detect clinically relevant protein biomarkers. For the issue, researchers have developed various methods to enhance binding kinetics between analytes and immobilized receptors on the surface of biosensors by reducing the boundary layer thickness. In the most case, flow in microfluidic channels are regarded as convective transport dominant stream and binding kinetics can be effectively augmented by increasing initial reactive flux, which is directly enhanced by the reduction of boundary layer thickness²⁵⁻²⁷. Lynn *et al.*²⁵ reported the enhancement of the detection limit by reducing the height of microfluidic devices.

Figure 1 The maximum binding rate vs. concentration for the detection of ssDNA involving the use of chambers with varying height ($Q = 20 \mu\text{l/min}$)⁶⁵.

Fig. 1 represents the relationship between the maximum binding rates and target concentrations of ssDNA with varying chamber heights. They have showed that lowering the height of a microchannel from 47 μm to 7 μm leads to the decrease in the maximum binding rates by a factor of 4. Simply reducing the height, however, may induce excessively high pressure drop ($\Delta P \propto \Delta h^{-2}$)^{9,10} that could cause breakage of the bonding between microfluidic channel above a certain bonding strength which limits practical use of the microchannel³². Another approach to enhance binding kinetics is the electrokinetic manipulation of biomolecules. Several groups reported that the binding kinetic can be increased by 3 or higher orders of magnitude by pre-concentration of charged molecules toward sensing surface using dielectrophoresis (DEP) technique²⁹⁻³¹. Fig. 2 shows schematic diagram and field-emission SEM image of the integrated device and the concept of DEP interaction of target molecules with on-off AC excitation conditions.

Figure 2 a) Schematic diagram of the integrated device of nanoelectronic with electrokinetic device, b) top view of field-emission SEM image for the integrated device, c) cross-section view of a microfluidic channel in no AC excitation condition and d) cross-section view of a microfluidic channel in AC excitation condition³⁰.

Gong³⁰ used streaming DEP with AC-field application to increase sensitivity up to 4 orders of magnitude thanks to corresponding electrostatic contribution to the binding affinity. In a similar way, Nafion nanochannel is used as an electrokinetic biomolecule pre-concentrator for the initial concentration increase of target biomolecules in target solution. Xichen *et al.*²⁸ represented that 200 nl of 5000-fold concentrated target solution can be achieved within 5 min. Actually, the performance of the pre-concentration method is enough for the extremely low level detection of biomarkers. However, such methods require electrodes on the sensor

chip and additional electronic devices for the generation of DEP force and electro-osmotic force, which limits universal application to different types of biosensors. Therefore, it is desirable to develop a straightforward and simple microfluidic method that can be directly integrated with any sensor platforms and induce enhanced performances for target applications.

2.2 Self-powered actuators & valves

The implementation of several pumps and valves onto a microfluidic component are essential for a sample preparation step of diagnostic process due to its multi-step protocol (e.g. separation, lysis and purification). Fluidic pumps and valves are, however, often require power sources and make the whole system bulky and complex. Therefore, for POC testing applications, several research groups have been tried to replace the active components with self-powered components. Firstly, capillary force has been widely used as actuation sources to transport samples in POC diagnostic systems³⁸⁻⁴¹.

Figure 3 (A) Schematic diagram of fabrication process of 3D μ PAD. (B) Image of a basket weave system 10 s after adding red, yellow, green, and blue dyes to the sample reservoirs. (C and D) Images taken 2 (C) and 4 (D) min after adding the dyes. (E) Cross section of the device showing a bridge channel between the top and bottom channels of paper. (F) Cross section of the device showing three layers with orthogonal channels (green and red) in the top and bottom layers of paper. (G) Cross section of the device showing the layers and the distribution of fluid (and colors) in each layer of the device shown in (D)³⁹.

Fig. 3 represents fabrication process and demonstration of a 3D μ PAD. Actually, Microfluidic Paper-based Analytical Devices (μ PADs) have strong attractiveness on bio-analytical applications in low-resource environment with their low-cost and simple fabrication^{39, 40}. They are, however, hard to control the flow rate of samples and to perform purification step of sample preparation. Gravitational force is well known for an actuating source in mechanical field, even fluidics in vertically oriented microchannel⁶⁶⁻⁶⁸.

Figure 4 Integrated T-sensor design operated by gravitational force. A sample, a reagent and a reference solution are put into the top left reservoir, the top middle reservoir, and the top right reservoirs, respectively, with known concentration of analyte. A photograph in the center showing such a T-sensor in operation, standing vertical⁶⁶.

Fig. 4 indicates typical gravity-driven T-sensor which is used to separate particles from a mixture. The gravity-driven pump provides simple and cost-effective fabrication process. This has, however, difficulties such as control of flowrates and performance of complicated

fluidic processes. Several research groups have developed finger pumps as a self-powered pump on POC devices⁴⁵⁻⁴⁷.

Figure 5 Photograph of a simple finger pump for three reagents⁵⁹.

Fig. 5 shows a finger pump with three reservoirs and a wicking pad. The pumps are operated with direct pressure by pushing elastic polymers implemented on inlets of POC devices. While the pumps have advantages in low-cost and simple process of fabrication, they are limited to use in low flowrate or precise control required reaction steps. Together with the development of self-powered pumps, various types of self-powered valves have been developed for microfluidic devices⁵¹⁻⁵⁶. Microvalves are urgent as an active component in bio-analytical process to control fluid flows by opening or closing fluidic passageways. The valve operation is strongly related with actuation mechanism and its application. Therefore, it is necessary to develop straightforward and simple self-powered controllable pump with a suitable microvalve to establish equipment-free POC diagnostic system.

2.3 Non-instrumented nucleic acid amplification

For nucleic acid-based POC diagnostic system, target amplification step is required to achieve clinically relevant sensitivity for the detection of extremely low concentration DNA. The target amplification is frequently carried out by an enzymatic reaction based method such as polymerase chain reaction (PCR). Heating elements, however, are essential due to the thermal cycling process of the PCR. Unlike the conventional PCR, isothermal amplification methods enable to amplify DNA at one constant temperature. Therefore, by replacing PCR with isothermal amplification methods, equipment requirements can be reduced or eliminated and it is more advantageous to establish POC diagnostic platform. Several isothermal methods such as loop-mediated isothermal amplification (LAMP), recombinant polymerase amplification (RPA), rolling circle amplification (RCA) and helicase dependent amplification (HDA) have been frequently used for the DNA-based POC diagnostic applications⁵⁷⁻⁶². Liu *et al.*⁶⁴ has been reported non-instrumented isothermal nucleic acid amplification device that exothermic reaction is used as a heating element for the LAMP of DNA.

Figure 6 Schematic exploded view of the water-activated, self-heating, non-instrumented cartridge for LAMP of nucleic acids⁶⁴.

Fig. 6 shows the structure of water-activated, self-heating, non-instrumented cartridge. In this paper, they used filter paper as automated water carrier with controlling water flow rate and exothermic reaction rate by adjusting width of the paper and used molded paraffin frame to maintain the required temperature. It has great benefit to achieve the LAMP of DNA without any additional heating elements. However, one of desires is that operating temperature and

time (60 ~ 65 °C for 60 min) for LAMP is higher and longer than RPA (37 ~ 42 °C for 25 ~ 35 min). As a result, when the LAMP cartridge is replaced with the RPA cartridge, total size of RPA cartridge is expected to be smaller due to less required particles and water and temperature maintenance is much easier than LAMP cartridge without the paraffin frame. Therefore, it is necessary to develop non-instrumented RPA cartridge and strategy for the integration of the RPA component with a microfluidic component which performs sample preparation step for the total DNA analysis from a sample to answer.

Chapter 3:

3. Enhancement of Label-free Optical Biosensor in Rotationally Focused Flow-based Microfluidic System

In this chapter, I introduce a T-shaped microfluidic device with a rotationally focused flow (RFF) method for the detection limit enhancement of a label-free optical biosensor. In this method, two fluids with different density are used to induce reduced boundary layer thickness without increasing pressure drop. The RFF method allows simple way to increase sensitivity without additional instruments. For the demonstration of the RFF method, three validation steps have been performed. Firstly, simulations using Computational Fluid Dynamics (CFD) ACE+ were carried out for the verification of the RFF and the observation of target molecule's behaviors. Secondly, the realization of the RFF in microfluidic devices was investigated by a fluorescence experiment and the results were then compared with simulation results. Lastly, the quantitative measurement of the enhanced detection limit by using the RFF flow method was carried out using label-free silicon microring resonator sensors.

3.1 Working Principle of RFF

Rotationally Focused Flow (RFF) Method

Figure 7 Schematic diagram of the RFF method's concept. An injected target fluid is rotated by adding a less dense fluid faster than the target fluid (e.g. $3Q : Q$). In cross-section views at various locations, clockwise rotation process is observed and injected analytes are rotationally focused near immobilized receptors onto a microring resonator by the relationship between flow rate and dynamic viscosity of a target fluid and a less dense fluid (e.g. $7h : 3h$).

Fig. 7 represents the schematic diagram of the RFF concept. The fluidic channel is simple T-shaped where two fluids with different density are introduced from two short ends and are flowed through main channel to another end. When a target fluid is injected from one of the inlets while a less dense fluid is injected from the other inlet, the self-rotational flow is formed and results in the reduction of the distance between target molecules and immobilized probe molecules on the sensor surface. In addition, clockwise rotation process is observed in a cross-section view at the middle of microchannel. According to characteristics of microfluidics, laminar flow with strong surface tension is expected in the rotated stream. This

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characteristic enables target molecules not only to be vertically focused to the bottom side in a microchannel with low diffusion, but also to be entrapped inside the target fluid.

In order to achieve higher probability of the binding performance between target molecules and immobilized probe molecules, the target fluids with biomolecules should be more hydrodynamically focused near the surface of sensors. Therefore, a less dense fluid is injected faster than a target fluid in order to increase the volumetric fraction ratio of the less dense fluid to the target fluid. This phenomenon is resulting in the enhancement of the binding probability as shown to the enlarged view of a sensing part in Fig. 7. The height of target fluids in RFF can be obtained by modified equation from Nguyen *et al.*⁶⁹;

$$H_R = \frac{Q_T \mu_L}{Q_L \mu_T + Q_T \mu_L} H_C \quad (1)$$

, where H_R is the height of target fluids in RFF, Q_T is the volumetric flow rate of target fluids, μ_T is the dynamic viscosity of target fluids, Q_L is the volumetric flow rate of less dense fluids, μ_L is the dynamic viscosity of less dense fluids and H_C is the height of a microchannel. In (1), SF is a typical flow method that target fluids are injected solo into a microchannel and it is made for the comparison of the suggested RFF method with current flow methods. In this work, I have fixed the ratio 3:1 between Q_L and Q_T and 5:4 between μ_L and μ_T for ethanol and target fluids diluted by water, respectively. Therefore, H_R is reduced to 3/10 of H_C by adding a less dense fluid with 3 times faster flow rate than target fluids based on (1). From the next section, the fixed ratio is used for all of simulations and experiments.

3.2 Method

3.2.1 CFD simulation

Behavior pattern expectations of biomolecules are important in controlling the biomolecules and they are directly concerned with the enhancement of the binding probability. Especially, in terms of microfluidics, the biomolecules are more easily controlled with the laminar flow in microfluidic devices by the high surface tension^{24, 70}. Based on the characteristics, fluidic simulations have been performed using CFD ACE+ software with two representative modules which were “Two-fluid” and “Spray”. “Two-fluid” module was chosen to apply two independent fluids with different densities and “Spray” module was used to assign particles as target biomolecules. In order to perform CFD simulation, a microchannel design of 300 μm of width, 200 μm of height and 4800 μm of length was selected. 200 μm of height was fixed as the maximum height of microchannel fabricated by UV-lithography⁷¹. 300 μm of width and 4800 μm of length were decided due to suitable aspect ratio (0.5 ~ 1) for the stable generation of hydrodynamic focusing⁷² and to accommodate existing silicon microring chip dimension, respectively.

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3.2.2 Materials

A Poly (dimethylsiloxane) (PDMS) kit was purchased from Dow corning (USA). Distilled (DI) water and phosphate-buffered saline (1X PBS) were purchased from Invitrogen (Carlsbad, CA, USA). 97% trichloro (3, 3, 3,-trifluoropropyl) silane was purchased from Fluka (Buchs, Switzerland). SU-8 2100 and SU-8 developer were purchased from Microchem (Newton, MA, USA). 3-aminopropyltriethoxysilane (APTES), bovine serum albumin (BSA), Isopropyl alcohol (IPA), 99.8 % ethanol, denatured ethanol and latex bead type of 1 μ m amine-modified polystyrene with fluorescent red were purchased from Sigma-Aldrich (St. Louis, MO, USA). Immunopure streptavidin and EZ-Link NHS-PEG₄-biotin were purchased from Thermo Scientific Pierce (Singapore). Biotin anti-human TNF- α was purchased from BioLegend (San Diego, CA, USA). Other chemicals were analytical reagent grade and were used as received. All samples and buffers were prepared using DI water and PBS.

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3.2.3 Design and fabrication

A simple T-shaped microchannel which has same dimension as designed in CFD simulation was printed onto a film photomask. PDMS microfluidic channels were realized by PDMS replica molding from SU-8/silicon masters that were fabricated by the conventional UV-photolithographic process using the film mask. For 200 μm of a target thickness, SU-8 2100 of a photoresist was poured onto 8-in. silicon substrate and spin-coated with the spin speed of 1,400 rpm for 60 s. The spin coated photoresist was then baked on a hotplate at 65 °C for 7 min and 95 °C for 40 min. The baked layer covered with the designed photomask was then exposed to 315 mJ/cm^2 dose of the UV light. In a post exposure baking process, the transferred photoresist was then baked on a hotplate at 65 °C for 5 min and 95 °C for 14 min. Finally, through a development process for 17 min using a SU-8 developer and rinse process by IPA and DI water, a SU-8 master for the replication of microfluidic devices was realized.

PDMS is the most common polymer for the fabrication of microfluidic devices because of those advantages such as simple and low-cost replication process. The fabricated SU-8/silicon master was coated for the silanization using 97% trichlorosilane during 30 min to prevent the adhesion between the master and PDMS replicas⁷³. A PDMS kit (sylgard 184 silicone elastomer kit) consists of a PDMS prepolymer (sylgard 184, part A) and a curing agent (sylgard 184, part B). The mixture of the PDMS prepolymer and the curing agent with 10:1 weight ratio was evenly stirred for 10 min using a glass rod. The PDMS mixture was then poured onto a SU-8/silicon master. The PDMS mixture on the master was then degassed inside a vacuum desiccator and cured for 1 h at 80 °C in a convection oven.

Figure 8 (a) Schematic diagram of O_2 plasma bonding process for the fabrication of a silicon based label-free optical biosensor which consists of a PDMS replica and a silicon based microring resonator. Images of; (b) fabricated PDMS/glass microfluidic device by replacing the silicon based microring resonator with the glass for dummy test and (c) the fabricated silicon based label-free optical biosensor.

Bonding processes using molded PDMS replicas with a slide glass or a silicon based microring resonator sensor chip were performed depending on the purpose of experiments (Fig. 8). The silicon based microring resonator has been fabricated from 220 nm thickness of SOI wafers with 2 μm of buried oxide (BOX) layer in our previous work^{73,74}. In brief, one linear input waveguide is connected with four microrings and each microring has a corresponded output waveguide. The dimension of racetrack-shaped microrings is 5 μm of radius with 2.042 of coupling length. In addition, the width of waveguides is 500 nm and the gap between a linear waveguide and a microring is 220 nm. A PDMS replica was pierced using a steel punch to connect tubes at inlet and outlet positions before a bonding process.

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Prepared PDMS replica and slide glass (or silicon based microring resonator) surfaces were clearly washed with running IPA and DI water, followed by drying under a nitrogen stream and by heating in a convection oven. They were then treated with the oxygen plasma to activate hydroxyl group for the adhesion using NT-2 plasma cleaning system (Anatech Ltd., CA) under 70 W of a treating power in 60 sccm of O₂ flow rate for 50 s. The treated PDMS replica was moved onto a slide glass after turning over and bonded with the slide glass for the verification of the RFF. In addition, in order to test the binding performance of the proposed binding method, a microchannel in the treated PDMS replica was aligned and bonded to the sensor chip by observing their positions through the inverted microscope with CCD camera (Fig. 8(a)). Finally, two types of microfluidic devices (Fig. 8(b) and Fig. 8(c)) have realized after a hardening process in a convection oven at 150 °C for 30 min.

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3.2.4 Flow experiment

In order to test the generation of RFF with the observation of biomolecule's behaviors in the fabricated PDMS microchannel, fluorescence experiment was performed using the solution of fluorescent polystyrene beads in PBS. The solution of fluorescent polystyrene beads and ethanol were stored in 1 mL of syringes separately and injected by two syringe pumps (Scientific Inc., model KD 200 series, USA) to control the fluids independently. Bottom view images of microchannels taken at time intervals using an inverted microscope (Olympus, model IX71, Japan) integrated with a CCD camera (Q imaging, model Rolera-XR, USA).

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3.2.5 Surface chemistry

For the non-specific binding of streptavidin, 3-aminopropyltriethoxysilane (APTES), NHS-PEG₄-biotin and bovine serum albumin (BSA) were treated using syringe pumps. The integrated PDMS/silicon based microring resonator device was firstly treated with oxygen plasma during the bonding process. It was then treated by injecting a solution of 2 % APTES in a mixture of ethanol/H₂O (0.95/0.05, v/v) via the left inlet using a syringe pump for 2 h, followed by thorough rinsing with ethanol. It was then treated through injection of 0.25 mg/mL solution of NHS-PEG₄-biotin in DI water for 1 h and rinsed with DI water in the same method. In addition, 0.1% of BSA in PBS was injected into the left inlet for 30 min to prevent non-specific binding at the surface of a silicon based microring resonator and rinsed with PBS.

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3.2.6 Quantitative Measurement

For the characterization of my system's performance quantitatively, optical measurement of wavelength shifts by biotin-streptavidin interaction in silicon based label-free optical biosensors which are assembled PDMS/silicon based microring resonators was carried out with different flow methods. Due to the highest non-covalent binding affinity ($K_D = 10^{-15}$ M), biotin-streptavidin interaction is frequently used to characterize the binding performance of biosensors⁷⁵. The equipment setup with LabVIEW program for the optical measurement and the optical sensing principle were described in our previous papers^{74, 76, 77}.

3.3 Results and Discussion

3.3.1 CFD simulation

Figure 9 CFD simulation results for investigations of; (a) the RFF generation by observing target molecule's behaviors with numbered cross-section views at the left inlet (1), the right inlet (2), the entrance of a mainchannel (3), 1 mm (4), 2 mm (5), 3 mm (6), 4.8 mm (7)

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distant from the entrance, (b) the safety from the denaturation of target molecules by ethanol at an outlet of a microchannel with 60 μm of a focused region, 18 μm of a diffusion region and 42 μm of the safe region thicknesses. The RFF was made by injecting 3 $\mu\text{l}/\text{min}$ of PBS (blue block) including polystyrene beads (ball typed dots) as a target fluid with 9 $\mu\text{l}/\text{min}$ of ethanol (red block) as a less dense fluid in the CFD simulation.

Fig. 9 represents CFD simulation results of the RFF. For the clear observation of biomolecule's behaviors, both of injected fluids were disabled in the result view except injected polystyrene beads as shown in Fig. 9(a). In cross-section views at various locations, rotation processes and vertically focused polystyrene beads (ball type dots) near the bottom side of a microchannel were observed. In that case, ethanol (red block) was added three times as much volume than polystyrene beads solution. Through the cross-section views, clockwise rotational flow stream and over half of hydrodynamic vertically focused biomolecules were investigated. It can be clearly seen that the RFF was already achieved at the front part of a microchannel outlet (between position 4 and 5) with the height of less dense fluids of ~ 140 μm and the height of the focused fluids of ~ 60 μm which agrees with theoretical values obtained using (1).

For an ideal choice of less fluid in the method, the density difference between two fluids must be large enough to ensure rapid fluid rotation in order to minimize diffusion between them. In this project, therefore, ethanol was selected ($\Delta\rho = 521 \text{ kg}/\text{m}^3$). One of major concerns for using ethanol could be the probability of denaturation of biomolecules⁷⁸ when two fluids are mixed. However, in typical microfluidic configuration, mixing a target fluid with an acidic liquid is unlikely to happen due to extremely low Reynolds number (Re) except a little diffusion²⁴. Therefore, the denaturation of input biomolecules can be avoided by controlling the diffusion layer thickness. The CFD simulation was carried out in order to confirm this phenomenon. Fig. 9(b) represents the backside view (position 7) of a

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microchannel in a CFD simulation result to investigate the diffusion layer thickness and a safe region from the denaturation. In this figure, red block, blue block and ball typed dots indicate ethanol, PBS and polystyrene beads, respectively. The simulation results show two distinct regions within focused region of RFF; a diffusion region where mixing between aqueous solution containing target molecules and ethanol occurs and a safety region (blue block) where biomolecules do not mixed with ethanol. The diffusion region forms beyond 42 μm above the bottom surface, which provides enough distance to prevent the diffusion of denatured molecules to the sensor surface. Therefore, the RFF method using ethanol is suitable to be used for my microfluidic device and the method is able to enhance their binding probability by reducing a distance between target molecules and immobilized probe molecules.

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3.3.2 Flow experiment using fluorescent beads

Figure 10 Bottom view images of microchannels taken by an inverted microscope; (a) the experimental result of a flow pattern which made by injecting 3 $\mu\text{L}/\text{min}$ of PBS including fluorescent polystyrene beads with 9 $\mu\text{L}/\text{min}$ of ethanol, (b) simulation result in the same geometric and flow conditions as the flow experiment and (c) overlapping image between both of results.

In this flow experiment, 10 $\mu\text{g}/\text{mL}$ solution of fluorescent polystyrene beads in PBS was injected to the right inlet with 3 $\mu\text{L}/\text{min}$ of volumetric flow rate and ethanol was injected to the left inlet with 9 $\mu\text{L}/\text{min}$ of volumetric flow rate. As shown in Fig. 10(a), the injected fluorescent polystyrene beads from the right inlet were flowed with becoming wider to the left side of a microchannel. According to a flow pattern of the injected fluorescent polystyrene beads, the flow is able to be perceived as a rotation flow by the density difference between the solution of fluorescent polystyrene beads and ethanol. In addition, the injected fluorescent polystyrene beads were not escaped from the flow boundary between the two fluids over time as shown in Fig. 10(a). Fig. 10(b) shows the bottom view of CFD simulation result in 300 μm of width, 200 μm of height and 4800 μm of length device design with the

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same flow condition as a flow experiment. For more accurate verification, a flow pattern of the fluorescent polystyrene beads in the experimental result (Fig. 10(a)) was overlaid with the CFD simulation result (Fig. 10(b)). Remarkably, the both images are perfectly aligned, which confirms the generation of the RFF as shown in Fig. 10(c).

3.3.3 Binding performance

Figure 11 Binding curves of; (a) wavelength shifts of 190 nM Streptavidin solution injected by the SF method, (b) the resonance shift values by SF method at 15 min (10 min after PBS washing) plotted against the different concentration of Streptavidin solution ranging from 9.5 nM to 1900 nM ($R^2 = 0.9838$), (c) wavelength shifts of 1.9 fM Streptavidin solution injected by the RFF method and (d) the resonance shift values by RFF method at 15 min (10 min after PBS washing) plotted against the various concentration of Streptavidin solution ranging from 0.19 fM to 190 pM ($R^2 = 0.9992$).

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The binding performance was investigated using silicon based microring sensor. Microring sensor is a label-free refractive index sensor by taking advantage of the evanescent field present near the ring resonator surface. Its resonant wavelength, λ , is directly related to the effective refractive index of surrounding area and the antibody-antigen interaction can be directly monitored by measuring the shift in the resonant wavelength, $\Delta\lambda$ ^{74, 76, 79}.

Using Streptavidin-biotin binding as a model case for antigen-antibody based sensor system, the binding performance of the RFF method was compared with that of the Single Flow (SF) method, where the streptavidin solution was injected into a microchannel without using a less dense fluid. The binding curve of 190 nM of Streptavidin solution injected by the SF method is shown in Fig. 11(a). The microchannel was first filled with PBS to obtain the baseline then the Streptavidin solution was injected at 3 $\mu\text{l}/\text{min}$ of a volumetric flow rate for 5 min followed by PBS washing for 10 min in order to remove any non-specific binding. The binding curves of different concentration of Streptavidin solution ranging from 9.5 nM to 1900 nM were obtained in similar manner and the resonance shift values at 15 min (10 min after PBS washing) are plotted against the concentration of Streptavidin solution in Fig. 11(b). The resonant wavelength shifts at 15 min were 25, 178, 385 and 590 pm corresponding to 9.5, 57, 190 and 1900 nM of Streptavidin solutions, respectively. From the graph, the tendency of conjugations is logarithmically proportional to the concentrations of Streptavidin solutions with a correlation coefficient in the least square method ($R^2 = 0.9838$).

RFF method is generated by adding a less dense fluid. In this experiment, ethanol was introduced as a less dense fluid to a microchannel. Fig. 11(c) represents a binding curve of 1.9 fM of Streptavidin solution injected by the RFF method. The microchannel was first filled with PBS to obtain the baseline, Streptavidin analytes was then injected with 3 $\mu\text{l}/\text{min}$ of a volumetric flow rate into the right inlet and ethanol was injected with 9 $\mu\text{l}/\text{min}$ of a

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volumetric flow rate into the left inlet. After 5 min of binding, the entire microchannel was washed with PBS for 10 min for the removal of non-specific binding. Generally, injected Streptavidin molecules are much more densely existed near sensor surface for the RFF method compared to the SF method. Consequently, the molecules could accumulate near the sensor surface and this could generate large amount of non-specific bindings and result in large drop-off in the wavelength shifts during PBS washing. The measured wavelength shifts at 15 min (10 min after PBS washing) by the biotin-Streptavidin interaction using the RFF method in various concentrations of Streptavidin solutions ranging from 0.19 fM to 190 fM were plotted as shown in Fig. 11(d). The resonant wavelength shifts were 260, 460, 646 and 862 pm corresponding to 0.19, 1.9, 19 and 190 fM of Streptavidin solutions, respectively. From the graph, the tendency of conjugations is logarithmically proportional to the concentration of Streptavidin with a correlation coefficient in the least square method ($R^2 = 0.9992$). Remarkably, the sensitivity of microring sensor with the RFF method was increased by 8-order of magnitudes compared to the SF method. This unexpected sensitivity enhancement cannot be simply explained by height reduction effect of hydrodynamically focused target fluids. In latter section of this paper, I will discuss more on the probable causes of remarkable sensitivity enhancement.

Binding curves for both methods were well fitted with the Langmuir model with correlation coefficients from plotted graphs. For the Langmuir model, the apparent dissociation constant (K_d) is defined as⁷⁴

$$\Delta\lambda = \lambda_{\max} \frac{C_s}{K_d + C_s} \quad (2)$$

where $\Delta\lambda$ is the measured resonant wavelength shift, $\lambda_{\max}$ is the maximum wavelength shift for bound Streptavidin to immobilized biotin onto a microring resonator, C_s is the concentration of Streptavidin solution and K_d is the apparent dissociation constant.

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In this experiments, over 190 fM of Streptavidin concentrations, wavelength shifts were varied in 800 to 920 pm. According to the data, I can estimate $\lambda_{\max}$ as 860 pm. K_d between Streptavidin molecules and immobilized biotins onto a microring resonators were calculated using (2) to be $4.12 (\pm 0.53) \times 10^{-7}$ M for the SF method and $2.82 (\pm 3.12) \times 10^{-15}$ M for the RFF method. This result shows the significant enhancement of the sensitivity and detection limits by the RFF method.

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3.3.4 Biological activity test

Table 1 Measured values of wavelength shifts by various combinations of Streptavidin solutions with flow methods in each testing procedure and calculated ratios by $\Delta\lambda_2/\Delta\lambda_1$ as described in Fig. 12(b)

Flow types	~ 5 min		~ 15 min		~ 20 min		Ratio ($\Delta\lambda_2/\Delta\lambda_1$)
	Solution	$\Delta\lambda$	Solution	$\Delta\lambda_1$	Solution	$\Delta\lambda_2$	
SF	190 nM of SA	99.7 pm	PBS	98 pm	190 nM of biotin-TNF α	53.5 pm	0.54
RFF	0.19 fM of SA	1400 pm	PBS	239.4 pm	190 nM of biotin-TNF α	127.4 pm	0.53
RFF	1.9 fM of SA	1425.7 pm	PBS	872.7 pm	190 nM of biotin-TNF α	465.3 pm	0.53

Figure 12 (a) Schematic diagram of experimental procedures to investigate the biological activity of conjugated Streptavidin to immobilized biotins onto a microring resonator by injecting Streptavidin solution, PBS and biotin-TNF α solution and (b) the binding curves of the biotin-Streptavidin interaction using various concentrations of Streptavidin solutions (red line; 0 ~ 5 min) with different flow methods (solid line; 190 nM Streptavidin solution with SF method, dot double-dashed line; 0.19 fM Streptavidin solution with RFF method and dashed

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line; 1.9 fM Streptavidin solution with the RFF method) followed by PBS washing (blue line; 5 ~ 15 min) and subsequent interaction between bound Streptavidin and biotin-TNF α (green line; 15 ~ 20 min).

In order to confirm that enhanced apparent sensitivity of RFF method is based on real increase in the sensitivity not due to other factors including non-specific binding of denatured protein clusters, I investigated the biological activity of bound Streptavidin molecules using biotinylated anti-human TNF- α (biotin-TNF α) as a secondary probe. Fig. 12 shows binding procedures and the binding curves of the biotin-Streptavidin interaction using various concentrations of Streptavidin solutions (red line) with different flow methods followed by PBS washing (blue line) and subsequent interaction between bound Streptavidin and biotin-TNF α (green line). The schematic of the detailed binding procedures is shown in Fig. 12(a). The binding curves for 190 nM of Streptavidin solution using the SF method, 0.19 fM and 1.9 fM of Streptavidin solution using RFF method are shown in Fig. 12(b). Numerical values of wavelength shifts at 5 min (after Streptavidin binding), 15 min (after washing) and 20 min (after biotin-TNF α binding) are listed in Table 1. The wavelength shifts at 15 min by the biotin-Streptavidin interaction were 98, 239.4 and 872.7 pm for 190 nM of Streptavidin/the SF method, 0.19 fM of Streptavidin/RFF method and 1.9 fM of Streptavidin/RFF method, respectively. After injecting 10 μ g/mL of biotin-TNF α for 5 min, the resonant wavelengths were shifted further as 53.5, 127.4 and 465.3 pm, respectively due to the binding between bound Streptavidin and biotin-TNF α . High concentration of biotin-TNF α was used to ensure all bound Streptavidin molecules are fully conjugated with biotin-TNF α . In principal, the resonant wavelength is proportional with the mass change of analytes bound to receptors onto the sensor surface⁷⁴. In this experiment, I used 26 kD of biotin-TNF α and 53 kD of Streptavidin. Based on the molecular weight ratio, the ratios of wavelength shifts by the

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Streptavidin-TNF- α with the wavelength shifts by the biotin-Streptavidin interaction in cases of 190 nM of Streptavidin/SF method, 0.19 fM of Streptavidin/RFF method and 1.9 fM of Streptavidin/RFF method were 0.54, 0.53 and 0.53, respectively (Table 1). This result clearly shows that all bound Streptavidin molecules using RFF methods are biologically active and verifies the enhanced sensitivity induced by RFF method.

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3.3.5 Potential migration of biomolecules by Dean vortex

Figure 13 Images of PMMA microfluidic channel assembled silicon label-free optical biosensor (top), 200 μm thickness of double sided tape attached PMMA microchannel layer fabricated by laser cutting machine (bottom left) and 3 mm thickness of PMMA channel cover layer including three holes fabricated by laser cutting machine (bottom right).

Figure 14 Comparison bar graph of wavelength shifts by binding Streptavidin to biotins immobilized onto silicon based microring resonators via SF (25 pm), VFF (240 pm) and RFF (913.5 pm) methods at 15 min (9.5 nM Streptavidin solution injection step for 5 min and PBS washing step for 10 min).

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As I discussed earlier, drastic enhanced sensitivity by the RFF method is beyond expectation that one would have from increased target analyte flux toward sensor surface caused by simple channel height reduction effect. In order to compare the effect of RFF method to the effect of the channel height reduction to the sensitivity, I made a simple microfluidic structure to create the vertically focused flow (VFF). For the operation of VFF method, PMMA straight microchannel with two inlets and one outlet in a line was designed (Fig. 13) and fabricated using laser cutting machine (Universal laser systems, USA). Streptavidin solution was injected to the first inlet with 3 $\mu\text{l}/\text{min}$ of volumetric flow rate and PBS was injected to the second inlet with 9 $\mu\text{l}/\text{min}$ of volumetric flow rate to achieve the same focusing rate of target solution as RFF method (Fig. 13). Fig. 14 indicates the wavelength shifts of Streptavidin bound to biotins immobilized onto silicon based microring resonators. The resonant wavelength shifts were measured with the injection of 9.5 nM of Streptavidin solutions via SF, VFF and RFF methods for 5 min and the washing step using PBS for 10 min. The wavelength shifts for 9.5 nM of Streptavidin solution at 15 min of SF, VFF and RFF methods were 25, 240, 913.5 pm, respectively. From the result, the wavelength shift of the VFF method was 10.4 times higher than the SF method. It is satisfied with theoretical approach.

$$Pe = Q/WD \quad (3)$$

, where Q is the volumetric flow rate, W is the width of a microchannel and D is the diffusivity of the analyte. Based on (3), Pe for the SF method (Pe_S) is 1667 where Q_S is 3 $\mu\text{l}/\text{min}$. In a similar way, Pe for the VFF (Pe_V) and RFF method (Pe_R) are 6667 where Q_V and Q_R is 12 $\mu\text{l}/\text{min}$. The binding probability is strongly affected with total steady-state flux to the sensor surface. In addition, when $Pe \gg 1$, the flux is influenced by the depletion zone which is decided by sensor sizes and height of channel ($\eta = L_s/H$)⁸⁰. According to my sensor

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dimension, the flux would be 4 ~ 10 times larger than SF⁸⁰. There, however, was a huge difference of the wavelength shifts between the VFF method and the RFF method, even though both flow methods have the same focusing rate of the target solution. Therefore, I should consider other additional causes for migrations of biomolecules for the drastic enhancement of sensitivity by the RFF method.

Expected particle migrations with Dean vortex in Rotationally Focused Flow

Flow as in twisted channel from the view point of target solution

Figure 15 Schematic diagram of expected particle migrations happened by the Dean vortex in RFF at the viewpoint of target fluids. Drawn illustration of particles migrations with the Dean vortex by RFF along different positions in a microchannel; from position 2 to 3 (A), from position 3 to 5 (B) and from position 5 to 7 (C) as defined in Fig. 9(a).

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Among various secondary flows, the transverse motion of particles by the Dean vortex is well-known to have significant effect on the enhancement of the sensitivity⁸¹⁻⁸³. Generally, particle migrations in laminar flow are changed when fluids flow in curved, bended or twisted structures. Inside the structures, particles are forced from the center to the outward side by centrifugal force or torsional force with a double symmetric vortex upward and downward⁸¹⁻⁸⁴. Fig. 15 illustrates the generated vortex and how migrations of particles would be happened in the RFF method. In the clockwise rotating region, the target fluid with biomolecules is forced according to the direction of rotation. In this case, the transverse vortex motion of biomolecules are expected as in a twisted channel without passing through the interface of two fluids based on high surface tension and diffusion limited transportation of microfluidic characteristics as shown in Fig. 15 and could result in the active migration of biomolecules closer to the sensor surface. Therefore, the Dean vortex could be one explanation for drastic enhanced sensitivity of RFF method. However further investigation should be carried out in the future in order to seek theoretical and experimental proof.

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4. Self-powered Switch-controlled Nucleic acid Extraction System

In this chapter, I report a self-powered switch-controlled nucleic acid extraction system (SSNES) for the sample preparation step of a POC diagnostic process. The restoring force of the compressed air inside a blocked syringe was used as actuating energy to achieve equipment-free vacuum actuator. Before the prototype fabrication of the SSNES, the working principle of a self-powered vacuum actuator was suggested and validated by numerical modelling and experimental testing. Then, the portable and disposable SSNES was realized by assembling three sets of the vacuum actuator, the switchgear, and microfluidic channels. In the DNA extraction process, DMA, which was characterized in our previous work⁸⁵, was used as a non-chaotropic reagent to eliminate some required injection steps for the extraction process and to derestrict material selection of DNA extraction devices. Finally, the performance of the SSNES was demonstrated with the genetic analysis of extracted DNA using *HRAS* gene and the result was investigated with gel electrophoresis.

4.1 Scheme of SSNES

Main component of Self-powered Switch-controlled Nucleic acid Extraction System (SSNES)

Figure 16 Schematic diagram for structures and simplified operation procedures of the SSNES. (Left part) a self-powered vacuum actuator made of two disposable syringes (working and actuating). The generation of potential energy via pushing up syringe ends held by a syringe binder and fixing at a supporting slot by a stopper. (Middle part) a multi-function switchgear component including a Venus symbol shape of switchgear (Reagent reservoir, push-button for the vacuum actuator and on-off valve function). The operation of switchgear component by sliding the Venus symbol shape of switchgear to connect (on) or disconnect (off) the outlet of reservoir and the inlet of mainchannel. (Right part) an assembled unit of SSNES by inserting the self-powered vacuum actuator into the switchgear component. The working process of the unit of SSNES as followed sequences; i) removal of a stopper by pushing the switchgear, ii) descent of two bound syringes due to the restoring force of the compressed air within the blocked syringe, iii) generation of negative pressure in the opened syringe and iv) pull of reagent fluid from the reservoir with flowing through the mainchannel to the opened syringe.

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The structure of the SSNES is largely separated into two components. Fig. 16 represents the schematic diagram for structures and simplified operation procedures of the SSNES. The first component of the SSNES is a vacuum actuator made of two disposable syringes, which are indicated at the left side of the schematic diagram. The actuator consists of an opened syringe, a blocked syringe, a syringe binder, a stopper, and a container with supporting slot for the stopper and flanges of the syringes. The opened syringe and the blocked syringe act as a working syringe and an actuating syringe in the system, respectively. Two syringes are held together by a syringe binder which joins the plunger ends and the flanges are fixed by slots on the side wall of the container to work as a part. Then, in order to generate potential energy, the syringe binder is pushed up and fixed at a supporting slot by a stopper. The vacuum actuator is operated by the generated negative pressure due to the restoring force of the compressed air inside the blocked syringe.

The second component is a Venus symbol shape of switchgear where the ellipse part is used as the reagent reservoir and the cross part is used as push-button for the vacuum actuator. The vacuum actuator is activated by pushing in the stopper to fall off from the supporting slot. When the switchgear is pushed in, the outlet of the reagent reservoir is aligned to the underneath of the liquid inlet of the mainchannel, which allows the reagent to flow into the mainchannel under negative pressure of the actuator. The detail schematic diagram is shown in Fig. 16. For on-off valve function, the switchgear is slid to connect (on) or disconnect (off) both coupling holes. The vacuum actuator and the switchgear are assembled together to make one unit of the SSNES as shown in the right side of Fig. 16. In a SSNES, when the switchgear part is pushed in to fall off a stopper, the plungers of two bound syringes descend due to the restoring force of the compressed air within the blocked syringe and consequently negative pressure is generated in the opened syringe, which is connected to

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the outlet of the mainchannel via a tygon tubing. Finally, under the negative pressure, the reagent fluid is pulled from the reservoir and flows through the mainchannel to the opened syringe. A rubber O-ring is placed in between coupling holes of the reservoir and the mainchannel in order to prevent the leakage of the reagent and to keep the vacuum pressure within the entire unit.

4.2 Method

4.2.1 Modelling of self-powered vacuum actuator

Figure 17 (a) Schematic diagram of single blocked syringe to demonstrate a plunger motion by restoring force of the compressed air after pushing plunger into the blocked syringe and (b) free body diagram with parameters used in modelling process to determine applied forces to a plunger inside a cylinder of syringe.

Prior to the experiments with the prototype, I first examined the effect of volume of compressed air and compressibility on the velocity of descending plungers and the flow rate of pulled reagent using a theoretical model. Fig. 17(a) represents the schematic diagram of a blocked syringe to demonstrate a plunger motion with the compressed air generated by pushing plunger into the syringe. In order to derive the equation of plunger motion, energy conservation in case of adiabatic was considered as

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$$\Delta W = \Delta KE + \Delta PE \quad (3)$$

where ΔW is the work generation by the expanded air, ΔKE is the kinetic energy change and ΔPE is the potential energy change. Due to extremely lower value of ΔPE than ΔW , ΔPE can be ignored and ΔW ($\Delta W = ma\Delta s$) is directly converted to ΔKE ($\Delta KE = m(v_l^2 - v_p^2)/2$) without ΔPE as

$$a\Delta s = (v_l^2 - v_p^2)/2 \quad (4)$$

where v_l is the later velocity of a plunger, v_p is the previous velocity of a plunger, a is the acceleration of a plunger and Δs is the location change of a plunger. In addition, Δs is defined as

$$\Delta s = s_l - s_p \quad (5)$$

where s_l is the later location of a plunger and s_p is the previous location of a plunger. This simple equation (4) is well-known as Torricelli equation⁸⁶ which is used to find later velocity without having known time intervals.

Fig. 17(b) is a free body diagram to determine applied forces to a plunger inside a cylinder. As shown in Fig. 17(b), net force applied to a compressed plunger is represented as follows

$$\Sigma F = F_g - F_f = ma \quad (6)$$

where

$$F_g = P_g A_p \quad (7)$$

where

$$P_g = (P_e - P_{atm}) \quad (8)$$

where F_g is the resultant force generated by gas, F_f is the friction force of rubber covered plunger with inner wall, m is the mass of plunger, P_g is the applied total pressure to plunger,

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A_p is the cross-section area of plunge, P_e is the expansion pressure of compressed gas and P_{atm} is the atmospheric pressure. F_g consists of P_g and A_p as defined in (7). In addition, P_g is mainly influenced by P_e and P_{atm} as shown in (8).

When compression or expansion process takes place with adiabatic condition inside well-sealed cylinder, P_e varies with the volume of the compressed air by the polytropic process equation of ideal gas, $PV^{1.4} = \text{Const}'$. In addition, the motion of a plunger by compressed air will be stopped at the final location of the plunger (s_f) when P_e approaches to P_{atm} . For well-sealed cylinder, s_f is almost same as the initial location before compressing plunger. In the case, P_e along with s_l is defined with known values of P_{atm} and s_f as

$$P_e A_p^{1.4} s_l^{1.4} = P_{atm} A_p^{1.4} s_f^{1.4} \quad (9)$$

$$P_g = \frac{P_{atm} (s_f^{1.4} - s_l^{1.4})}{s_l^{1.4}} \quad (10)$$

According to the above relationship, P_g is redefined as (10) by substituting P_e from (9) into (8).

As working space is miniaturized, the contact force becomes more dominant for motion of the plunger than the body force and is composed with complicated factors. Especially, the friction force of the rubber sealed plunger in circular cylinders varies with velocity and pressure of an object as follows

$$F_f = C \sqrt{\frac{\mu v_l}{P_g}} + D v_l \quad (11)$$

where μ is the dynamic viscosity of flowed fluid in friction region⁸⁷. Constants of C and D for the friction force are determined as

$$C = 1.26 \times 10^{-6} \pi B d E \sqrt{\epsilon^2 \epsilon} \quad (12)$$

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$$D = \pi \mu D_r l / \delta \quad (13)$$

where B is the bore size of a cylinder, d is the diameter of rubber seal, E is the young's modulus of rubber, ε is the elastic strain rate of a rubber seal, D_r is the diameter of a plunger rod, l is the contact length between a cylinder and a rubber sealed plunger and δ is the clearance gap between a cylinder and a plunger ((12), (13)).

Unlike Xia *et al*'s study⁸⁷, in my system, the rubber friction of the plunger should be doubled since an actuating part (i.e. blocked syringe) and a working part (i.e. opened syringe) need to act in tandem in order to function as a vacuum actuator as described in the previous section. Therefore from here on, the constants C and D will be replaced with $2C$ and $2D$. By replacing F_g and F_f in (6) with (7) and (11), a is rewritten as (15) and substituted into (4) as

$$v_l^2 - v_p^2 = 2\Delta s \left(P_g A_p - 2C \sqrt{\frac{\mu v_l}{P_g}} - 2D v_l \right) / m \quad (14)$$

where

$$a = \left(P_g A_p - 2C \sqrt{\frac{\mu v_l}{P_g}} - 2D v_l \right) / m \quad (15)$$

After transposing and raising (14) to eliminate square root, quartic equation with respect to v_l is defined as

$$\begin{aligned} v_l^4 + \frac{4\Delta s v_p^2}{m} v_l^3 + \left(\frac{16\Delta s^2 D^2 - 2v_p^2 m^2 - 4\Delta s m P_g A_p}{m^2} \right) v_l^2 \\ - \left(\frac{8\Delta s m D P_g + 16\Delta s D P_g^2 A_p + 16\mu \Delta s^2 m C^2}{m^2 P_g} \right) v_l + \left(\frac{m^2 v_p^4 + 4\Delta s m P_g A_p v_p^2 + 4\Delta s^2 P_g^2 A_p^2}{m^2} \right) = 0 \end{aligned} \quad (16)$$

Figure 18 Result plots of theoretical results for flow rates of pulled fluid ($Q = v_l A_p$) according to the location of compressed plunger ($s_c = s_f - s_l$) from -10 mm (pushed plunger in 10 mm from the initial location after blocking syringe head) to 0 (the blocked position of syringe head to create air chamber) using; (a) different volume of syringe sets (actuating & working) as 3 ml (red), 5 ml (blue) and 10 ml (grey) syringes and (b) different initial locations of plungers in a set of 3 ml BD syringes as 58 mm (grey), 48 mm (blue) and 38 mm (red) and (c) theoretical results for flow rates of pulled fluid verses the (actual) locations of a plunger from 0.0481 m to 0.058 with 0.0017 increment (blue) using a set of 3 ml syringes and its comparison result with experimental flow rates (red) at the same condition.

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Table 2 Parameter list of geometric values and material properties for BD syringes used in modelling and experiments.

Syringe type	Parameter					
	A_p [mm ²]	B [mm]	d [mm]	D_r [mm]	E [GPa]	ϵ
3 ml	58.9	8.66	0.001	0.5	30	0.34
5 ml	114	12.06	0.001	1	30	0.34
10 ml	165	14.5	0.001	1.1	30	0.34
Syringe type	Parameter					
	μ [Pa·s]	l [mm]	m [g]	P_{atm} [Pa]	δ [mm]	
3 ml	1.86E-05	3	1.2	101300	0.33	
5 ml	1.86E-05	3.7	2.25	101300	0.66	
10 ml	1.86E-05	3.8	3.35	101300	0.726	

Fig. 18(a) and (b) show the plots of theoretical results for the flow rates of pulled fluid ($Q = v_l A_p$) versus the location of compressed plunger ($s_c = s_f - s_l$) using (16). In this work, I selected three commonly used BD disposable syringes, 3, 5 and 10 mL and other parameters were experimentally determined or obtained from the BD syringes specifications (See Table 2 for values). I set the initial location of the plunger to 0 (the initial location means the location of the plunger when the syringe head is blocked in order to create air chamber) and the starting location to -10 mm (the plunger is pushed in 10 mm from the initial location after the syringe head is blocked).

The flow rates of pulled fluid by working syringe for 3, 5 and 10 ml syringe are compared in Fig. 18(a). Even though three sets of syringes have different dimensions, the flow rates of different syringe sets are within narrow range from 25 μ l/s to 35 μ l/s at the maximum speed. Therefore I chose 3 mL syringes for the vacuum actuator from here on. Fig. 18(b) shows the flow rate of pulled fluid depending different initial locations of plungers in a set of 3 ml BD syringes. The flow rates are decrease when the initial location of plunger is

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farther from the syringe head (38 vs 48 vs 58 mm) since the ratio of the volume of compressed air to that of the air chamber in the blocked syringe decreases (0.26 vs 0.21 vs 0.17). These results show that the volume as well as the flow rate of the pulled liquid can be easily controlled by selecting syringes in different sizes of the syringe and the initial location of the plunger of the self-powered vacuum actuator. In this project, I chose the initial plunger location of 58 mm since the flow rate is suitable for my DNA extraction system.

For the verification of the theoretical result, I experimentally measured the flow rate of reagent using the self-powered vacuum actuator made of a set of 3 ml syringes. The flow rate versus the (actual) location of plunger (s_l) from the theoretical calculation using (16) and the experimental data are compared in Fig. 18(c). For the theoretical calculation, I set the value of location change, Δs , as 0.0017 m which is one increment of 3 ml BD syringe. Finally, v_l was calculated by (16) and the flow rates were v_l multiplied by cross-section area of syringes. For experimental set-up, 3 ml of blocked BD syringe was compressed by pushing a plunger and released. The plunger motion was recorded by a digital camera. The velocity of a plunger was obtained using elapsed time for one increment movement from the recorded video and the flow rates of pulled fluid was calculated using the velocity and the cross-section area of the cylinder. The theoretical and experimental data agree well with each other.

4.2.2 Microfluidic channel with manual on-off valve

Figure 19 (a) Image of a PMMA microchannel attached switchgear component consisting of two movable switchgears and two-inlet meandering microchannel. Rubber O-rings were inserted below two inlets of a microchannel to effectively transmit applied negative pressure from a vacuum syringe pump to reagents. Images of functional test results of the switchgear component under continuously applied negative pressure with various operating conditions; (b) off-on condition by pushing the right switchgear, (c) off-off condition by pulling both switchgears and on-off condition by pushing the left switchgear and (d) on-on condition by pushing the right switchgear together with the left switchgear. The right reservoir was immediately pulled to test shut-off ability of a switchgear based injection controller with the initial point of air gap in (c). For on-on condition, red/blue color inks were evenly introduced from both reservoirs.

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Sequential injections of reagents are necessary for multi-step reactions for microfluidic device operation. For the sequential injections, several independent reservoirs with corresponding valves are urgent. Therefore, I suggested poly (methyl methacrylate) (PMMA) switchgear fabricated by a CO₂ laser cutting method and tested functions of sequential injection and valve. Fig. 19 represents the PMMA prototype of the microchannel attached switchgear component and several combinations of operating conditions. The switchgear component consists of movable switchgears and a body part including working space for the switchgears and rubber O-rings (Fig. 19(a)). On the switchgear, the designed microchannel was attached by a double-sided tape. In addition, to effectively transmit applied negative pressure from a vacuum syringe pump to a reagent, rubber O-rings were inserted between the inlet of a microchannel and the outlet of a reagent reservoir and held by metal screws and nuts. Fig. 19(b) ~ (d) show combinations of operating conditions such as; (b) off-on condition, (c) off-off and on-off conditions and (d) on-on condition. As shown in Fig. 19(b), a blue color ink was flowed into a microchannel when the inlet of microchannel and the outlet of switchgear are connected under continuously applied negative pressure from a vacuum syringe pump. In order to test shut-off ability of a switchgear, the switchgear was immediately pulled for the disconnection of the coupling holes (off-off condition). As a result, the initial point of air gap in Fig. 19(c) indicated the off-off condition. By pushing the left switchgear, red color ink was injected into a microchannel (Fig. 19(c); on-off condition) and red/blue color inks were evenly introduced from both reservoirs after inserting the right switchgear (Fig. 19(d); on-on condition). Through the functional testing, the on-off valve function of a switchgear was investigated and the switchgear would be used for a pump operated by negative pressure.

4.2.3 Design and fabrication

Figure 20 Illustrated parts breakdown of the SSNES (c) and its components; a self-powered vacuum actuator component (a: an actuating syringe, a working syringe, a syringe holder, a stopper, a syringe binder assembly and four syringe container walls), a switchgear component (b: a lower cover, an intermediate part, an upper cover with coupling holes, Venus symbol shape of switchgear assemblies, rubber O-rings, screws and nuts), detailed parts of the switchgear assemblies (d: a base layer, a coupling channel layer and a reagent reservoir layer) and a microfluidic component (e: an APTES treated slide glass, two coupling layers, a binding channel layer and a mixing channel layer). The self-powered vacuum actuator is constructed by fitting convex-groove structures and other components are assembled by attaching double sided tapes.

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intermediate part which includes the movement space for the switchgear assemblies were fabricated by cutting 2 mm thickness of a PMMA sheet (Fig. 20(b)). They were assembled by attaching 0.1 mm thickness of double sided tapes (3M, USA) on both sides of the intermediate part and by fastening bolts with nuts. Before assembling, switchgear assemblies were placed in the movement space of the intermediate part with rubber O-rings. The detailed composition of the switchgear assembly is a base layer, a coupling channel layer and a reagent reservoir layer as shown in Fig. 20(d). The dimension of reagent reservoir depends on the volume of a target reagent. Base, coupling channel and reagent reservoir layers were fabricated using 0.5 mm, 0.5 mm, 1 mm thicknesses of PMMA sheets, respectively and assembled by 0.1 mm thickness of double sided tapes attached on both sides of the coupling channel layer. Above mentioned, microfluidic component should include three independent inlets and outlets, a mixing channel and a binding channel for DNA extraction. Therefore, the microfluidic component was composed as five layers shown in Fig. 20(e) and detailed design and pathways of reagents were described in Fig. 21; from the top, 1st layer - an APTES treated slide glass, 2nd layer - 1 mm width and 0.5 mm thickness of a meandering channel as the binding channel, 3rd layer - 0.2 mm thickness of a coupling layer, 4th layer - 1 mm width and 0.5 mm thickness of a spiral channel as the mixing channel and 5th layer - 0.2 mm thickness of a coupling layer. In order to enhance the performance of the DNA binding, the APTES treated slide glass was fitted with an isolated binding channel. In addition, double sided tapes attached PMMA sheets in both sides were cut together along channel designs and remaining sticky regions were covered by coupling layers. Finally, SSNES was realized by inserting three self-powered vacuum actuator components into a switchgear component and by attaching a microfluidic component onto the front side of the switchgear component with a spacer.

4.3 Experimental

4.3.1 Materials

Distilled (DI) water and phosphate-buffered saline (1X PBS) were purchased from Invitrogen (Carlsbad, CA). 3-aminopropyltriethoxysilane (APTES), bovine serum albumin (BSA), dimethyl adipimidate (DMA), sodium bicarbonate, agarose, ethidium bromide (EtBr) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lysis buffer (AL buffer and proteinase K), 10× PCR buffer, MgCl_2 , deoxynucleotide triphosphate, Taq DNA polymerase were purchased from Qiagen (Hilden, Germany). Other chemicals were analytical reagent grade and were used as received. All reagents and buffers were prepared using DI water and PBS.

4.3.2 DMA based DNA extraction using a disposable microfluidic chip

In this work, I performed DMA-based DNA extraction in the microfluidic device. DMA is a non-chaotropic DNA capturing reagent, where its bi-functional imidoesters are reversibly covalently binding with the amine groups on the sticky ends of the fragmented DNA⁸⁵. In DMA-based DNA extraction process, the cell lysis and pre-modification steps can be carried out as a single step in a mixing channel. In addition, for urine sample, a separation channel or a separation technique is not required to be integrated to a microfluidic component for a DNA extraction process since urine is relatively clean compared to blood with abundant red blood cells. Therefore, I designed a spiral micromixer as a microfluidic component for cell lysis and pre-modification steps. For the purification of extracted DNAs, binding DNA and washing the bound DNA are necessary. In this case, amine modification is frequently used on the surface of a binding channel to capture the DNA. In this work, 3-aminopropyltriethoxysilane (APTES) solution is used for the amine modification. In addition, covalent bonds of silane in APTES solution are easily formed with silanol groups contained surface such as glass or silicon. Thus, I choose a slide glass as a binding region and modify the surface by APTES. The process of amine modification with APTES was performed as follows: i) a slide glass was treated with oxygen plasma to activate silanol groups by NT-2 plasma cleaning system (Anatech Ltd., CA) under 100 W of a treating power in 80 sccm of O₂ flow rate for 60 s, ii) the activated glass was immersed in 2 % APTES solution in 95 % ethanol (v/v; 5 % H₂O with 95 % ethanol) for 2 h, iii) the APTES treated glass was washed with ethanol and DI water for 5 min in three times and dried with a nitrogen stream and iv) the surface was cured at 120 °C for 15 min in a convection oven (Mettler, Frankfurt, Germany). In order to enhance binding efficiency, I designed a wide meandering channel and covered the modified slide glass onto the isolated meandering channel.

4.3.3 Operating procedure

Figure 22 Photographs of the assembled SSNES with (a) working principle of the SSNES as followed sequences; i) fall of stopper by pushing switchgears with prominent ends (switch), ii) descent of syringe binder by compressed air pressure and iii) pull of reagents from the reservoirs passing through a microchannel by negative pressure in the connected condition and (b) procedure instructions of DNA extraction with different roles of reservoirs and storages in the system. Three reservoirs containing; (RI) the mixture of lysis buffer (50 μ l of AL buffer with 5 μ l of proteinase K) with 100 μ l of a DMA agent, (RII) 300 μ l of a PBS buffer and (RIII) 150 μ l of an elution buffer. DNA extraction process is started from putting 50 μ l of urine into RI and three solutions inside reservoirs are sequentially injected. At the end of the process, each 50 μ l of eluted DNA is separately collected in SI, SII and SIII as shown in the enlarged view (red color box).

Fig. 22 shows the photographs of the assembled SSNES. When switchgears are pushed in, stoppers, held in support slots, falls off by the prominent ends of switchgears. As a result, a syringe binder is descended by compressed air pressure and reagents are pulled from the reservoir passing through a microchannel by negative pressure (Fig. 22(a)). Fig. 22(b) shows the finished prototype of SSNES. RI, RII, and RIII, represents the first, the second and

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the third reservoirs for reaction agents, respectively. RI contains the mixture of lysis buffer (50 μ l of AL buffer with 5 μ l of proteinase K) with 100 μ l of a DMA agent for the cell lysis and DNA binding steps. In addition, 300 μ l of a PBS buffer is put into RII to ready for washing and purification of the sample and 150 μ l of an elution buffer (pH 10.6, 10 mM sodium bicarbonate) is put into RIII to ready for the elution of extracted DNAs. Finally, RI, RII and RIII are covered with polycarbonate tapes (PC tapes) (3M, USA) to prevent the contamination of buffers and agents before operating. For the collection of extracted DNA, a thin plastic container is attached to the third working syringe. The container is divided into three section (50 μ l each) in order to investigate the amount of extracted DNA in different portion of elution buffer (front (SI), middle (SII) and end (SIII)) exit from the microchannel.

The development of the SSNES aims to be user-friendly system which can be dealt by non-professional user with simple instruction. Typically, urine can be easily self-collected by users at home and therefore a suitable sample for point-of-care diagnosis. The operation procedure for the SSNES is as follows; Firstly, the user is to detach a PC tape from RI and add 50 μ l of urine into RI using a disposable pipette and then push in RI. When the RI is pushed in, the self-powered vacuum actuator I is initiated and start pulling the reagent from RI. The mixture of a lysis buffer, a DMA agent and urine is injected into the first inlet of microchannels and the solution is mixed in the spiral micromixers. The lysis reaction and simultaneous binding of DMA and extracted DNA occurs during this step. While the mixture solution is passed through a binding channel, the DNA/DMA complexes are captured onto APTES treated slide glass. After passing through the binding channel, the rest of the mixture is flowed into a working syringe in the first vacuum actuator. At this point, the user should pull out RI to complete the first step. Then the user is to detach a PC tape from RII and push in the RII. In the similar way as the first step, 300 μ l of a PBS buffer is introduced into the

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second inlet of microchannels to wash away non-specific and unbound molecules. Then, the waste is flowed into a working syringe in the second the vacuum actuator. For the last step of the DNA extraction process, the user pushes RIII in after pulling RII out and detaching a PC tape from RIII. 150 μ l of an injected elution buffer is flowed through a binding channel to elute DNA by breaking the covalent bonds between amine groups (of DNA as well as APTES) and DMA. Each 50 μ l of eluted DNA is separately collected in SI, SII and SIII as shown in the enlarged view (red color box) of Fig. 22(b). Entire process takes within 10 min. In short, the user would operate the SSNES by simple steps of detaching tapes, adding urine, pushing and pulling switchgears. It provides quite easy process and automatically guided flows to destinations (waste or storage) via separating actuation elements for the user-friendly sample preparation of POC diagnostics.

4.4 Results and Discussions

4.4.1 Downstream analysis of extracted DNAs

Figure 23 Genetic analysis of extracted DNAs with *HRAS* gene (450 bp) by conventional PCR and gel electrophoresis. Images of (a) Collection of eluted DNA from the first 50 µl of eluted DNA (SI), the second 50 µl of eluted DNA (SII), the third 50 µl of eluted DNA (SIII) and master mix without DNA as a negative control (N). (b) Electropherogram results of stained PCR amplicons using SI, SII, SIII and N according to different sized molecules with EtBr on agarose matrix. Extraction processes and visualizations were repeated three times and the results were confirmed with similarity.

In order to confirm DNA extraction performance of the SSNES, the downstream analysis of extracted DNAs was carried out with *HRAS* gene which is one of DNA biomarkers for bladder cancer using a PCR technique⁷⁷. The eluted DNA was amplified using primers for *HRAS* gene (Forward: 5'-TTTCCCATCACTGGGTCAT-3', Reverse: 5'-GCCTCTCCCTGGTACCTCTC-3') by conventional PCR and confirmed the results with gel electrophoresis. The eluted DNA is collected from SI, SII and SIII as shown in Fig. 23(a). Firstly, conventional PCR was performed with Taq PCR core kit. The reaction mix included 2.5 µl of 10× PCR buffer, 2 µl of 2.5 mM MgCl₂, 2 µl of 0.25 mM deoxynucleotide

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triphosphate, each 2 μ l of 25 pmol forward and reverse primers for *HRAS* gene and 0.5 μ l of Taq DNA polymerase (1 unit). The amplification of the master mix was performed with 35 cycles at 95 $^{\circ}$ C, 60 $^{\circ}$ C and 72 $^{\circ}$ C for 30 s, 30 s and 30 s, respectively. After 35 cycles, an elongation step was carried out at 72 $^{\circ}$ C for 7 min. Secondly, the PCR amplicons were visualized by gel electrophoresis. The gel was formed by curing 2 g/ml (2 %) of agarose pre-polymer with 1.5 μ l of ethidium bromide (EtBr) for 60 min. Fig. 23(b) represents the electropherogram result of stained PCR amplicons according to different sized molecules with EtBr on agarose matrix. M, SI, SII, SIII and N lanes in the result indicate DNA ladder, amplified DNA samples from SI, SII and SIII and no DNA template as a negative control, respectively. Band intensities directly indicate the quantity of *HRAS* genes in PCR amplicons. From dark bands for all of extracted DNAs (SI, SII and SIII) unlike a negative control (N), I can confirm the existence of abundant *HRAS* genes in extracted samples and the successful DNA extraction using the SSNES. In addition, extraction processes and visualizations were repeated three times and the results were confirmed with similarity. Therefore, I expect that the SSNES can be widely used as user-friendly operated and disposable self-powered DNA extraction system and the syringe based vacuum actuator would be easily utilized for diverse applications with various microchannels as a self-powered fluidic pump.

Chapter 5:

5. Non-Instrumented DNA Analysis System

In this chapter, I report a non-instrumented DNA analysis system (NIDAS) for DNA-based POC diagnostic process. Hydration-reactive powder mixture which is used as a portable heating element was selected and produced hydrogen gas by adding water was used as actuating energy to achieve a self-powered actuator. As a valve, commercially available optical adhesive films were used with attaching onto or detaching from holes of microchannels. Based on the actuation concept, the working principle of non-instrumented gas-driven microfluidic system was suggested and gas producibility (in terms of flow rates and uniformity) and its long-lasting ability were experimentally evaluated. For non-instrumented DNA amplification, exothermic reaction based recombinant polymerase amplification (RPA) device was suggested and the amplification performance was validated by gel electrophoresis. Then, portable and disposable NIDAS was realized by assembling hydration reaction based self-powered actuator, DNA extraction microfluidic channel and exothermic reaction based RPA device. In the DNA extraction process, DMA, which was characterized in our previous work⁸⁵, was used as a non-chaotropic reagent to eliminate some required injection steps for the extraction process and to derestrict material selection of DNA extraction devices. Finally, detailed experimental procedure was described with colored dyes and the performance of the NIDAS was investigated with *EGFR* gene.

5.1 Scheme of non-instrumented gas-driven microfluidic system

Metal alloy particles can produce heated gases by reacting with water. The produced gases are potentially used as not only a heat source, but also an actuation source. Especially, precisely controlled and long-lasting production of gases can be made by well-designed composition of metal alloy mixture with other chemical ingredients and it provides a high performance self-powered micropump. In addition, the gas flow can be controlled with attaching cover tapes to or detaching cover tapes from holes of a microchannel (inlet or outlet) for the switching of on-off condition and flow direction.

Figure 24 Schematic diagram of working principle for a hydration reaction based self-powered actuator; i) applying water & attaching cover tape and ii) producing hydrogen gas by hydration reaction & pushing reagent and basic design concept for non-instrumented gas-driven microfluidic system. Formation of hydrogen gas from the self-powered actuator located in the inlet of a microchannel by applying water and covering the inlet hole. Sequential injection of pre-inserted reagents (red-blue-green) with air spacers between reagents through reaction zone to the outlet of the microchannel. Occurring reactions of reagents in the reaction zone according to the injection sequence.

Fig. 24 represents the schematic diagram of working principle for a hydration reaction based self-powered actuator (i and ii) and basic design concept for a non-instrumented gas-driven microfluidic system. At the beginning of actuator operation, cover tapes are detached from the inlet of a microchannel, sequentially. Then, water is dropped onto the powder mixture for the hydration reaction using a micro-pipette (i). With the hydration reaction, hydrogen gas is immediately formed and by attaching new cover tape onto inlet hole, the formed hydrogen gas within the limited space of the microchannel pushes reagents along a microchannel to an outlet (ii).

Based on the principle, I can design a self-powered gas-driven microfluidic device as shown in Fig. 24. The powder mixture is put in a deep container located at the foremost end of microchannel. Before covering the upper layer of a microchannel, I would place reagents in segmented positions of the microchannel with enough clearances according to reaction sequence. Chemical treated or receptor immobilized part is attached onto the opened region of a reaction zone in the microchannel to perform various bio-analysis processes after covering the upper layer. At the last step for the preparation of the microfluidic device, a hole which is located in the next above the powder mixture is covered with a cover tape to prevent pre-reaction of powder mixture with water in air.

As above mentioned about the working principle of a hydration reaction based self-powered actuator, gas is formed via the detachment of cover tape from the inlet of a microchannel, the injection of water into the container of powder mixture. In order to transport pre-inserted reagents to a reaction zone in the microchannel, inlet hole is again covered by attaching new cover tape. The pre-inserted reagents with trapped air between reagents would be sequentially flowed and passed away the reaction zone with specific reactions corresponded to treated chemical or immobilized receptor. In this process, I can

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separate wastes and test samples by blocking or opening outlets with cover tapes, as needed.

5.2 Material selection

Distilled (DI) water and phosphate-buffered saline (1X PBS) were purchased from Invitrogen (Carlsbad, CA). 3-aminopropyltriethoxysilane (APTES), bovine serum albumin (BSA), dimethyl adipimidate (DMA), sodium bicarbonate, agarose, ethidium bromide (EtBr) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lysis buffer (AL buffer and proteinase K) was purchased from Qiagen (Hilden, Germany). Portable heating element was purchased from Outing (Seoul, Korea). Rehydration buffer, dried enzyme pellets and magnesium acetate (MgAc) were purchased from TwistDX (Cambridge, UK). Other chemicals were analytical reagent grade and were used as received. All samples and buffers were prepared using DI water and PBS.

5.3 Hydration Reaction based Self-powered Actuator

5.3.1 Selection of powder mixture

The performance of self-powered pumps or actuators are evaluated by how easy the control of flow rate is, how uniform the flow rate is and how long the operation lasts. For hydration reaction based gas-driven pumps, the composition of powder mixture, amount of powder and amount of water would be directly involved in the production rate and duration of gas and it would affect the operating flow rate and time of the pumps. In this work, I choose a specific powder mixture which is used as a portable heating element for ready-to-eat products and it is composed of aluminum (Al; 47%), calcium hydroxide (CaO; 30%), calcium carbonate (CaCO₃; 11%), sodium carbonate (Na₂CO₃; 10%) and sodium hydroxide (NaOH; 2%). The mixture of CaO and Al possibly produces heated gases by reacting with water. During the hydration reaction, CaCO₃, Na₂CO₃ and NaOH play a representative role to increase reaction (operating) time.

5.3.2 Gas producibility and its long-lasting ability tests

Figure 25 Images of; (a) three straight microchannels with $1 \times 1 \times 25 \text{ mm}^3$ volume for the flow rate measurement of formed gas by hydration reaction and (c) a long microchannel with $1 \times 1 \times 1000 \text{ mm}^3$ volume for the determination of flow rate uniformity and the duration of hydration reaction. Graphs of gas flow rate changes; (b) along the microchannel with different amounts of the powder and (d) over time with 10 mg of the powder.

With the fixed composition of the powder, the flow rates of reagents can be changed by different amounts of the powder or water. In hydration reaction, variations of powder amount is more effective to change the flow rates rather than water amount. Therefore, I used different amounts of the powder and 100 μl of water for hydration reaction to demonstrate corresponding flow rates in a simple straight channel (Fig. 25(a)). Test devices were fabricated by a CO_2 laser cutting/engraving machine (Universal laser systems, AZ, USA) using PMMA sheets and a test device consists of an upper cover layer which is engraved with a millimeter ruler and has three sets of inlets and outlets for three microchannels, a channel

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layer which includes three microchannels designed with $1 \times 1 \times 25 \text{ mm}^3$ (width $\times$ height $\times$ length) of dimension and three reagent reservoirs with ellipse shape for each channel, a lower layer which includes 8 mm diameter of circular cylinder shaped container for powder mixture, a plat lower cover layer. While the assembling process, 20 μl of green colored dyes were placed on reagent reservoirs before attaching the upper cover layer.

In order to test the effect of solute amount difference in hydration reaction, I separately put 1, 5 and 10 mg of the powder into inlets of three microchannels in a test device and put 30, 50 and 100 mg of the powder into another test device. Then, hydration reactions were started by injecting 100 μl of water into powder containers and covering inlet holes with cover tapes and formed gases pushed in dyes through channels to outlets of microchannels. The flow motion was recorded by a digital camera. The velocity of fluid was obtained using elapsed time for 1 mm movement from the recorded video and flow rates of fluid were calculated using the velocities and the cross-section area of microchannels. The flow rates was plotted according to the amounts of the powder used to hydration reactions as shown in Fig. 25(b). From the graph, results showed that larger amount of the powder generates higher flow rates. In addition, the flow rates can be easily controlled with proper amount of the powder depending on purposes (mixing, binding or sorting) of the applications for relatively short length of a microchannel required applications (less than 25 mm).

In order to use the gas-driven pump for multi-step reactions, flow rate uniformity and long-lasting ability are necessary to be investigated. For the investigation, I designed and fabricated 1 m length of microchannel and chose 10 mg of the powder with the assumption that lower amount of the powder has relatively high uniformity of flow rates (Fig. 25(c)). In the same way as previously, 10 mg of the powder was placed in the powder container and it was activated by 100 μl of water after covering an inlet hole with a cover tape. The flow motion was recorded with 1 min of time interval for 2 hours and the velocity of fluid was

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obtained via flow distance per min from the recorded video. Then, flow rates of fluid were calculated and plotted with time as shown in Fig. 25(d). In the figure, the measured flow rates show that gas forming process is maintained by hydration reaction between extremely low amount of the powder (10 mg) and small water drop (100 μ l) with narrow range of flow rates (4 ~ 8 μ l/min) for over 2 hours except for 10 min from the initial reaction. In the special application which needs to avoid rapidly changing region from 0 to 10 min, I can cover an inlet hole with a cover tape at 10 min after putting water into the powder container.

5.4 Exothermic reaction based recombinant polymerase amplification device

Figure 26 (a) Image of structure of the non-instrumented RPA device. Adding a test sample into the left reaction reservoir and a solution for negative control into the right reaction reservoir and attaching an optical adhesive film onto reaction chambers. (b) A graph of temperature variation plot with time using different combination of exothermic reactive powder amounts (0.5 g and 0.4 g) and paper strip widths (3 mm and 5 mm). Initiation of exothermic reactive powder by wicking water to the powder container 5 min after applying water to a water reservoir. 0.5 g amount of the powder and 3 mm width of the paper strip met required temperature and time for the RPA of DNA. (c) Image of the RPA device with decided condition captured during the RPA. (d) Image of gel electropherogram result indicating DNA ladder (M), amplified test sample (T) and negative control (N).

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The recombinant polymerase amplification (RPA) method is one of isothermal amplification methods, especially it is performed at relatively low temperature (37 ~ 42 °C) during shorter process time (25 ~ 35 min) without thermal cyclers. In the experiment, the specific composition of powder mixture in the previous section was used as the solute of exothermic reaction for the heat-up process in the RPA method and water was automatically carried with a Whatman filter paper (Sigma-Aldrich, USA) to the powder container. The RPA device consists of two main parts; upper reaction chamber part and lower container part as shown in Fig 26(a). All layers of the RPA device were fabricated by CO₂ laser cutting/engraving method using PMMA sheets and assembled by attaching double sided tapes between layers. The container part includes a container layer with two large holes for a powder container (width: 20 mm, length: 20 mm and height: 3 mm) and a water container (10 mm × 20 mm × 3 mm) and a base layer with an engraved groove for a filter paper. In the reaction chamber part, two floated reaction chambers and a wide hole for the water container were deeply engraved and cut through on a reaction chamber layer, respectively, and the layer was covered with a thin top layer which was cut through with four holes for a gas vent, two reaction chambers and a water container.

In the exothermic reaction, paper width and powder amount are key factors to achieve the desired temperature and duration of the heat-up process. Accordingly, temperature variations in time were plotted with different amount powders (0.4 and 0.5 g) and difference paper widths (3 and 5 mm) as shown in Fig. 26(b). For the temperature measurements, I inserted a digital probe thermometer with timer (Oneida, USA) into a reaction chamber during the heat-up process and read temperatures from the thermometer per a minute. In the experiments, temperature increase is started at 10 min after adding 500 µl of water into the water container of the RPA device and the maximum temperature and over time of exothermic reaction are varied with different experimental conditions (pale green, pale blue

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and pale red lines). Based on the result graph, I realized that the maximum temperature and the reaction over time can be controlled by the width of filter paper (i.e. flow rate of water) and the ratio of total amount with the flow rate, respectively. Consequently, for RPA method (37 ~ 42 °C for 25 ~ 35 min), I chose 0.5 g of powder and 3 mm width of filter paper.

In order to confirm the performance the device, RPA of nucleic acids was carried out with human genomic DNA using the reagents and protocols described in TwistAmp Basic kit (TwistDX, UK). The optimized protocol is as follows; i) 29.5 µl of rehydration buffer, each 2.4 µl of 10 mM forward and reverse primers (forward:5'-GCA GCA TGT CAA GAT CAC AGA TTT TGG GCT and reverse:5'-CAT GTG TTA AAC AAT ACA GCT AGT GGG AAG GCA, 240 bp), 10.7 µl of distilled (DI) water and 2.5 µl of 50 ng/µl human genomic DNA for a test sample or 2.5 µl of nuclease-free water were mixed in 200 µl tubes and the mixtures were vortexed. Two of 47.5 µl reaction mixtures were mixed with dried enzyme pellets which includes dNTPs, adenosine59-triphosphate, phosphor-creatine, creatine kinase, and components of the proteins gp32 (single-stranded DNA binding protein), uvsX (recombinase), and the uvsY (recombinase load factor). In order to initiate the reactions, 2.5 µl of magnesium acetate (MgAc) was added into each tube. Each 50 µl of the final reaction mixture was separately put into reaction chambers. Finally, reaction chambers were covered with cover tapes and reaction mixtures were incubated for 40 min from adding 500 µl of water to a water container (Fig. 26(c)).

The RPA amplicons were visualized by electrophoresis technique using 1 % of agarose gel with 1.5 µl of ethidium bromide (EtBr). Fig. 26(d) shows the electropherogram result of stained RPA amplicons with EtBr onto agarose matrix according to different sized molecules. In the result figure, three vertical lines indicate DNA ladder (M), amplified test sample (T) and no DNA template (N). For P and N lines, band intensities in the position of

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red box directly represent the quantity of *EGFR* genes in RPA amplicons. Therefore, through the dark band in T line unlike N line, I could realize that the RPA of nucleic acids is successfully performed in my RPA device.

5.5 Non-Instrumented DNA Analysis System

5.5.1 Design and Fabrication

Figure 27 Schematic structural diagram of a non-instrumented DNA analytical system (NIDAS); i) reagent reservoir component, ii) DNA extraction component, iii) DNA amplification & detection component and iv) water & powder container component.

Fabrication of all components by cutting/engraving PMMA sheets and double sided tapes using CO² laser machine and assembly process of components performed with double sided tapes. v) Cover tapes, bridge channels made by cutting optical adhesive films.

As above mentioned, I designed an integrated system which can perform DNA extraction, amplification and read-out without any additional instruments using the self-powered hydration reaction based gas-driven pump and the non-instrumented exothermic reaction based RPA device. The integrated system, non-instrumented DNA analytical system (NIDAS), consists of four main components: i) reagent reservoir, ii) DNA extraction, iii) DNA amplification & detection and iv) container components as shown in Fig. 27. First, reagent reservoir component was designed as elliptical capsule shape of 9 reservoirs (8

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reservoirs with 40 μl volume and a reservoir with 30 μl volume) connected in a line for the storage and separation of reagents depending on their purposes. In addition, air in empty reservoirs act as spacers between neighboring reagents to prevent mixing each other in the sequential injection process. Second, in order to perform DNA extraction process (e.g. lysing, washing, elution steps), I designed 4 mixing channels with spiral shape (1 mm width) and a long binding channel with meandering shape (1 mm width). In addition, one end outlet of the binding channel was connected with a channel toward a waste container and the other outlet was connected with a broken channel near reaction chambers. Third, the waste channel and the broken channel were engraved on the top of a reaction chamber layer. As represented in the previous section, three floated reaction chambers with each 60 μl volume for RPA were deeply engraved from the top of the reaction chamber layer. Lastly, a container of powder for exothermic reaction and a container of water with same dimension as the non-instrumented RPA device and a container of powder for hydration reaction with $6 \times 6 \text{ mm}^2$ circular area was designed in the container component.

All of designed layers with double sided tapes were cut or engraved by CO_2 laser machine. From the bottom side, a base layer, a container layer and a reaction chamber layer were fabricated and assembled using 3 mm thickness of PMMA sheets and 0.1 mm thickness of double sided tapes, respectively. Then, the reaction chamber layer was covered with a doubled sided tape and 0.5 mm thickness of a PMMA sheet. For the reagent reservoir component, 1 mm thickness of a PMMA sheet was cut and it was attached onto the cover layer of reaction chamber with a double sided tape. In order to connect the reagent reservoir layer and the DNA extraction layer, an intermediate layer was fabricated using 0.5 mm thickness of a PMMA sheet and attached between the layers with double sided tapes. On the top of the integrated system, $50 \times 24 \text{ mm}^2$ sized glass, which was functionalized with amine group, was attached. In addition, four different designs of detachable channels and covers

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were cut by CO₂ laser cutting/engraving machine using optical adhesive films (Invitrogen, USA) (Fig. 27(e)). Finally, the NIDAS was realized by assembling all components with double sided tapes which were attached between components and attaching four detachable channels and covers onto their proper positions.

5.5.2 Preparation

DNA analysis process is started from DNA extraction and it is ended with the detection of amplified target DNA through DNA amplification. In order to obtain high-purity DNA, binding DNA extracted from cells using dimethyl adipimidate (DMA) and washing the unbound DNA and other molecules with PBS are necessary in the DNA extraction step. One of strengths of using DMA as a DNA binding agent is that DMA solution can be mixed with a lysis buffer without inhibition, thus the solutions can be stored together in a reservoir. As mentioned in the previous section, all reagents, which are used for the DNA analysis, are inserted to reservoirs and reaction chambers according to reaction sequences in the fabrication step. Firstly, I placed 11 μl of lysis buffer (10 μl of AL buffer with 1 μl of proteinase K) and 20 μl of 25 mg/ml DMA solution for cell lysis and pre-modification of extracted DNA in the first reservoir of 9 reservoirs near the outlet of a reagent reservoir channel (green colored dye). Secondly, I separately placed 120 μl of PBS in fourth, fifth and sixth reagent reservoirs (each 40 μl of PBS) for the washing step of the DNA extraction (blue colored dye). Thirdly, I inserted 30 μl of 10 mM sodium bicarbonate (pH 10.6 elution buffer) in ninth reagent reservoir for the elution of bound DNA (red colored dye). Fourthly, 36.8 μl of RPA master mixes which was used in the test of non-instrumented RPA device without adding templates (DNA and DI water) were separately inserted to three reaction chambers and 13.2 μl of DI water was only added to the uppermost reaction chamber as a negative control. Lastly, the reaction chambers were covered with an optical adhesive film.

Bi-functional imidoesters of DMA reversibly-covalently bind amine groups on the functionalized surface and the sticky ends of the fragmented DNA. Therefore, I modified glass surface with amine groups using APTES which is a well-known silane-coupling agent and covered a binding channel by the glass. Briefly, the APTES treatment was performed as

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followed sequences; i) oxygen plasma was used to activate silanol groups on a slide glass using NT-2 plasma cleaning system (Anatech Ltd., CA), ii) the activated glass was immersed with 2 % APTES solution in 95 % ethanol for 2 h, iii) the glass was washed with ethanol and DI water for 5 min in three times and dried with a nitrogen stream and iv) finally, curing of the surface was carried out in a convection oven (Mettler, Frankfurt, Germany) at 120 °C for 15 min.

5.5.3 Experimental procedures

Figure 28 Images of analysis process of DNA from urine sample using the NIDAS; (a-i) detaching an optical adhesive film from the inlets of hydration reactive power container and test sample reservoir, (a-ii) adding urine sample to the reservoir, (a-iii) applying water to the powder container and (a-iv) attaching new adhesive film to initiate the hydration reaction based self-powered actuator, (b-i) initial condition of the NIDAS, (b-ii) starting sequential injection and reaction, (b-iii) flowing the wastes out (lysis buffer & DMA & urine sample and PBS), (c-i) opening another outlet by detaching cover film from reaction chambers, (c-ii) changing flow direction of eluted DNA solution toward reaction chambers, (c-iii) separating the solution evenly to two reaction chambers, (c-iv) mixing the solutions with RPA reagents, (d-i) detaching bridge channel to isolate the reaction chambers, (d-ii) attaching slide glass with double sided tape onto reaction chambers, (d-iii) adding water to the water container and (d-iv) incubating test samples for 35 min.

Fig. 28 represents DNA analysis process in the NIDAS using colored dyes. At the beginning of the process, urine sample was applied as followed order (a); i) an optical

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adhesive film was detached from the holes above the powder container and the first reagent reservoir, ii) 10 μl of test sample was introduced to the first reagent reservoir using 10 μl of a disposable pipette, iii) 100 μl of DI water was added to the powder container using 100 μl of a disposable pipette and iv) the holes were again covered with an optical adhesive film at 10 min after injecting DI water. As a result, hydrogen gas was formed by the hydration reaction and the gas started to push reagents in sequence from the mixture of urine sample, lysis buffer and DMA (green colored dye) to elution buffer (red colored dye) (Fig. 28(b)). In spiral channels, the cell lysis and binding of DMA with extracted DNA simultaneously occurred. While the mixture was passed through a long meandering channel, the DNA/DMA complexes were captured by the amine group of APTES treated slide glass. According to the sequential flow, PBS (blue colored dye) was secondly flowed through the long meandering channel to wash away non-specific and unbound molecules. In case of a microchannel which has multi-outlets, flow directions of fluids can be easily controlled by adjusting hydrodynamic resistances ($R = 12\mu\text{l} / wh^3$) among microchannels. In this work, in order to separately guide waste fluids and extracted DNA fluids, I designed two microchannels with different lengths to make the hydrodynamic resistance difference. In addition, by blocking reaction chambers (i.e. outlets of relatively shorter channel), I made to flow two solutions (green and blue colored dyes) toward a waste container as shown in Fig. 28(b-iii). For the next step, in order to collect extracted DNA, I detached an optical adhesive film from the reaction chambers when the waste fluid was completely passed through the meandering channel. Together with higher hydrodynamic resistance of a waste channel, due to higher hydraulic head of the waste fluid in a waste container, an elution buffer was flowed to reaction chambers which have relatively lower pressure through the meandering channel with eluted DNA by breaking the covalent bonds of amine groups between DNA and APTES as shown in Fig. 28(c). During the process, I observed that the eluted DNA fluid was evenly

split to two reaction chambers by the same pressure drop from a junction to outlets of the chambers (Fig. 28(c-ii ~ iv)). Fig. 28(d) shows the operation process of RPA in the NIDAS. For the minimization of thermal losses via air in microchannels, short bridge-channel made of designed optical adhesive films was detached from the reaction chambers (Fig. 28(d-i)) and a slide glass with $22 \times 22 \text{ mm}^2$ area was again attached onto the chambers with a double sided tape (Fig. 28(d-ii)). Finally, by adding 500 μl of water to a water container using a disposable pipette, exothermic reaction of the powder was started (Fig. 28(d-iii)) and DNA was incubated with RPA reagents for 40 min to amplify extracted DNA (Fig. 28(d-iv)).

5.5.4 Performance test of the NIDAS

Figure 29 Image of gel electropherogram result indicating DNA ladder (M), amplified test samples (T1 and T2) and negative control (N).

In order to investigate the performance the NIDAS, RPA of nucleic acids was carried out with EGFR gene using the reagents and protocols described in TwistAmp Basic kit (TwistDX, UK). The detailed process is same as described in Sec. 5.4. Mixtures of RPA reagents with extracted DNAs, T1 and T2, and a solution of RPA reagent without DNA, N, were incubated for 40 min to amplify extracted DNA. T1, T2 and N samples were visualized by electrophoresis technique using 1 % of agarose gel with 1.5 μ l of ethidium bromide (EtBr). Fig. 29 shows the electropherogram result of stained RPA amplicons with EtBr onto agarose matrix according to different sized molecules. For T1, T2 and N lines, band intensities in the position of red box directly represent the quantity of *EGFR* genes in RPA amplicons. Therefore, through the dark band in T1 and T2 lines unlike N line, I could realize that the DNA analysis (extraction, amplification and detection) from urine is successfully performed in the NIDAS.

Chapter 6:

6. Conclusion and Future Work

6.1 Conclusion

The purpose of this dissertation is the development of; i) a microfluidic system to enhance the sensitivity of protein biomarker based biosensors for the protein-based POC diagnosis of infectious diseases and ii) a fully disposable system which consists of a controllable self-powered actuator, DNA extraction channel component, non-instrumented RPA device with colorimetric detection method for the DNA-based POC diagnosis of infectious diseases.

In Chapter 3, I presented the RFF method to enhance the sensitivity of label-free optical biosensors for the detection of low concentration protein biomarkers. The RFF were made by adding a less dense fluid horizontally and the hydrodynamic focused rate can be controlled with changing the volumetric fraction ratio between the target fluid and the less dense fluid. These phenomena were resulting in the enhancement of the binding probability and the sensitivity. For the investigation of fabricated microfluidic devices with the RFF method, CFD simulations, flow experiments, and quantitative measurements were performed. The formation of RFF and behaviors of biomolecules inside a simple T-shaped microchannel were investigated in CFD simulations and fluorescent bead experiments. The CFD simulation confirms the hydrodynamic focusing of target fluids (3/10 of the channel height) when the volumetric flow rate ratio of less dense fluid, ethanol, to the target fluid is fixed to 3:1 which agrees with theoretical expectation. For the quantitative characterization of binding performances, optical measurements of silicon based label-free optical biosensors were

carried out using biotin-Streptavidin as a model antigen-antibody system with different flow methods. The results showed drastic improvement (8-order of magnitudes) in the sensitivity of the biotin-modified microring sensor in the RFF method compared to the SF method, where only target fluid was injected to the microchannel. For both flow methods, the tendency of conjugations between streptavidin and immobilized biotin was logarithmically proportional to the concentrations of Streptavidin solutions. However, the apparent dissociation constants obtained from the Langmuir models were to be $4.12 (\pm 0.53) \times 10^{-7}$ M for the SF method and $2.82 (\pm 3.12) \times 10^{-15}$ M for the RFF method. I conducted a series of experiments including bioactivity testing and comparison with the VFF method, which has purely the height reduction effect and verified the real effect of the RFF method for significant improvement in the sensitivity of the optical sensor. I speculate that the transverse motion of particles by the Dean vortex can be one of the dominant effects to the sensitivity enhancement. Although further investigations are required to proof this new phenomenon theoretically and experimentally, the RFF method offers a simple and effective way to enhance the sensitivity of label-free optical sensor and can be widely applied to any biosensor without requiring additional instruments.

In Chapter 4, I present the development of a self-powered switch-controlled nucleic acid extraction system (SSNES) that can extract DNA from body fluids in 10 min with simple operation steps. The entire system is mainly made of disposable syringes and plastic substrates and could be manufactured at low cost. The SSNES consists of a series of self-powered vacuum actuator and switchgear unit. The syringe based vacuum actuator is used to generate negative pressure to pull samples and reagents through the microchannel. The flow rate and volume of pulled reagent for each unit can be controlled by selecting different size of the syringe and the initial location of the plunger of the vacuum actuator. On-off valve function is provided by a Venus symbol shape of switchgear which also can function as

reagent reservoir and start-button for the vacuum actuator. The end-user can easily operate the SSNES by simple steps of detaching tapes, pushing and pulling switchgears for clinical sample processing. My device has great potential to be used for the POC applications because it is disposable, self-powered, fast, inexpensive and simple to use. Furthermore, its simple structure makes it easy to integrate with other LOC systems for POC molecular diagnostics.

In Chapter 5, I present the development of a non-instrumented DNA analysis system (NIDAS) that enable to carry out total analysis of DNA biomarkers from sample to answer within 1 hour. The entire system is mainly made of disposable plastic substrates, powder mixtures and tapes, thus it has high price competitiveness. All of reagents are inserted to the NIDAS in the fabrication stage. The hydration reaction based self-powered actuator which is located in a foremost side of microchannel is used to sequentially push in pre-inserted reagents through binding zone toward the outlet of microchannel. The flow rates generated by the self-powered actuator is uniform with narrow distribution and its operating time long-lasting enough (over 2 hours) for POC application. On-off valve function is provided by optical adhesive films with attaching to or detaching from the holes (inlets and outlets) of microchannels. In addition, the performance of exothermic reaction based non-instrumented RPA using same powder mixture which is used in the self-powered actuator has been successfully determined. Finally, the NIDAS would be the best solution for the POC total analysis of DNA.

6.2 Future Works

6.2.1 Integration strategy for the establishment of POC protein-based diagnostic system.

For the analysis of protein biomarkers, an optical instrument and active fluid components are necessary. Especially, for the establishment of POC infectious disease diagnostic system, bulky and complex components should be replaced with minimized, non-instrumented or self-powered components.

The POC protein-based diagnostic cartridge would consist of:

- i) The first layer is PDMS microfluidic component which consists of hydration reaction based self-powered actuator and a microchannel designed for the generation of the RFF,
- ii) The second layer is a multiplexed silicon waveguide type biosensor chip that has been developed and fabricated is integrated with the microfluidic layer.
- iii) Inexpensive LED with IC chip are chosen as a light coupling and IC components and implemented to the bottom side of the cartridge.

Finally, the integrated system is designed to be a disposable cartridge which consists of the components with a protector.

6.2.2 Naked-eye detection of the NIDAS's results

For the establishment of POC diagnostic device, the characterization procedure of the device is needed to be performed by naked-eye detection of amplified target DNA. Therefore, I will develop the naked-eye detection method with RPA amplicons and it will be applied to the NIDAS.

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