Lab on a Chip

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Influenza A Virus-Specific Aptamers Screened by Using an Integrated Microfluidic System

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The influenza A virus is a notorious pathogen that causes high morbidity, high mortality, and even severe global pandemics. Early and rapid diagnosis of the virus is therefore crucial for preventing
10 and controlling any influenza outbreaks. Recently, novel nucleic acids-based affinity reagents called aptamers have emerged as promising candidates in diagnostic assays, as they offer several advantages over antibodies, including *in vitro* selection, chemical synthesis, thermal stability and realtively low costs. Aptamers with high sensitivity and specificity are generated via Systematic Evolution of Ligands by Exponential Enrichment (SELEX), a process that is currently time-
15 consuming, as well as labor- and resource-intensive. In this study, an integrated microfluidic system was developed and was successfully applied to screen a specific aptamer for the influenza A/H1N1 (InfA/H1N1) virus in an automated and highly efficient manner. The selected aptamer was implemented in a magnetic-bead assay, which demonstrated specific and sensitive detection of InfA/H1N1 virus, even in biological samples such as throat swabs. Consequently, this specific
20 aptamer presents a promising affinity reagent for the clinical diagnosis of InfA/H1N1. This is the first demonstration for screening influenza virus-specific aptamers using the microfluidic SELEX technology, which may be expanded for the rapid screening of aptamers against other pathogens for future biomedical applications.

Introduction

25 Of the causative agents of infectious diseases, such as viruses, bacteria, fungi, and parasites, viruses are particularly notable in their ability to cause severe diseases and even global pandemics because viruses are readily transmissible from person to person through aerosolized secretions or bodily contact. In particular, the
30 influenza virus, which causes an upper respiratory illness that may lead to bronchitis, pneumonia, meningitis, or even death [1], is notorious for its high morbidity and mortality and ranks as one of the most contagious diseases. For example, the influenza virus was responsible for historic pandemics such as the Spanish Flu,
35 which resulted in 40-million deaths from 1918 to 1920 [2], the Hong Kong Flu, which caused 1-million deaths during 1968-1969 [3], and the more recent H5N1 bird Flu outbreak that occurred in 2004 [4]. In general, 250,000 to 500,000 people around the world die from influenza infection each year, and this figure can
40 potentially rise to the millions during pandemic years [5]. The influenza virus is generally classified as type A, B or C according to their nucleoproteins (NP) and matrix proteins (M1) [6]. Within influenza types, type A is further classified in subtypes by their 17 variations of the hemagglutinin proteins (HA) and 10

45 variations of the neuraminidase proteins (NA) [7]. In April 2009, a novel influenza strain emerged in several countries that was a swine-origin influenza (SOIV) and incorporated partial genes from pigs, birds, and humans [8]. This is known as the 2009 pandemic influenza A (H1N1) and immediately become a
50 worldwide public health concern. After the H1N1 2009 influenza virus pandemic outbreaks, the general public began to take note on the impact that viruses have in causing serious outbreaks of which influenza infections are the most prevalent. Therefore, it is essential to quickly and accurately diagnosis influenza to prevent
55 and control the spread of potentially pandemic-causing strains for many health center for disease control (CDC).

Current diagnosis for an influenza infection ranges from viral culture to serological, antigen or nucleic acids detection. Among these methods, the detection of the virus antigen using
60 influenza-specific antibodies, such as the immunofluorescence test (IFT), the enzyme-linked immunosorbent assay (ELISA), and rapid diagnostic kits for influenza infections are the most commonly adopted methods in hospitals and research laboratories for the detection of Influenza viruses in clinical samples [9,10].
65 Despite their usefulness, the antibodies used in these methods suffer from several drawbacks, such as sensitive to changes in

temperature and humidity, poor batch-to-batch reproducibility, and high risks for cross-reactions [11]. Additionally, recent research indicates that the diagnosis of influenza A/H1N1 virus using clinical specimens in antibody-based rapid test kits have demonstrated the broad range of sensitivity from 10 to 69% [12, 13]. Therefore, a more robust and reliable affinity reagent is critically needed in order to fulfill the purpose of diagnosing Influenza, as well as controlling and preventing another Influenza pandemic.

Aptamers are single stranded RNA or DNA oligonucleotides that can bind to and recognize various kinds of targets ranging from small molecules, proteins, to whole cells with high specificity and affinity [14]. The screening process is known as Systematic Evolution of Ligands and Exponential Enrichment (SELEX), which is an "*in vitro*" selection process [15]. This technology was first developed in the 1990's, which performs the simultaneous screening of more than 10^{15} individual nucleic acid molecules [16]. Aptamers have been viewed as effective substitues for antibodies, with properties that help overcome the disadvantages of antibodies. For example, one of the main advantages of aptamers is that they could be selected by an in vitro process, which can be performed accurately and reproducibly by obviating living organisms. Furthermore, they also possess a higher affinity and specificity, a thermal stability, could be rapidly chemically synthesized with dramatically lower costs, and may induce minimal immune responses. More importantly, the high purification of apatamers can be produced and may decrease the batch-to-batch variations within high yields [17]. Due to these characteristics, aptamers appear to be an excellent candidate for replacing antibodies in diagnostics and therapeutic technologies [18]. However, the traditional SELEX process requires skilled technicians performing 10 to 20 rounds of time-consuming and labor-intensive tasks in specialized laboratory settings. Therefore, there is a need for the development of a new platform that can perform the entire SELEX process within a shorter period of time to facilitate the rapid screening of novel aptamers [19].

Recently, microfluidic technology has been explored with the aim to miniaturize biomedical and chemical analysis systems and has demonstrated superior performance to its large-scale counterparts [20]. Currently, micro-scale bio-systems have become available to perform several crucial operations on a single, integrated microfluidic device including sample pretreatment, transporting, mixing, performing reactions, separation and detection [21,22]. For example, a widely used application in biotechnology is a magnetic bead-based system that can immobilize either target molecules or affinity reagents on the surfaces of beads. These magnetic beads can be easily manipulated, sorted, and concentrated within a microfluidic chip. For instance, microfluidic devices comprised of both a magnetic bead-based module and a microfluidic control module to screen specific ligands against a particular target has been explored [23]. This new screening process utilizes magnetic beads conjugated with specific proteins that incubate with the random, single standed DNA (ssDNA) pool. Applying an external magnetic field gradient allows the aptamers-magnetic bead complexes to be efficiently extracted from whole ssDNA pool [24]. Recent studies have shown that the magnetic bead-based approach implemented on microfluidic systems has significantly improved the sensitivity and specificity of the screening process [25]. Moreover, fast screening of aptamers could be successfully demonstrated [26]. Taking advantage of the benefits from miniaturization and from microfluidic chips that integrate the SELEX process could lead to clinically relevant application in diagnostics. This study therefore presents an integrated, magnetic bead-based microfluidic system for the screening of specific aptamers for the infA/H1N1 virus. The selected aptamers show the potential for better-performing affinity reagent in the diagnosis of an influenza infection. This systems integrates the entire SELEX process by combining aptamer incubation, extraction and nucleic acid amplification processes. All the fluidic samples were mixed rapidly and manipulated precisely in an automatic manner, and selected aptamer can be easily coupled in an assay to detect infA/H1N1 with high sensitivity and high specificity from clinically relevant matrices.
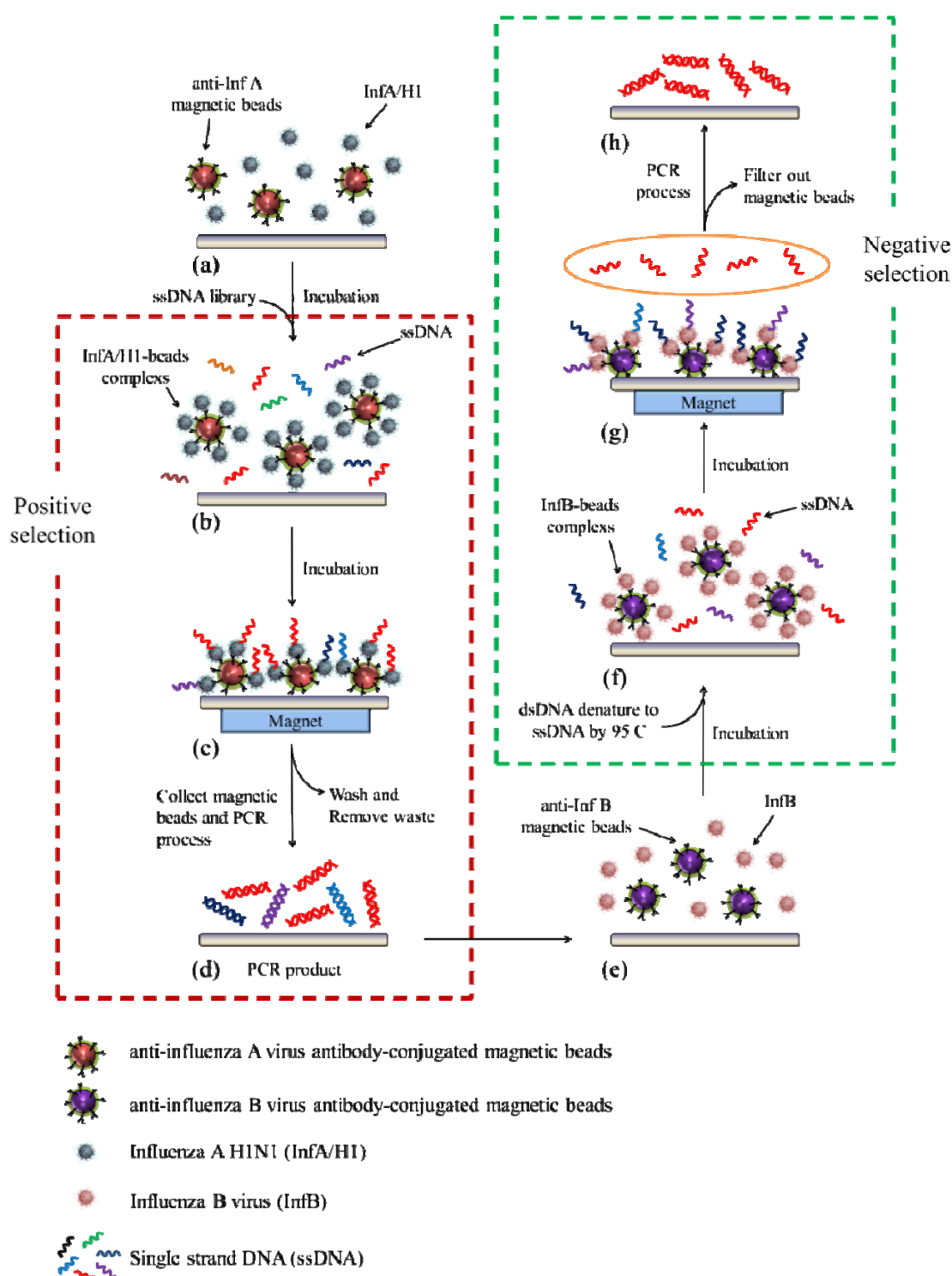
Fig. 1: A schematic illustration of the screening process for specific aptamers performed on the integrated SELEX microfluidic chip. For positive selection, the ssDNA library was adhered to the InfA/H1N1 to form aptamers-virus-beads complexes. Then, non-bound ssDNA was removed using a washing buffer. Captured ssDNA were collected using magnetic beads and amplified by PCR. For negative selection, the other types of influenza virus such as InfB, along with anti-NP-B mAb magnetic beads to form InfB-beads complexes. The PCR products were further incubated with InfB-beads complexes to filter out the ssDNA that could bind to both InfA/H1N1 and InfB. The supernatant of non-captured ssDNA was then amplified by PCR for the subsequent selection.

Materials and methods

Overview of microfluidic SELEX against influenza A/H1N1 Virus. In this study, an integrated microfluidic system was developed to perform the entire SELEX process for
5 screening specific aptamers for InfA/H1N1. The microfluidic SELEX presented in this work entailed a system consisted of several modules on an integrated SELEX chip, which included a microfluidic control module (composed of micropumps, micromixers and microvalves), a magnetic
10 bead-based aptamer extraction module, and a polymerase chain reaction (PCR) module for incubation, extraction and amplification processes. In addition, to obtain highly specific aptamer for InfA/H1N1, the microfluidic SELEX presented in this work alternated five rounds of positive and
15 negative selections, as shown in Figure 1. The ssDNA library pool contains a 40-nucleotide (nt) random sequence in the center flanked by two 16-nt PCR primer regions (5'-GGCAGGAAGACAAACA-N40-GGTCTGTGGTGCTGT-3', UNI-ONWARD CO., Hsinchu, Taiwan). To initiate the
20 positive selection, the target InfA/H1N1 virus (64 hemagglutinin unit (HAU), 10 µL) was first captured onto specific anti-nucleoprotein (NP) -A monoclonal antibody (mAb)-conjugated magnetic beads (4×10^8 beads/mL, 10 µL) to form virus-bead complexes. The ssDNA library (10 µM,
25 10 µL) was then incubated with virus-bead complexes in the incubation chamber of the SELEX chip. During the incubation, the mixture was mixed by utilizing a suction-type micromixer (described in the following section). InfA/H1N1-specific aptamers were captured by the virus-bead
30 complexes for the positive selection. The virus-bead complexes, along with bound ssDNA, were then collected by applying a magnetic field at the bottom of the incubation chamber, while unbound ssDNA molecules were washed away by a washing buffer ($1\times$ phosphate buffered saline (PBS), pH 7.4, 100 µL)
35 during a 5-minute washing step by activating a suction-type, pneumatic micropump. Next, the captured ssDNA was transported by the micropump to the PCR chamber of the SELEX chip for amplification, which completed a round of positive selection. For the negative selection, the amplified
40 products were incubated with other types of influenza viruses such as influenza B (InfB, 64 HAU, 10 µL) coated on magnetic beads ($4\times10^8$ beads/mL, 10 µL). After incubation, the captured ssDNA was pelleted using a magnetic field. The supernatant of non-captured ssDNA was then transported into
45 the PCR chamber for amplification. With alternating positive and negative selection processes, the screened aptamers exhibit high affinity and high specificity to the target virus (InfA/H1N1).

50 **Chip design.** In order to perform microfluidic SELEX, several modules were integrated within the microfluidic chip, including suction-type, pneumatic micropumps and micromixers, microvalves, loading chambers, a PCR module, and a magnetic bead-based aptamer extraction module. As schematically
55 shown in Figure 2a, the microfluidic SELEX chip contained several chambers for PCR reagents, influenza virus, double-distilled water (ddH$_2$O), waste, and washing buffer ($1\times$ PBS).
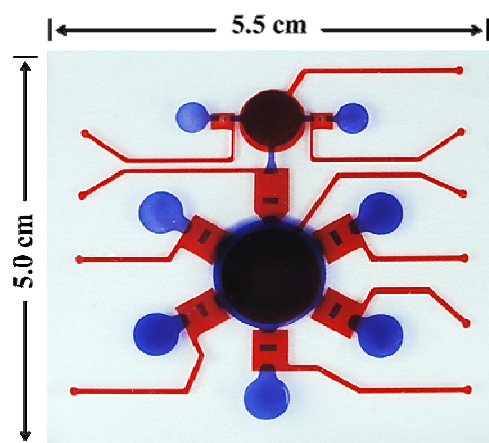
(a)
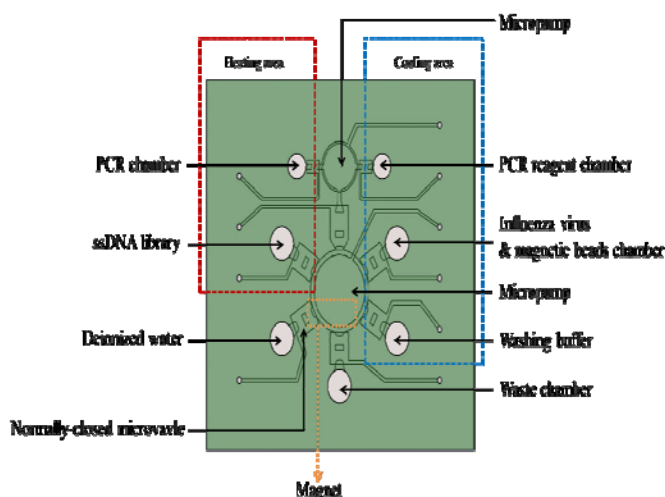
60
(b)
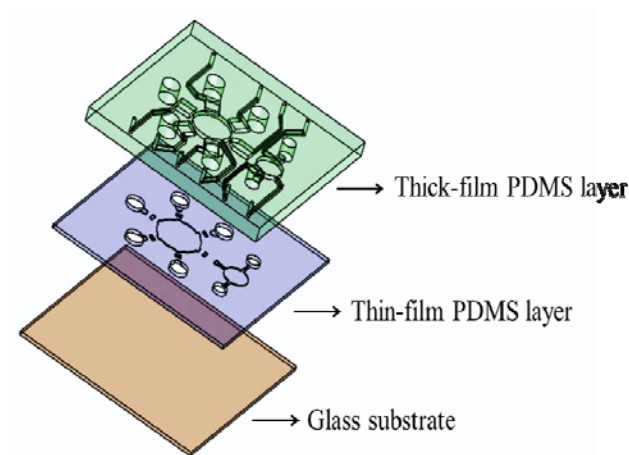
(c)

Fig. 2: (a) A schematic illustration of the integrated microfluidic chip. (b) An exploded view of the integrated microfluidic chip. It consisted of two PDMS layers and one
5 **glass substrate. (c) A photograph of an assembled integrated microfluidic SELEX chip. The dimensions of the chip are measured to be 5.5 cm × 5.0 cm.**

This chip also contained a PCR reagent chamber, which allows
10 one to perform extraction of selected ssDNA and amplification of nucleic acids. This microfluidic SELEX chip consisted of two polydimethylsiloxane (PDMS, Sylgard 184A/B, Dow Corning Corp., USA) layers that include a thin-film liquid channel layer and a thick-film air chamber layer. A glass
15 substrate (0.7 mm, G-Tech Optoelectronics Corp., Taiwan) was used as the base for the microfluidic chip and was bound with the two PDMS layers (Figure 2b). The microfluidic control module consisted of membrane-type micropumps/micromixers and the normally-closed microvalves automatically performed
20 the SELEX process [27]. In addition, an external temperature controller was used to perform PCR. Note that two Peltier coolers were placed underneath the microfluidic SELEX chip to perform PCR and to avoid degradation of the PCR reagents in this study. Accordingly, the microfluidic chip was divided
25 into two regions; the heating area provided accurate thermal control for PCR and the cooling area helped storage of reagents. The pneumatic micropumps/micromixers were activated by applying the negative gauge pressure that produces a suction force injection into the air chamber, which actuated the
30 pneumatic devices for delivery and mixing of liquid or sample [28]. Additionally, the normally-closed microvalves prevented fluid backflow during the SELEX process. It consists of a floating-block structure with a membrane that

resides inside the sample fluid microchannel and an air
35 chamber placed over the microchannel [28]. These generate the memebrane deflection such that the sample fluid flows through the microvavles in only one direction and thus prevents backflow. Detailed information about the operating principle of the micropumps and the micromixers can be found
40 in the Supplementary Information (Supplementary Figure 1).

Fabrication of the microfluidic chip. The microfluidic chip was fabricated using the soft lithography microfabrication process (34). Briefly, the master molds for casting the thick
45 PDMS layer and the thin PDMS layer were first formed on polymethymethacrylate (PMMA) substrates using a computer-numerical-control (CNC) machine (EGX-400, Roland Inc., Japan) equipped with a 0.5-mm-diameter drill bit [29]. All the microfluidic structures were replicated using a PDMS casting
50 technique form the molds on PMMA. First, the elastomer (Sylgard 184A silicon elastomer, Dow Corning, USA) and curing agent (Sylgard 184B elastomer curing agent, Dow Corning, USA) of the PDMS were mixed well in a 10:1 ratio by weight and then was placed in a vacuum chamber for 30
55 minutes to remove any air bubbles generated during the mixing process. The well-mixed PDMS was poured onto the master mold of PMMA substrates, and cured at 80°C for 2 hours to completely form the microfluidic structures and entirely evaporate the solvent. Finally, the PDMS layers and the glass
60 substrate were surface-treated with oxygen plasma and manually bonded to form the complete microfluidic chip [29]. The dimensions of the microfluidic SELEX chip were measured to be 5.5 cm in length, 5.0 cm in width, and 0.5 cm in depth (Figure 2c).

65

The suction-type microfluidic control module. A suction-type micropump/micromixer is essential in this work because it enables the rapid delivery and mixing of variable amounts of biological samples and reagents. There are
70 several advantages for the suction-type micropumps/micromixers, such as the prevention of cross-contamination and the increased pumping rate under the higher back pressure [30]. The upward deflection of the PDMS membranes along the microchannel is due to the
75 supplied negative gauge pressure into the air chamber, and the pneumatic-like effect generates the fluid flow through the

microchannel. Hence, the performance of the micropump is essential to drive the liquid forward within the integrated microfluidic chip. In this work, the volume of ddH$_2$O would be transported by the applied air pressure difference of the
5 suction micropump for calibrating the pumping rate. After observation and calculation, the pumping rate would be determined in the integrated microfluidic chip. In this study, the microfluidic SELEX chip also required an active micromixer that performs the incubation process for influenza
10 virus, ssDNA library and magnetic beads. Therefore, another suction-type, pneumatic micropump was also used as a micromixer when negative gauge pressure was supplied to the normally-closed microvalve and the PDMS membranes (Supplementary Figure 1). An optical image of mixed dyes and
15 deionized (DI) water was observed using a high-speed charge-coupled-device (CCD) camera (MC1311, Mikrotron, Germany) to characterize the mixing performance. Two solutions containing 100 µL of DI water and 20 µL of red ink were first loaded into the reagent chambers. After applying negative
20 gauge pressure, the normally-closed microvalve and the PDMS membranes of the micropump were deflected up to drive the DI water and red ink into the mixing chamber for mixing. Then, the negative gauge pressure (air gauge pressure: -70 kPa) was supplied to the air chamber through the connecting air channel
25 at a driving frequency of 1 Hz for the PDMS membrane deflection to trigger flow transportation. Consequently, the DI water and red ink were mixed completely within 2 s. In order to estimate the mixing performance, the captured images were analyzed using a digital image processing technique. The
30 mixing index of the micromixer calculated from the measured concentration profile across the mixing chamber in the horizontal (X) planes, is defined as follows [31]:

$$\sigma(A) = \left(1 - \frac{\int_A |C^+ - C_\infty^+| dA}{\int_A |C_0^+ - C_\infty^+| dA}\right) \times 100\%$$

(1)

In this formula, $\sigma(A)$ is the mixing index considering the local normalized concentration (C^+) across the area of the
35 mixing chamber (A). C_0^+ is the initial normalized concentration, where C_0^+ is 0 for completely unmixed and 1 for completely mixed. C_∞^+ denotes the completely mixed state, equating to 0.5 in the concentration profile. Samples that are

mixed completely have a value of σ that is set to 100%. In
40 contrast, a σ of 0% reprensents that the samples are completely unmixed.

Preparation of virus strains. In this study, inactivated influenza A/H1N1 virus (InfA/H1N1, 97N510H1), and
45 influenza B virus (InfB, 94N399IB) were obtained from The Department of Microbiology and Immunology, National Cheng Kung University, Taiwan. All protocols for collecting influenza viral samples from patients could be found in a previous work [34]. Both of these viruses were cultured in Mardin-Darby
50 canine kidney (MDCK) cells. The culture medium supernatant would be harvested to collect the virus particles and frozen at -80°C until used. The initial titers of both influenza A and influenza B virus stocks were 64 HAU. Subsequently, these two types of viruses were used in the microfluidic SELEX
55 process. Other inactivated viral strains, including influenza A/H3N2 virus (InfA/H3N2, A/California/7/2004), dengue virus type 2 (DENV-2, PL046), and enterovirus type 71 (EV-71, MP4), were prepared and generously provided by the Department of Microbiology and Immunology, National Cheng
60 Kung University, Taiwan. The initial titer and concentrations of these three virus strains were 64 HAU, 5×10^6 plaque-forming unit (PFU), and 2×10^7 PFU, respectively. These viral strains were used for verifying the specificity of the influenza A/H1N1 virus-specific aptamer.

65

Antibodies and immunomagnetic beads. In order to capture different types of influenza viruses, primary mouse anti-influenza A virus nucleoprotein monoclonal antibodies (anti-NP-A mAb, H16L-10-4R5 cell line (HB-65), ATCC, USA) and
70 primary mouse anti-influenza B virus nucleoprotein monoclonal antibodies (anti-NP-B mAb, influenza B nucleoprotein (B017), GeneTex Co., USA) were utilized in this study [33]. These anti-NP-A/ or B mAbs were conjugated on epoxy-coated magnetic beads (diameter (Ø) of the beads = 4.5
75 µm, Dynabeads® M-450 Epoxy, Invitrogen Co., USA) for screening specific InfA/H1N1 aptamers during the microfluidic SELEX process. Note that the surface of the magnetic beads were coated with an epoxy amino group, thiol and hydroxyl functional groups, which were used to conjugate with the
80 specific anti-NP-A/ or B mAbs (Department of Microbiology and Immunology, National Cheng Kung Universuty, Tainan,

Taiwan). Detailed information about the operating principle of conjugation protocol can be found in the Supplementary Information.

PCR protocol for the microfluidic SELEX. The extracted ssDNA pool from each repeated SELEX round was amplified by on-chip PCR. The PCR mixture reaction contained 5 μL of extracted ssDNA pool from the SELEX process, 3 μL of 10×X PCR buffer (JMR Holdings Co., UK), 0.25 μL of 25 mM MgCl$_2$, 0.5 μL of 2.5 mM dNTPs, 0.5 μL of Superthermo Gold Taq DNA polymerase (5 U/μL, JMR Holdings Co., UK), and specific SELEX primers, which included 0.5 μL of SELEX forward primer (5'-GGCAGGAAGACAAACA-3', final concentration= 0.5 μM), 0.5 μL of SELEX reverse primer (5'-ACAGCACCACAGACCA-3', final concentration = 0.5 μM). PCR was performed at 95°C for 10 minutes for initially denaturing the double stranded DNA, and followed by 20 cycles of three steps, including denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. A final extension step was held at 72°C for 10 minutes for final elongation. The PCR products were visualized using polyacrylamide gel electrophoresis (PAGE), stained with ethidium bromide, and visualized in an ultraviolet (UV) image staining.

Flow cytometric analysis. A flow cytometry-based technique was used to determine the binding affinity for the selected aptamer. Specifically, various concentrations of fluorescein isothiocyanate (FITC)-labeled aptamers (Protech Technology Enterprise Co., Taiwan) were incubated with InfA/H1N1 virus (64 HAU, 10 μL) at 25°C for 30 minutes in darkness; then the affinity of virus-aptamer complexes were quantified by flow cytometry (Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA). All of these experiments were repeated three times. After subtracting the mean fluorescence value of the background fluorescence signal, the percent gated fluorescence signal above the background signal of virus-aptamer complex was used to calculate the equilibrium dissociation constants (K_d) of the InfA/H1N1-specific aptamer. The K_d was calculated by using GraphPad Prism 5.0 software according to the percent gated fluorescence signal of specific binding on the concentration of aptamers with the one site-specific binding

equation, $Y = BmaxX / (K_d + X)$, where the Y represents the bound fraction, the $Bmax$ is the saturated binding, and the X is the concentration of ligands [34].

Aptamer-based InfA virus detection assay. The successfully selected InfA/H1N1-specific aptamer was amine-modified (Protech Technology Enterprise Co., Taiwan) and conjugated onto carboxylic acid coated magnetic beads (1-μm diameter, Dynabeads® MyoneTM Carboxylic Acid, Invitrogen Co., USA). Detailed protocol for preparation of the influenza A/H1N1-specific aptamer conjugated magnetic beads can be found in the Supplementary Information. For the detection assay, 10 μL InfA/H1-specific aptamer conjugated magnetic beads (4×10^8 beads/mL) were incubated with 10 μL target virus or non-targets (InfA/H3N2 virus, InfB virus, DENV-2 virus, EV-71 virus, and human white blood cells (WBC, GAPDH)) in various biological matrices (1× PBS buffer, throat swabs, human sputum, and human serum) along with 80 μL ddH$_2$O in eppendorf tubes. The samples were mixed on a wheeling rotator (Intelli-Mixer RM-2L, ELMI Ltd, Latvia) at room temperature and 25 rpm for 30 minutes, allowing the target virus to be captured by the aptamer. Subsequently, the samples were magnetically trapped and washed by ddH$_2$O to remove non-reactant. Captured virus-aptamer-bead complexes were transferred into PCR tubes and heated at 95°C for 5 minutes for virus lysis. After lysing, reverse-transcription polymerase chain reaction (RT-PCR) reagents (KK4660 KAPA SYBR® FAST One-Step qRT-PCR kit, ABI Prism™ Co., USA) were then loaded into PCR tubes and a commercial PCR machine (Applied Biosystems® 2720 Thermal Cycler, USA) to perform one-step reverse-transcription and PCR amplification of released viral RNA. The 20 μL RT-PCR mixture was composed of 5 μL of magnetic bead-captured viral particles or tested WBC, 0.4 μL of deoxyuridine triphosphate (dUTP, 10 mM), 0.4 μL of KAPA RT Mix (50×), 10 μL of KAPA SYBR FAST qPCR Master Mix (2×), 0.4 μL of each forward and reverse primer for various targets [32,35-37], and 3.4 μL of ddH$_2$O. Primer sequences for RT-PCR are listed in the Supplementary Information (Supplementary Table 1). The RT-PCR process started with the complementary DNA (cDNA) synthesis step at 42°C for 5 minutes, followed by the thermocycling process. This included an initial denaturing step at 95°C for 5 minutes to denature the double strand DNA and

40 cycles of denaturing at 95°C for 3 s, annealing at 58°C for 20 s, and extension at 72°C for 1 s. The final extension step was carried out at 72°C for 7 minutes to finish the RT-PCR process. In order to confirm the accuracy for RT-PCR, purified

5 RNA or DNA control samples were performed concurrently with the tested virus or WBC samples. All viral RNA was extracted and purified from viruses via commercially available purification kits (PureLinkTM Viral RNA/DNA Mini Kit, Invitrogen Co., USA). Likewise, human chromosomal DNA

10 was extracted and purified from human WBC using commercial kits (PureLinkTM Genomic DNA Mini Kit, Invitrogen Co., USA) to serve as positive controls. The concentration of total RNA for InfA/H1N1, InfA/H3N2, InfB, DENV-2, EV-71, and human chromosomal DNA were

15 measured as 5.56, 8.30, 12.00, 6.36, 7.66, and 6.20 ng/μL, respectively. The 20 μL RT-PCR positive control mixture consisted of 1 μL of total viral RNA or human chromosomal DNA, 10 μL of KAPA SYBR FAST qPCR Master Mix (2×), 0.4 μL of dUTP (10 mM), 0.4 μL of KAPA RT Mix (50×), 0.4

20 μL of each specific forward/reverse primer, and 7.4 μL of ddH$_2$O. For the negative control, RNase-free water was used to replace viral RNA. Finally, the RT-PCR products were detected by slab-gel electrophoresis at 100 V for 40 minutes using a custom-made system, which integrated an optical

25 detection module. The fluorescence signal from DNA-dye complexes, which is a green light (λmax = 522 nm), is induced by a blue light source (λmax = 488 nm) used for detection on this custom-made system [38]. Viral RNA was extracted from InfA/H1N1 virus sample using the QIAamp Viral RNA Mini

30 kit (QIAGEN, Germany). The concentration of extracted RNA was measured by a spectrophotometer (Beckman, DU530 UV/Vis, USA). The RNA copy numbers were determined following a formula reported in a previous study [39]. The initial concentration of the InfA/H1N1 virus at 6.4×10^1 HAU

35 was determined to contain ~1.6×10^8 copies of viral RNA.

Results and discussion

Characterization of the microfluidic control module. The key microfluidic devices for performing automated SELEX and generating the influenza virus-specific aptamer are a

40 suction-type micropump and a micromixer [30]. The performance of these two devices were therefore first characterized. In this study, the optimization of operating

conditions is important to explore the relationship between the pumping rate and the applied air pressure. Figure 3a

45 shows the relationship between the pumping rate and the applied air pressure of the suction-type micropump.
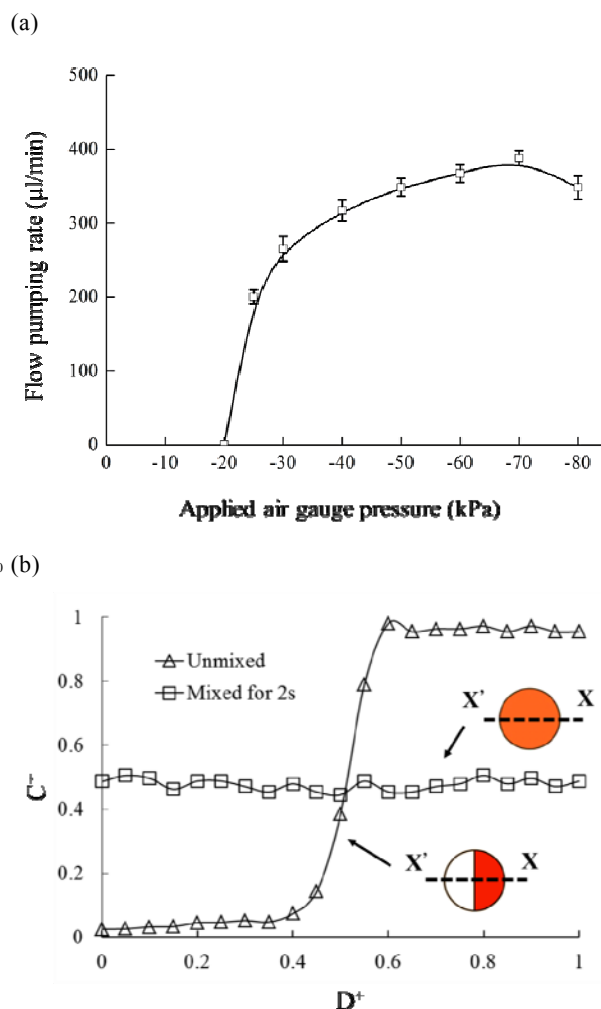
(a)

(b)

Fig. 3: (a) The relationship between the pumping rate and the applied air gauge pressure in the developed micropump.

(b) Sample mixing within the micromixer was

55 **demonstrated by comparing the mixing index (σ) before and after mixing. The mixing index was calculated from the normalized concentration profile (C$^+$) across the normalized horizontal location (X'/X) measured across the center of the mixing chamber (D$^+$). The normalized**

60 **concentration profile across the mixing chamber indicated that the micromixer efficiently mixed the ssDNA and the magnetic beads, enhancing the mixing index from 23% to 96% within two seconds.**

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Observations showed that the pumping rate increased with the increasing air gauge pressure. The average value of the pumping rate was measured as high as 388.24 ± 10.02 µl/min for the transport of biological samples and reagents and actuated at a gauge pressure of -70 kPa in subsequent experiments. With the purpose of determining the numerous steps for fluid transport and reproducibility, the pumping rate was chosen to a plateau at a high gauge pressure. Not that the error bars displayed in Figure 3a were obtained from five repeated measurements. The normalized concentration profile across the mixing chamber is shown in Figure 3b, where D^+ corresponds to the normalized location (X'/X) measured across the center of the mixing chamber and C^+ indicates the normalized concentration profile. This experimental result indicated that the DI water and red ink were mixed completely. In this study, the mixing index under the unmixed condition was measured as only 23% with no active mixing. Then, the mixing index with high mixing efficiencies was measured as 96 % within 2 s after the negative gauge pressure (air gauge pressure: -70 kPa) was supplied to the air chamber. As a result of this process, the mixing index increased from 23 % to 96 % with the PDMS membrane of the suction-type micromixer. The proposed suction-type microfluidic device provided a gentle and efficient mixing process by using active micromixers with an air gauge pressure of -70 kPa, and produced a mixing efficiency higher than 96% within 2 s.

Screening of influenza A/H1N1-specific aptamers in microfluidic SELEX. In the proposed SELEX process, positive and negative selections were used as alternate processes to screen highly specific aptamers for InfA/H1N1. The positive selection used magnetic beads that captured specific InfA/H1N1-ssDNA complexes and was amplified by using on-chip PCR. The size of specific amplified influenza aptamer was 72 base-pairs (bp) and was confirmed using PAGE after each positive round during the SELEX process. Figure 4a shows the electrophoretic results of the positive selection after continuous rounds in the SELEX process. Note that two bands appeared in lanes 1 and 2, indicating non-specific amplified products still existed in the first two SELEX processes.
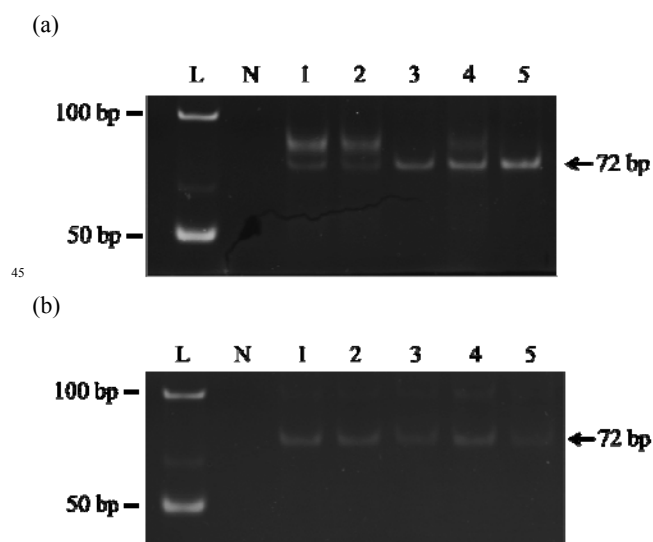
(a)

(b)

Fig. 4: The electrophoretic results showed the screening of the InfA/H1-specific aptamer after each round of SELEX using the microfluidic SELEX system, as indicated by the 72-bp amplified product. The presence of a 72-bp product indicated that the specific aptamers was amplified. Lanes 1-5 contained the PCR result after each round. (a) The results for the positive selection by performing continuous rounds in the SELEX process. (b) The results for the negative selection by performing continuous rounds in the SELEX process. Note that lane N used ddH$_2$O as the negative control, and lane L was the 50-bp DNA ladder.

However, the selected PCR products have been undergoing several rounds of screening leading to only one band, as shown in lanes 3 to 5, indicating that the selected PCR products was specific to InfA/H1N1. It indicates the successful screen of candidate aptamers from ssDNA library. The 72-bp PCR products indicated that aptamers were selected by different rounds. Lanes 1-5 presented the PCR results by continuous rounds of SELEX by positive selection. Note that lane N was the negative control using only ddH$_2$O for PCR process. The experimental results showed that the specific aptamer pool became enriched after each round of microfluidic SELEX. For the negative selection, specific viral aptamers were incubated with InfB captured by anti-NP-B mAb conjugated magnetic beads and unbound ssDNA in the supernatant was amplified by PCR and visualized using slab-gel electrophoresis. Figure 4b shows the PCR results of the negative selection, with lanes 1-5 loaded with different round after negative selection. Again, Lane N used ddH$_2$O as the negative control for PCR. The

amplified products after various rounds by SELEX negative selection for InfA/H1N1 could be successfully screened. Both of these experimental results indicated that the InfA/H1N1-specific DNA-aptamers were successfully extracted and amplified by the microfluidic SELEX chip. After the InfA/H1N1-specific aptamer screening, the extracted and amplified DNAs were cloned with the TOPO vector® system (pCR®2.1-TOPO®, 39. kb, Invitrogen Co., USA) and screened using a Luria-Bertani broth (LB)-ampicillin (100 μg/ml) plate with 2% (w/v) of the 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal). Note that the number of the clones demonstrated the strong affinity of the continuous specific aptamer with InfA/H1N1. A total of 28 plasmid DNAs with the InfA/H1N1-specific aptamer were transformed and the colonies were extracted and purified using the FavorPrepTM Plasmid DNA Extraction Mini Kit (Favorgen Biotech Co., Taiwan). Note that the screened 28 candidates have significantly alleviated the subsequent effort for the following sequencing process, when compared with the previous studies (over 100 candidates) [40]. One of the clones with a strong affinity was sequenced as follows.

5'-

GGCAGGAAGACAAACAGCCAGCGTGACAGCGACGC
GTAGGGACCGGCATCCGCGGGTGGTCTGTGGTGCTGT

-3'

The secondary structure of the InfA/H1N1-specific aptamer predicted by the MFOLD software (ver. 3.2) could be found in the Supplementary Information (Supplementary Figure 3) It should be noted that the proposed microfluidic SELEX process is more efficient than traditional SELEX, which is usually time-consuming and labor-intensive in their washing and incubation steps. Additional drawbacks are that manual operation may cause the loss of collected beads. Furthermore, it needs a number of large-scale equipment such as a PCR machine, shakers, and pipettes, and generally requires large reagent consumption. The entire traditional SELEX procedure takes at least 160 minutes and sample consumption is 30 μl. By using the proposed microfluidic SELEX chip, the entire procedure for SELEX takes only 80 minutes and requires only 10 μl of sample. Note that the microfluidic SELEX process only requires 5 rounds while the traditional SELEX usually takes 10-20 rounds. More detailed information about the comparison of the traditional methods and the microfluidic

system for the screening of aptamers could be found in the Supplementary Information (Supplementary Table 2)

The dissociation constant of the influenza A/H1N1-specific aptamer. The binding affinity of the selected aptamer was measured by a flow cytometry-based technique, where different concentrations of (FITC)-labeled aptamers were incubated with InfA/H1N1 and then quantified by flow cytometry. In this study, various concentrations of the FITC-labeled aptamer showed significantly higher fluorescence signal than the control sample. The estimated K_d value of the InfA/H1N1-specific aptamer was measured to be 55.14 ± 22.40 nM, which is comparable to the affinity of the influenza virus antibody ($10^{-6} \sim 10^{-8}$ M) [41]. Detailed information about the calculation of the dissociation constant could be found in Supplementary Information (Supplementary Figure 4).

Specificity tests of the influenza A/H1N1-specific aptamer. To demonstrate the functionality of the screened InfA/H1N1 aptamer, it was conjugated onto magnetic beads in a magnetic assay that was subsequently analyzed by one-step RT-PCR and post-reaction fluorescence detection. Using this assay, the specificity of the aptamer was determined. Aptamer-coated magnetic beads were challenged with different strains of viruses, including InfA/H1N1, InfB, InfA/H3N2, dengue virus type 2 (DENV-2), enterovirus type 71 (EV-71), and human WBC. After PCR reactions, which were supplemented with SYBR Green dye, the PCR amplicons were detected via the SYBR green fluorescence as measured by a photomultiplier tube (PMT).

(a)

(b)

(c)

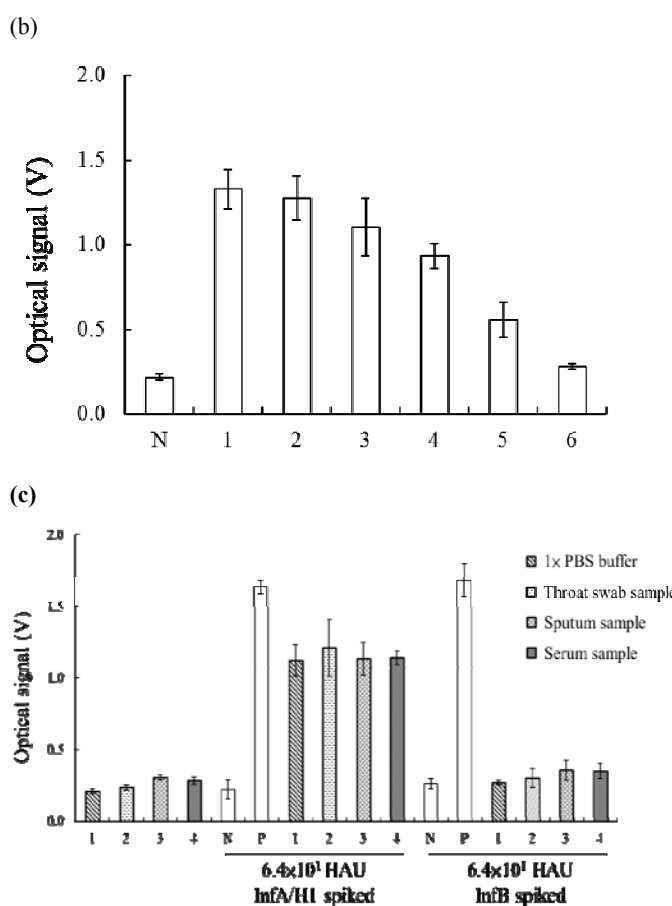6.4×10^1 HAU InfA/H1 spiked 6.4×10^1 HAU InfB spiked
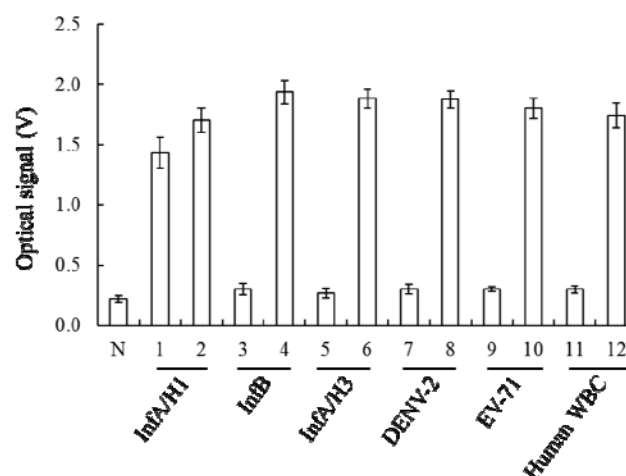
Figure 5: (a) The specificity results were further confirmed by the fluorescent signals acquired from the optical detection module. (b) The sensitivity test using the optical signal method for InfA/H1-specific aptamers by incubating 10-fold serial dilutions of InfA/H1, ranging from 6.4×10^1 to 6.4×10^{-4} HAU with aptamer-conjugated magnetic beads and RT-PCR. (c) The fluorescent signals from various biosamples spiked with InfA/H1 and InfB were first analyzed by the optical module. Higher fluorescent signals were observed when InfA/H1 was added into the biological samples.

Figure 5a shows that a high fluorescence signal (sample 1; 1.433 ± 0.128 V; n = 3) was observed from only the InfA/H1N1 sample, which is indicative of the specificity of the screened aptamer. Furthermore, fluorescence signals from other virus samples – InfB (sample 3), InfA/H3N2 (sample 5), DENV-2 (sample 7), EV-71 (sample 9), and human WBC (sample 11) (Figure 5a) – were all ~0.3 V, which is comparable to that of the no-virus negative control (sample N, 0.223 ± 0.027 V; n = 3). Importantly, the positive control associated

with each sample using extracted and purified RNA or DNA yielded high fluorescence signals (all > 1.7 V), as shown in samples 2, 4, 6, 8, 10, and 12 in Figure 5a, which indicated the accuracy of the PCR reactions. These results were confirmed by gel electrophoresis, as shown in the Supplementary Information (Supplementary Figure 2). The non-parametric Kruskal-Wallis one-way analysis of variance was performed to determine differences occurred between different tested samples. The analysis of specificity tests indicated that there were statistically significant differences between groups ($p < 0.0001$). Consequently, the p value less than 0.05 indicated the two different samples, including tested samples and negative control, have significant difference. In addition, the p value was measured to be 0.003 for specificity tests with fluorescence signals, and it indicated that have significant difference with negative control. While the InfA/H1N1-specific aptamer conjugated magnetic beads showed excellent specificity with a significant signal of 1.433 ± 0.128 V for InfA/H1N1, the other viruses gave signals that exhibited no difference from the negative controls. These results were confirmed by gel electrophoresis, as shown in the Supplementary Information (Supplementary Figure 5). It indicated that the InfA/H1N1-specific aptamer conjugated magnetic beads provide high specificity for InfA/H1N1, and are suitable for detecting or subtyping an influenza infection with reasonable accuracy.

Sensitivity tests of the influenza A/H1N1-specific aptamer. The sensitivity of the developed magnetic-bead assay was evaluated by detecting InfA/H1N1 virus with concentrations ranging from 6.4×10^1 to 6.4×10^{-4} HAU (ten-fold serial dilutions). The limit of detection (LOD) for the developed assay was determined from fluorescence signals measured by the optical detection module, as shown in Figure 5b, with samples 1 through 6 designating 6.4×10^1 through 6.4×10^{-4} HAU and sample N indicating the no-virus negative control. As the titer of InfA/H1N1 increased, the optical signals increased accordingly. Although the sample containing 6.4×10^{-4} HAU produced a fluorescence signal (0.281 ± 0.016 V) that was distinguishable from the negative control (0.218 ± 0.021 V), a student's t test revealed that these results were not significantly different ($p = 0.08$). Consequently, the LOD of the assay was determined to be 6.4×10^{-3} HAU. These results were confirmed by gel electrophoresis, as shown in the Supplementary

Information (Supplementary Figure 6). In addition, manual sensitivity tests were performed using the anti-NP-A mAb conjugated beads with InfA/H1N1 virus to compare with the InfA/H1N1-specific aptamer conjugated magnetic beads in 2% agarose gel, as shown in Supplementary Information (Supplementary Figure 6). The electrophoretic results demonstrated that the InfA/H1-specific aptamer showed 100-fold improvements in sensitivity when pegged against the anti-NP-A mAb in a similar RT-PCR-based detection assay. The LOD of this assay is $10^2 \sim 10^3$ times more sensitive than the HA inhibition assay for the detection of influenza viruses in conventional clinical diagnostics [42]. Moreover, by converting HAU to the copy number of viral RNA (see Supplementary Information), the LOD was calculated to be 1.6×10^4 copies. These experimental results showed an acceptable LOD in comparison with many reports or kits, with previous RT-PCR studies that used purified viral RNA samples showing the LOD of influenza detection between 10 and 10^4 copies [43,44].

Clinical utility tests of the influenza A/H1N1-specific aptamer. To test the clinical applicability of the selected InfA/H1N1-specific aptamer, its function in several biological samples were tested. Specifically, 6.4×10^1 HAU of InfA/H1N1 viruses were spiked in 1×PBS buffer and clinically relevant matrices including throat swab samples, sputum samples, and serum samples. Viruses were magnetically captured, amplified by RT-PCR, and analyzed by gel electrophoresis as described in previous sections. The results were shown in Figure 5c. The fluorescence signals from these biological samples were all > 1.1 V, indicating that the aptamer remained functional even in the biological samples. In contrast, when no virus or InfB viruses were spiked in these biological samples, the fluorescence signals were similar to that of the negative controls (all ~0.3 V). Similarly, these results were confirmed by gel electrophoresis, as shown in the Supplementary Information (Supplementary Figure 8).

According to these results, the InfA/H1N1-specific aptamer-based assay effectively detected InfA/H1N1 virus from clinically-relevant sample matrices and also displayed specificity against InfB viruses and background substances in biological samples.

Conclusions

In this study, a new integrated microfluidic chip with a magnetic bead-based approach was demonstrated to automatically perform the screening of specific aptamers using the SELEX technique. This is the first time that an integrated microfluidic system was reported to perform the entire SELEX process using viral particles of influenza virus to screen for the specific aptamer. With this approach, the InfA/H1N1-specific aptamer, which is a biomarker for an influenza infection, was successfully and rapidly isolated and enriched from a random nucleic acid library. Compared to traditional SELEX methods, this integrated virus-SELEX technique is smaller in size, and consumes less sample and reagents. The screening of specific aptamers could be automatically performed on a single miniature platform, which integrated devices for the incubation, extraction and amplification of nucleic acids. As a result, the entire SELEX process could be shortened to 60 minutes using this microfluidic SELEX chip, which is much faster than the conventional methods that require approximately at least 160 minutes. Moreover, the developed system performs the entire process automatically, making it less labor-intensive and greatly reducing the risk of contaminated results or infections to the operator. Furthermore, the selected aptamer has been further examined for its binding affinity and the specificity towards the target, and was sequenced and synthesized to test the capability of detection of the influenza A virus.

The specificity and sensitivity for the InfA/H1N1-sepcific aptamer was also validated using RT-PCR. The experimental results showed that InfA/H1N1 could be successfully distinguished from various types of viruses using the InfA/H1N1-sepcific aptamer. Furthermore, the LOD of the InfA/H1N1-sepcific aptamer was experimentally found to be 6.4×10^{-3} HAU, which is more sensitive than conventional clinical diagnostics. The aptamer also remained functional in biological samples including throat swabs, sputum, and serum. Therefore, this selected aptamer is well suited for the detection of an influenza infection in clinical applications. The developed microfluidic system provides a powerful tool for the rapid screening of analyte-specific aptamers for use in biomedical applications.

Acknowledgements

Notes

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The preliminary results in this paper have been presented at the 16th International Conference on Miniaturized Systems for Chemistry and Life Sciences, µTAS 2012 Conference, OKINAWA, Oct 28-Nov 1, 2012.

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