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Original Article

A Reagent-Ready-on-Chip Microfluidic Immunoassay System for Rapid Detection of Influenza A H1N1 and H3N2 Viruses

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Abstract Development of effective disease screening method is the best approach for the control of infectious diseases. However, conventional screening methods require to operate in well-equipped laboratory. That makes the screening operation expensive and time-consuming. In this work, a reagent-ready-on-chip microfluidic immunoassay system was developed and rapid detection of influenza A H1N1 and H3N2 viruses was demonstrated to show its simplicity and rapidity. The microfluidic system integrated 4 single-stroke thermopneumatic actuators and a reaction chamber. All related reagents were pre-installed in the system and manipulated automatically for indirect immunoassay. Detection of influenza A H1N1 and H3N2 viruses based on its internal structure protein, i.e., nucleoprotein (NP), was demonstrated using the system. Result was represented by colorimetric signal in the reaction chamber. The use of thermopneumatic actuation could achieve fluid manipulation without external assistive equipment and colorimetric result could be observed directly without the need of external dedicated detector. Influenza virus screening could be completed around 1 hour with less sample volume (20 μL) and reagent volume (50 μL). The system achieved one-step operation of immunoassay. It is highly integrated and has potential to be developed to portable devices to realize rapid diagnostics in remote environment and clinics without well-equipped facility.

Keywords: Microfluidic system, Immunoassay, Influenza virus, Portable device, Rapid diagnostics

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Introduction

Influenza viruses are highly infectious and may cause pandemic breakout. Based on the Centers for Diseases Control and Prevention, USA, they can be categorized into 3 types including influenza A, B, and C[1]. Among them, influenza A and B viruses cause seasonal epidemics and respiratory illness, and infection of influenza C virus may only result in minor symptoms. Furthermore, influenza A virus can be subtyped according to its antigenic properties of the outer surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). In recent years, influenza A H1N1 and H3N2 viruses are the major subtypes circulating in humans.

In the past century, millions of people worldwide have been killed by several major pandemic breakouts including Spanish flu (influenza A virus of H1N1 subtype) in 1918-1919[2] and Hong Kong flu (influenza A virus of H3N2 subtype) in 1968-1969[3]. More importantly, up to million people passed away in some pandemic years and that could be attributed to the spread of influenza viruses. Hence, the control of spread of

influenza viruses has become a global health concern. Currently, commercial diagnostic testing kit such as QuickVue Influenza A + B is available for rapid diagnostics; however, it was reported that the sensitivity is poor[4-6]. Therefore, the most reliable and sensitive diagnostic method of influenza virus is based on real-time reverse-transcriptase polymerase-chain-reaction (RT-PCR) currently[7]. The RT-PCR normally takes 6 hours to be completed and requires to be operated in a high standard laboratory. For rapid diagnostic applications, it is more appropriate to the devices that are portable, fast response, simple operation, and inexpensive[8]. Development of the rapid and sensitive diagnostic devices has pressing needs for remote environment and clinics with lack of well-equipped facility[9,10].

Microfluidic systems, also well known as "lab-on-a-chip" or "micro-total-analysis-system", for clinical diagnostic applications has been extensively reported recently[11-14]. They are envisioned as miniaturized platforms integrating with entire bio-analytical protocols traditionally performed in biological laboratories. Recently, detection of influenza viruses has also been demonstrated based on microfluidic technology[15-18]. Microfluidic devices that employ polymerase chain reaction (PCR) have been developed for the identification of the influenza viruses[15,16]. These devices integrated fluidic and thermal components such as heaters, temperature sensors, and addressable valves. Although they provided highly sensitive detection, sample preparation from either clinical or field was a significant issue. Purification of nucleic acid (RNA or DNA) was required before loading sample to these devices. Alternatively, immunoassay is well-recognized serological diagnostic method for the detection of influenza viruses[19-21]. The principle is based on the specific binding feature of an antibody to its antigen. Microfluidic device that employs immunoassay has been demonstrated for the rapid diagnosis of influenza A virus[17]. This device was embedded with a micromixer driven by compressed air; hence, that involved a number of pneumatic connections. Although these excellent developments are much more simplified than traditional bio-analytical instruments, but they are still not readily accessible to untrained personnel and not appropriate for rapid diagnostic applications in remote environment and clinics[22]. For example, users are required to sequentially apply sample and related reagents to the microfluidic devices. Moreover, the operation of immunoassay involves a series of processes such as incubation and washing steps. External fluidic driving mechanisms are commonly required to manipulate reagents in such microfluidic devices[23]. That is not practical for the operation of rapid diagnostic devices.

In this work, a reagent-ready-on-chip microfluidic immunoassay system was developed in order to achieve one-step operation of diagnostics. Rapid detection of influenza A H1N1 and H3N2 viruses was demonstrated to show its simplicity and rapidity. In the operation of immunoassay, each reagent is required to be applied once and a continuous pumping is not necessary. Such that, we packaged all reagents on-chip and each reagent was dispensed once to the reaction chamber in order to eliminate the manual loading of reagents, external fluidic driving mechanisms, complicated tubing connections, significant waste of reagents, and potential contamination. The system integrated 4 single-stroke thermopneumatic actuators and a reaction chamber. Reagents including blocking buffer, primary antibody, secondary antibody, signal amplification reagent were pre-installed in the reagent reservoirs of the thermopneumatic actuators. User only applied sample to the reaction chamber and reagents were sequentially manipulated to the reaction chamber according to the pre-set computer program. Indirect immunoassay was automatically performed and colorimetric result could be obtained in the reaction chamber based on the protocol of using gold nanoparticles (AuNP) and gold enhancement (GE)[24]. Detection of influenza A H1N1 and H3N2 viruses based on its internal structure protein, i.e., nucleoprotein (NP), was demonstrated using the system. Results represented by colorimetric signals showed the influenza virus screening can be quantitatively performed with less sample volume (20 μL), less reagent volume (50 μL), and shorter period of time (around 1 hour). The proposed reagent-ready-on-chip microfluidic system is highly integrated. It has potential to be developed to portable devices to realize rapid diagnostics in remote environment and clinics without well-equipped facility.

Results and Discussion

Fluid Manipulation of the Microfluidic System

The microfluidic system mainly consisted of 4 thermopneumatic actuators and a reaction chamber, as shown in Figure 1. Thermopneumatic actuation is based on air expansion in the sealed air chamber when temperature is elevated. By the ideal gas equation, expanded volume of the air chamber is linearly proportional to the elevated temperature. Hence, fluid encapsulated in the reagent reservoir can be manipulated by the direct control of the driving voltage across the heater. In order to investigate the temperature variations on the mem-

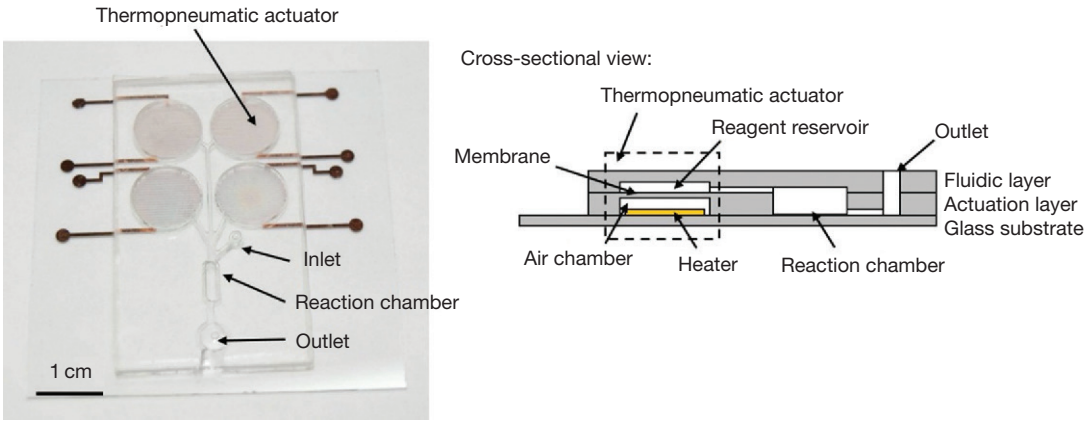


Figure 1. The microfluidic system integrating with 4 thermopneumatic actuators and a reaction chamber. (Left) Photographic image of the microfluidic system. (Right) Cross-sectional illustration of the microfluidic system. The dimensions of the microfluidic chip are listed below. The air chamber was 10 mm in diameter and 700 μm in height. The reagent reservoir was 10 mm in diameter and 700 μm in height. The connecting microchannel was 500 μm in width and 400 μm in height. The reaction chamber was 5 mm in length, 2 mm in width, and 1.4 mm in height. The membrane thickness was 300 μm.

brane, voltages of 17, 20, and 23 V were respectively applied to the heater and temperature variations on the membrane were recorded by an infrared thermometer. Note that the fluidic layer was not bonded to the actuation layer in order to have precise measurement of the membrane temperature. Also, the membrane temperature was not equal to the heater temperature because air and PDMS material are not good at thermal conduction. The objective of the study of temperature variations on the membrane was to understand the temperature affecting the reagent in the reagent reservoir. The temperature responses are shown in Figure 2(a). Obviously, higher driving voltage could generate higher temperature. More importantly, the temperature had instant response and elevated rapidly in the starting 2 min after the voltage application. That indicated large portion of the reagent can be rapidly pumped out from the reagent reservoir after the voltage application and the reagent affected by temperature can be minimized.

The thermopneumatic actuator was evaluated by the measurement of volume flow speed under different driving voltages. The speed was defined as the fluid volume in the reservoir divided by the time required for draining the entire fluid. The experimental result was plotted in Figure 2(b). Higher driving voltage generated higher volume flow speed and the correlation is highly linear with an R-square value of 0.98. Result revealed that PDMS material is appropriate to be used for the frequently deformed membrane.

The microfluidic system consisting of 4 thermopneumatic actuators and a reaction chamber was demonstrated on sequentially manipulating 4 fluids to the chamber. A sequence of photographs was captured and

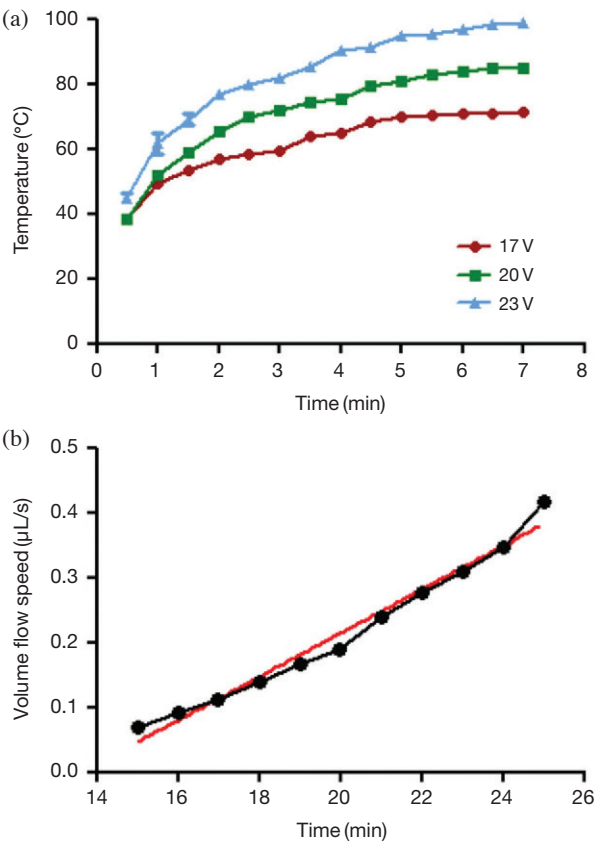


Figure 2. Characteristics of the thermopneumatic actuator. (a) Temperature responses on the membrane of the actuator under different driving voltages, i.e., 17, 20, and 23 V. (b) Correlation between volume flow speed and driving voltage of the actuator. The speed was defined as the fluid volume in the reservoir divided by the time required for draining the entire fluid. The solid line was plotted by linear regressive approximation.

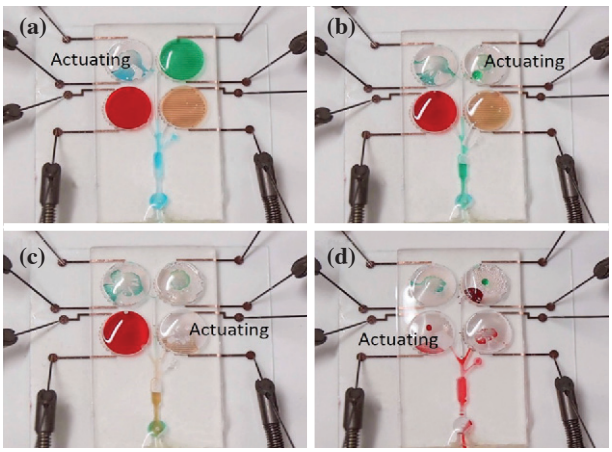


Figure 3. A sequence of photographs showing sequential manipulation of 4 fluids to the reaction chamber from (a) upper left, (b) upper right, (c) lower right, to (d) lower left. The driving voltage of the thermopneumatic actuators was 20 V.

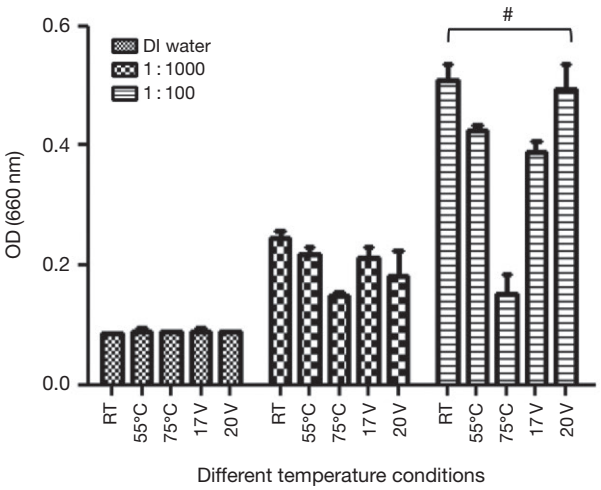


Figure 4. Investigation of the protein binding affinity at elevated temperature. Mouse IgG and anti-mouse IgG conjugated to AuNP were used as antigen-antibody pair. Antigen was applied to the wells of standard 96-wells microplate for immobilization. Then, different concentrations, i.e., 0 (DI water; control), 1 : 1000 (1000 times dilution), and 1 : 100 (100 times dilution), of antibody solutions were treated under different temperature conditions. Five temperature conditions were included and divided into reference group, i.e., RT, 55°C, and 75°C, and study group, i.e., 17 and 20 V. The treated antibody solutions under different conditions were respectively applied to the wells of the microplate. After signal amplification, the binding affinity could be represented by optical density (OD) of the reacted solution. Error bars represent standard deviations of the mean with $n > 3$. The notation of # indicates data without statistical significance ($p > 0.05$) analyzed by one way analysis of variance (ANOVA).

is shown in Figure 3. In order to observe the fluid manipulation process, four colored solutions were respectively loaded in the reagent reservoirs. By applying driving voltage of 20 V across the heater of its actuator, solution was pumped out from the reagent reservoir to the reaction chamber. Based on the pre-set computer program of the voltage application, four solutions were sequentially manipulated by the corresponding thermopneumatic actuators.

Investigation of the Protein Binding Affinity at Elevated Temperature

Because protein degradation may be induced at elevated temperature, protein binding affinity was investigated under different temperature conditions. In the design of the microfluidic system, reagent was stored in the reservoir above the heater. When driving voltage was applied to the heater, noticeable temperature elevation of the reagent was induced and that might influence the property of the protein in the reagent. As discussed earlier, large portion of the reagent can be rapidly pumped out from the reagent reservoir after the voltage application. That can minimize the affection of protein binding affinity. However, protein concentration in the reagent and driving voltage of the heater were optimized in order to further eliminate the affection of protein binding affinity. In this study, mouse IgG and anti-mouse IgG conjugated to AuNP were used as antigen-antibody pair because their binding affinity was confirmed. Antigen was applied to the wells of standard 96-wells microplate overnight for immobilization. Then, different concentrations, i.e., 0 (DI water; Control), 1 : 1000 (1000 times dilution), and 1 : 100 (100 times dilution), of antibody solutions were treated under different temperature conditions. Five temperature conditions were included and divided into reference group, i.e., RT, 55°C, and 75°C, and study group, i.e., 17 and 20 V. In the reference group, antibody solutions in eppendorf were respectively stored at certain temperature for 10 min. In the study group, antibody solutions were respectively collected from the outlet of the thermopneumatic actuator driven by certain driving voltage. Next, the treated antibody solutions under different conditions were respectively applied to the wells of the microplate for 1 h. The GE solution was utilized for signal amplification. The binding affinity could be represented by optical density (OD) of the reacted solution and read on a microplate reader (ELx800, BioTek, USA), as shown in Figure 4. Obviously, in RT condition, higher concentration of antibody generated higher signal. In the reference group, protein binding affinity significantly decreased with temperature ele-

vation from RT, 55°C, to 75°C. That showed temperature elevation highly influences the protein characteristics. In the study group, there was no statistical difference of protein binding affinity in the 1 : 100 antibody solutions between incubated at RT and collected from the actuator driven by 20 V. That implied the antibody solution under such condition was high enough in concentration and fast enough to be pumped out from the reagent reservoir after the voltage application.

Detection of Influenza A H1N1 and H3N2 Viruses

Detection of influenza viruses based on its internal structure protein, i.e., NP, using the microfluidic system was demonstrated to show the practicality. The original virus titer was 2.7×10^6 pfu/mL and serial dilutions of virus were used to show the detection. Sample containing influenza A virus was added to the reaction chamber. Next, blocking buffer, rabbit anti-NP antibody, anti-rabbit IgG conjugated to AuNP, and GE solution were sequentially and automatically manipulated to the chamber for the indirect immunoassay. The driving voltage of the thermopneumatic actuators was 20 V. The immunoassay results of the detection of influenza A H1N1 and H3N2 viruses is respectively shown in Figure 5 and Figure 6. Results indicated that the primary antibody of anti-NP antibody could detect the presence of both influenza A H1N1 and H3N2 viruses. The colorimetric signal increased with the increase of the virus concentration. The entire process of the on-chip indirect immunoassay took around 1 hour with less sample volume of 20 μL and reagent volume of 50 μL.

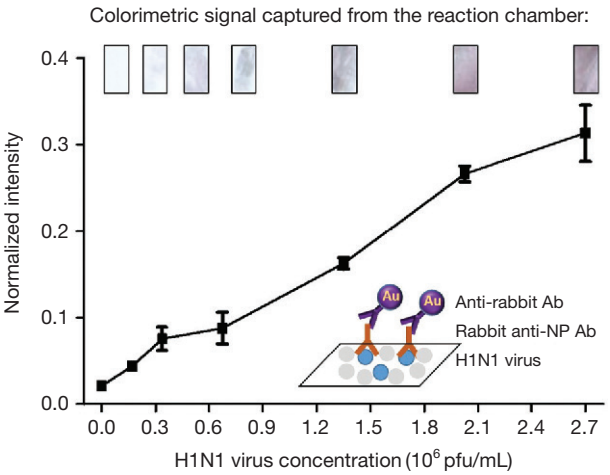


Figure 5. Detection of influenza A H1N1 virus captured by anti-NP antibody. The driving voltage of the thermopneumatic actuators was 20 V. Error bars represent standard deviations of the mean with $n > 3$.

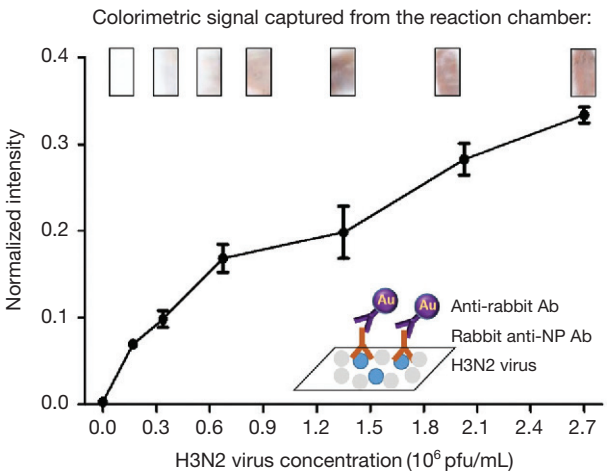


Figure 6. Detection of influenza A H3N2 virus captured by anti-NP antibody. The driving voltage of the thermopneumatic actuators was 20 V. Error bars represent standard deviations of the mean with $n > 3$.

Conclusions

A reagent-ready-on-chip microfluidic immunoassay system was developed and rapid detection of influenza A H1N1 and H3N2 viruses circulating in humans was demonstrated using the system. Reagents were preinstalled in the system and manipulated automatically by on-chip thermopneumatic actuators. Indirect immunoassay was performed to detect influenza viruses. Result was represented by colorimetric signal in the reaction chamber. The system could achieve one-step operation of immunoassay which is important to untrained personnel for rapid diagnostic applications in remote environment and clinics. Influenza virus screening could be completed around 1 hour with less sample volume (20 μL) and reagent volume (50 μL). The reagent-ready-on-chip microfluidic immunoassay system is highly integrated and has potential to be developed to portable devices to realize rapid diagnostics in remote environment and clinics without well-equipped facility.

Materials and Methods

Chemicals and Reagents

Primary antibody, i.e., rabbit anti-NP antibody, was raised in-house and kindly provided by Prof. Shin-Ru Shih at Chang Gung University. Secondary antibody,

i.e., anti-rabbit IgG conjugated to AuNP and anti-mouse IgG conjugated to AuNP, mouse IgG, and 3-aminopropyltriethoxysilane (APTES) were purchased from Sigma, USA. GE solution for the development of AuNP was purchased from Nanoprobes, USA. Glutaraldehyde (GA) was purchased from Bionovas, Canada. The washing buffer was phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) with 0.05% v/v Tween 20. The blocking buffer consisted of 0.05% v/v Tween 20 in 1 × PBS with 1% w/v fat-free milk powder. All experiments were performed at room temperature (RT; 22-25°C) if there is no particular statement.

Preparation of Influenza Viruses

Two strains of influenza A viruses including a laboratory strain of A/Puerto Rico/8/34(H1N1) and a clinical strain of A/Taiwan/3446/02(H3N2) (originally isolated by Department of Laboratory Medicine, Chang Gung Memorial Hospital, Linkou, Taiwan) were amplified by either 10-day embryonic eggs or Madin-Darby canine kidney (MDCK) cells. The amplified virus stocks were titrated by plaque formation assay with MDCK cells. To disrupt the viral particles, the virus stocks were treated in 0.2% NP40 solution and kept at 4°C for 10 min. The indicated amounts of treated influenza virus stocks were then applied to the microfluidic system for further experiments.

Design of the Reagent-ready-on-chip Microfluidic Immunoassay System

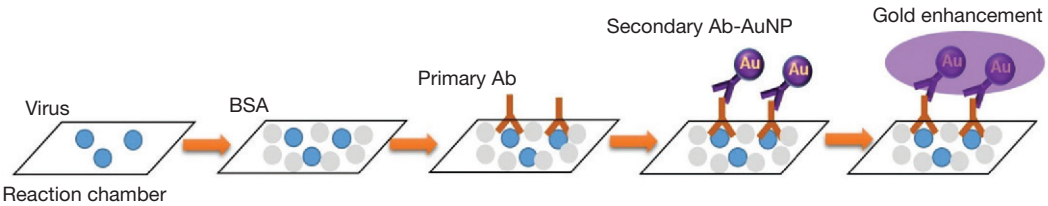
The microfluidic system mainly consisted of 4 thermopneumatic actuators and a reaction chamber. The system was assembled by 3 layers named as glass substrate, actuation layer, and fluidic layer. Four copper heaters (Cr/Cu: 500/2000Å) were fabricated on the glass substrate by standard microfabrication technique including metal deposition and lift-off processes. The actuation and fluidic layers were made of polydimethylsiloxane material (PDMS; Sylgard® 184, Dow Corning, USA) and fabricated by soft lithography. The microfluidic system was assembled by bonding 3 layers together using oxygen plasma.

A thermopneumatic actuator consisted of a copper heater, an air chamber (Ø: 10 mm; Height: 700 μm), a membrane (Thickness: 300 μm), a reagent reservoir (Ø: 10 mm; Height: 700 μm), and a connecting microchannel (Width: 500 μm; Height: 400 μm). The actuation mechanism using thermopneumatic principle can generate large membrane movement to induce pressure for fluidic manipulation with relatively low driving voltage and simple driving electronics[25-27]. Before the experiment, reagent was pre-installed in the reagent reservoir. When a voltage was applied to the heater, temperature in the air chamber increased to induce expansion of the chamber. Then, the membrane was deformed and squeezed the reagent in the reagent reservoir. Hence, the outflow volume of the reagent was the expanded volume of the air chamber. Consequently, the reagent was pushed to the reaction chamber through the microchannel. The actuator was used for single-stroke manipulation of reagent from the reagent reservoir to the reaction chamber.

The reaction chamber (Length: 5 mm; Width: 2 mm; Height: 1.4 mm) was the site for performing indirect immunoassay. After surface modification of the glass surface of the chamber, sample containing influenza virus was manually applied to the chamber. Then, reagents including blocking buffer, primary antibody, secondary antibody, GE solution were sequentially introduced by the thermopneumatic actuators according to the pre-set computer program. In between each application of reagent, washing buffer was applied by the syringe pump from the inlet to wash the reaction chamber. Consequently, colorimetric result of the indirect immunoassay could be obtained in the reaction chamber. In our design, we packaged all reagents on-chip in order to enhance the practicality. Since washing buffer is not assay-dependent and the usage volume is large for each assay, storage externally can reduce the size of the microfluidic system. Hence, user is required to apply sample to the reaction chamber only. The remaining protocol will be handled automatically in order to achieve one-step operation of diagnostics. That is practical for untrained personnel and is appropriate for rapid diagnostic applications in remote environment and clinics without well-equipped facility.

Surface Modification of Glass Surface for Protein Immobilization

Glass surface of the reaction chamber was utilized as the site for indirect immunoassay. In order to securely capture protein, surface modification is necessary to activate functional groups of the glass surface. The protocol followed previously reported method[28] and is briefly described. The glass substrate was cleaned in a 70 : 30 (v/v) mixture of H_2SO_4 and H_2O_2 for 30 min. After washing several times with deionized (DI) water and drying in oven at 80°C, the glass substrate was bonded with the actuation layer. Then, 5% (v/v) APTES in acetone was applied to the glass surface of the reaction chamber for 30 min. Next, it was rinsed thoroughly in acetone and distilled water, dried in nitrogen flow,



Scheme 1. Protocol of the indirect immunoassay.

and baked in oven at 80°C for 2 h. The APTES-treated glass surface became hydrophobic, supporting the fact that the surface contact angle was around 60°[29]. Then, 2.5% (v/v) GA in PBS was applied to the APTES-treated glass surface of the reaction chamber for 2 h. The GA reacted with amino group on the glass surface and worked as a cross-linker between the protein and glass surface. After carefully rinsing with distilled water and drying, the fluidic layer was bonded with the actuation layer to construct the microfluidic system.

On-chip Indirect Immunoassay

Before performing the indirect immunoassay, four reagents including blocking buffer, primary antibody, secondary antibody, and GE solution were respectively installed in 4 on-chip reagent reservoirs by using syringes manually. Adhesive tapes were attached and sealed the injection holes in order to prevent leakage and evaporation. Then, the reagent-ready-on-chip microfluidic immunoassay system was ready for the rapid diagnostics.

The protocol of indirect immunoassay is illustrated in Scheme 1. User applied 20 μL sample containing influenza virus to the reaction chamber through the inlet of the system and the sample was incubated for 15 min. Influenza virus could be covalently immobilized on glass surface via the GA cross-linker. Blocking buffer was then manipulated by the thermopneumatic actuator to the chamber and incubated for 15 min. The active site on the glass surface was blocked in order to reduce the non-specific signal. Next, reagents containing primary antibodies, secondary antibodies, and GE solution were sequentially manipulated to the chamber and incubated for 15 min each. In between each manipulation of the reagents, washing buffer was applied to the chamber by an external syringe pump and washed thoroughly. Hence, result of the indirect immunoassay was represented by the colorimetric signal of the reaction chamber.

Conventionally, enzyme and its reactive substrate are used for the signal amplification of the immunoassay result, e.g., horseradish peroxidase (HRP) and tetramethylbenzidine (TMB). This amplification system may have high sensitivity, but enzymatic activity is likely to be degraded because any slight change in their conformation would severely impact its function. The objective of this work is to develop a rapid diagnostic device for remote environment and clinics without well-equipped facility. The use of enzyme for signal amplification may not be appropriate for such purpose. In contrast, AuNP is a kind of metallic nanoparticles and provides stable property and long shelf life. To generate detectable colorimetric signal, GE solution was used to enlarge the AuNP physically. The enlarged AuNP became visible and the immunoassay result could be directly represented by the color intensity. Comparatively, AuNP/GE system was suggested to provide a more robust signal amplification and be appropriate for rapid diagnostic applications.

Analysis of the Immunoassay Results

The colorimetric signal of the immunoassay was captured by a regular camera and analyzed by ImageJ computer software. Eight-bit grayscale image was used to quantify the gray level of the colorimetric signal. The immunoassay result was represented by normalized intensity:

$$Normalized\ intensity = \frac{255 - graylevel}{255}$$

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