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Simultaneous and automated detection of influenza A virus hemagglutinin H7 and H9 based on magnetism and size mediated microfluidic chip

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\textbf{A B S T R A C T}

Influenza viruses with multiple subtypes have highly virulent in humans, of which influenza hemagglutinin (HA) is the major viral surface antigen. Simultaneous and automated detection of multiple influenza HA are of great importance for early-stage diagnosis and operator protection. Herein, a magnetism and size mediated microfluidic platform was developed for point-of-care detection of multiple influenza HA. With multiplex microvalves and computer program control, the detection process showed high automation which had a great potential for avoiding the high-risk virus exposure to the operator. Taking advantage of magnetism and size mediated multiple physical fields, multiple influenza HA could be simultaneous separation and detection depended on different-size magnetic beads. Using high-luminance quantum dots as reporter, this assay achieved high sensitivity with a detection limit of 3.4 ng/mL for H7N9 HA and 4.5 ng/mL for H9N2 HA, and showed excellent anti-interference ability and good reproducibility. These results indicate that this method may propose new avenues for early detection of multiple influenza subtypes.

1. Introduction

Infectious viruses, such as HIV, Ebola virus, severe acute respiratory syndrome (SARS) and so on, have a great threat to human health and life and brought devastating effects on social and economic development [1–3]. Among them, influenza A virus has become one of the most prevalent infectious diseases and caused highly infectious respiratory illness [4], which are estimated to result in about 3–5 million cases of severe illness, and about 290 000–650 000 respiratory deaths [5]. Influenza A subtypes are classified by the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins expressed on the surface of virus particles. To date, 15 antigenic subtypes of HA and 9 antigenic subtypes of NA are discovered throughout the world [6]. HA is the major viral surface antigen and plays in an important role in virus invasion and propagation with the interaction between glycans on the host cell, which causes influenza virus rapidly spreading infection throughout the world [7,8]. Influenza virus with H7 and H9 HA subtypes can cause human-to-human, human-to-animal transmissions [9,10]. These kinds of transmissions make the point-of-care detection and operator protection very important [11–13]. Therefore, simultaneous and automated detection of multiple influenza HA subtypes are needed for the diagnosis and treatment.

In recent years, many magnetic-based sensitive assays have been developed for influenza A detection, due to the unique properties of magnetic materials like high surface-to-volume, easy modification, convenient manipulation [14–21]. Nowadays, most of magnetic-based assays are limited by large samples, low level of automation and time-consuming process. Moreover, it is difficult to simultaneously use different-size magnetic beads (from nanometers to micrometers) in biological applications due to their similar magnetic magnitudes. Microfluidic technology has many unique advantages over conventional analytical techniques, which could reduce sample and reagent consumption, high throughput, miniaturization, multiple physical field manipulation, and the potentiality of automation [22]. Thus, many
efforts have been devoted to the development of magnetic-based assays on microfluidic chips [4,23–26]. However, these assays on microfluidic chips usually need fabrication methods like soft lithography, fine magnetic field manipulation, and single-size magnetic bead. Therefore, new methods that can overcome this problem are urgently needed for the simultaneous and automated detection of multiple influenza HA subtypes on the microfluidic chip.

In our previous work, we have developed some biomedical applications based on magnetic beads on the microfluidic chip [4,22,27–31], and also dedicating ourselves to the construction of microfluidic devices [32–34]. On this previous basis, a magnetism and size mediated multiphysics field microfluidic chip was constructed for simultaneous and automated detection of multiple influenza HA subtypes (Scheme 1). The unique advantages of our proposed strategy can be listed as follows: First, assisted by multiplex microvalves and computer program, the detection process showed high automation which was helpful to protect the operator from virus infection. Second, multiple physical fields, which consisted of magnetism mediated magnetophoretic separation and size mediated signal detection, enhanced the complex sample treatment ability and realized the multiple influenza HA subtypes simultaneous detection only by different-size magnetic beads. Third, using high-luminance quantum dots as reporter enhanced the detection sensitivity. This method can quantitatively detect H7N9 and H9N2 HA with a detection limit of 3.4 ng/mL and 4.5 ng/mL, respectively. In addition, the assay was robust, and had showed high specificity, anti-interference ability and good reproducibility. In this way, this method opens up new avenues for virus subtypes analysis and expands the simultaneous application of different-size magnetic beads on the microfluidic chip.

2. Experimental

2.1. Materials and instruments

PVC glass sandblasting protective film (P-B50) was purchased from Vary Era (Shanghai) Industry Co., Ltd., China. 1 × PBS (pH 7.4) were obtained from Life Technologies, USA. DI water was generated by a MILLI-Q system (Millipore, MA, USA). Bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A quantum dots 605 streptavidin conjugate (SA–QDs) was purchased from Wuhan Jiayuan Quantum Dots Co., Ltd. The antibodies and HA protein for H7N9 and H9N2 were purchased from Sino Biological (Beijing). Magnetic beads were brought from BaseLine Chromtech Research Center (Tianjin).

Fluorescence images were recorded by a CCD camera (QImaging optiMOS) mounted on an inverted fluorescence microscope (Olympus IX83). Microchannels were made by a CNC engraving and milling machine (JDLGC16 A8, Jingdiao Group Co., Ltd.). The type of the engines using in the home-made vacuum pump were MS-150-24. Pneumatic solenoid valves (ITV-1030, SMC Corporation, Japan). WD-V350 small laminating machine was obtained from Hangzhou Caide.
2.2. Fabrication of the automated microfluidic chip

First, two glasses were covered with PVC glass sandblasting protective film through small laminating machine to protect the glass during chemical etching, and then the protective film on the glass was treated by the laser engraving to imprint the structure of microchannel. After that, the parts of film treated by laser were peeled off the glass for etching. The etching rate was related to the concentration of reagents, humidity and temperature, so the rate should be calculated before the microchannel etching according to CNC computer engraving machine measurements. During etching, the protective film on the glass was orderly removed to obtain the different microchannel heights. After chemical etching, the chip should be quickly rinsed in ultrapure water to prevent the etched glass slag from condensing. Second, the etching microchannel further processed to generate the inlets and outlets via CNC computer engraving machine. Then glasses was immersed in a solution of sulfuric acid for 2 h to make the glass surface hydrophilic, which is favorable for bonding. After washing with deionized water, glasses were bound in the water and heated at 80 °C for more than 2 h on a micro-controlled digital display hot plate to completely evaporate the water vapor in the chip. Finally, the chip is placed in a ceramic fiber...
muffle furnace for high-temperature sintering for more than 24 h, obtaining the magnetism and size mediated microfluidic chip.

On the other side, the fabrication of microvalves was according to following steps. PDMS prepolymer was spin-coated on a PET (polyethylene terephthalate) sheet, and then kept at 75 °C for 1 h to generate the PDMS cured membrane. The gas channels on upper glass were fabricated by chemical etching with 200 μm in depth and 600 μm in width. The upper glass and PDMS membrane were bound together with the aid of a corona discharge treatment, baked at 80 °C for 30 min. Then the lower glass layer was bonded to the other side of PDMS membrane with the upper glass layer in the same way to obtain the whole microvalves chip, and the fluidic channels on lower glass were generated by chemical etching.

2.3. Program of the multiplex microvalves microfluidic chip

The operation panel in multiplex microvalves chip was controlled by LabVIEW, and the console computer program was written by Keil.
μVision4 on the STM32F107VCT6 main control chip. The command was transmitted to the circuit board through the serial communication, which was used for controlling switch of air pressure through multi-channel pneumatic solenoid valves. The magnitude of air pressure could be changed by the voltage of the electro-pneumatic regulator. Finally, the air pressure pressed the PDMS membrane in the multiplex microvalve chip to block the circulation of the liquid control reagent, realizing automated sample injection.

2.4. Preparation of immunomagnetic beads

Magnetic beads were transformed into immunomagnetic beads (IMBs) via antibody modification. Anti-H7N9 and H9N2 HA antibodies were immobilized on the surface of magnetic beads through cross-link the amines of the antibodies with the carboxylic acid groups. 5 μm and 10 μm MBs were dispersed through ultrasound instrument, and 20 μL MBs were taken out respectively. The MBs were further washed by PBS and separated by a magnetic scaffold. Then the carboxylic acid groups on the MBs were activated in 10 mg/ml EDC and 5 mg/ml NHS with gentle shaking for 30 min at room temperature. After activating, the MBs were washed three times by PBS, and 2 μg antibodies were added and reacted with the activated MBs for 4 h to form the immune magnetic beads. After reaction, the IMBs were washed by PBS for three times and stored in 4 °C for use.

To verify the modification of H7N9 and H9N2 antibody on the IMBs surface, FITC (fluorescein isothiocyanate) -conjugated AffiniPure goat anti-mouse IgG was diluted and incubated with the IMBs for 30 min. After incubation, the IMBs were washed by PBS three times and observed on the inverted fluorescence microscope.

2.5. Conjugation of biotin to antibodies

The antibodies were reacted with sulfo-NHS-LC-biotin to form the biotin modified antibodies. 0.1 mg sulfo-NHS-LC-biotin was dissolved into 90 μL ultrapure water, and then 2 mg antibodies were added in the sulfo-NHS-LC-biotin solution. They were incubation for 4 h with shaking to modify the biotin on the antibodies. After incubation, the surplus sulfo-NHS-LC-biotin was removed through a desalting NAP-5 column to obtain the biotin modified antibodies.

2.6. Sensitive assay of influenza viruses

The sample and detection reagents were stored in the reagent bottles, and the multiplex microvalves were used to control the reagents flowing into the chip. Influenza virus HA for H7N9 and H9N2 was diluted in a series concentration. 100 μL HA sample and 100 μL IMBs were reacted and flowed into the microchannel at a rate of 5 μL/min, which the IMBs tagged virus were separated from a complex matrix. After that, the IMBs tagged virus were captured in the sizes mediated detection zones, where 10 μm IMBs were captured in the first zone with minimum height 7 μm, and 5 μm IMBs were trapped in the second zone with minimum height 4 μm (Fig. S1). To track the size separation effectiveness, 10 μm IMBs modified with red fluorescence and 5 μm IMBs modified with green fluorescence were observed in the inverted fluorescence microscope. Then the biotin conjugated antibodies were flowed into the chip and reacted with the IMBs tagged viruses in the detection zones. Finally, SA-QDs were injected the chip via microvalve controlling and incubated with the complex to form the QDs labelled immune sandwich complex. The fluorescence intensity of QDs was acquired by a charge coupled device (CCD) to determinate the HA concentration.

3. Results and discussion

3.1. Characterization of the IMBs

Magnetic beads were transformed into immune magnetic beads (IMB) after antibodies modification on their surface. To verify the modification of the H7N9 and H9N2 antibody, the immune
fluorescence was used to test the modified IMBs via FITC (fluorescein isothiocyanate) -conjugated AffiniPure goat anti-mouse IgG incubation. As shown in Fig. 1A–C, the green fluorescence of FITC exhibited on anti-H7N9 antibody modified IMBs, while no green fluorescence was observed on the MBs (Fig. 2D–F). As shown in Fig. S2A–F, the IMBs of H9N2 showed the same phenomenon. These results illustrated that the IMBs were successfully coated with anti-H7N9 and anti-H9N2 antibodies, and the native active conformations were well-retained after modification.

3.2. Automated detection platform

The detection platform consisted of multiplex microvalves manipulation zone, magnetophoretic separation zone and size mediated detection zone. Among them, different detection reagents were stored and controlled by multiplex microvalves with a computer program, which greatly reduced the contact with the high-risk influenza HA (Fig. 2A). The multiplex microvalves were established by inserting a polydimethyldilsiloxane (PDMS) membrane into two glasses, which are cheap and simple without any complex silicon-based device or soft lithography. The detailed fabrication and characterization of multiplex valves were reported in our previous work [35]. After the magnetic-based immunoassay with multiplex microvalves controlling, IMBs-tagged influenza HA was flowed into the microchannel of separation zone at a rate of 5 μL/min. With the function of magnetization, IMBs-tagged HA were been immunomagnetic separation in the lateral direction of the microchannel. A uniform magnetic field gradient was applied to the microchannel of separation zone to contribute the immunomagnetic separation (Fig. 2B). The detailed magnetic field analysis, assisted by finite element calculation, was described in supporting information section S.1. The effectiveness of separation was observed by the microscopic image (Fig. 2C), and further tracked by the fluorescent labeled micropherses and recorded in a movie attached as a supporting information. In the movie, different-size magnetic beads captured in the different size mediated zones were clearly observed. After immunomagnetic separation, the IMBs-tagged complex was trapped in corresponding detection zone based on different-size magnetic beads (Fig. 2D), and further reacted with biotinylated antibody and SA-QDs to generate immune-sandwich complexes, obtaining the detection signal. The velocity of flow was optimized. As shown in the Fig. S3, the fluorescence intensity reached a maximum when the flow of biotinylated antibodies was 5 μL/min, therefore, 5 μL/min was the best flow rate. Fig. 2E showed the whole detection platform, making the detection with a high automation.

3.3. Sensitive assay of H7N9 and H9N2 HA

A sensitive immunosensor was constructed for dual influenza HA detection. The immune-sandwich complexes were enriched in the detection zones based on their size difference, and their concentration was directly related to the QDs fluorescence signal. The fluorescence signal was acquired via CCD and quantitatively processed through IPP software. As shown in Fig. 3A, both of the fluorescence intensity in two detection zones didn’t have obvious signal at the concentration of 0 ng/mL, while the intensity gradually enhanced with the concentration increasing of the target sample viruses (10, 20, 50, 100, and 500 ng/mL) (Fig. 3B–F). Fig. 3G showed that the fluorescence intensity reached a plateau toward H7N9 HA after 100 ng/mL, and the intensity increased slowly after 100 ng/mL for H9N2 HA (Fig. 3I). A linear relationship for H7N9 and H9N2 HA was obtained in a wide range of 5–100 ng/mL, with a linear correlation coefficient (R²) of 0.985 and 0.996 (Fig. 3H, J). According to IUPAC standard detection limit (LOD) calculation method [36], the LOD was calculated to be 3.4 ng/mL for H7N9 HA and 4.5 ng/mL for H9N2 HA.

Compared with the previous work on the detection of influenza viruses [4,18], this detection method had a highly automated ability to complete the whole detection process, which conducted the sample introduction, separation and detection on the chip through the control of a computer program. Meanwhile, multiple influenza HA subtypes could be simultaneously detected only by using different-size magnetic beads, enriching the simultaneous application of different-size magnetic beads in biological detection.

3.4. Specificity, precision, and reproducibility of the magnetism and size mediated assay

To further investigate the analytical performance, the specificity for H7N9 HA was tested by using the H9N2 HA, hepatitis B virus (HBV) and Ebola as a control. As shown in Fig. 4A, the fluorescence intensity of H7N9 HA was higher than other viruses. And the specificity of H9N2 HA was also tested by comparing with H7N9 HA, HBV and Ebola. Fig. 4B showed that the fluorescence intensity of H9N2 HA was higher than other viruses. And the assay was further tested in a complex matrix (liver, lung and fowl dung). As shown in Fig. 4C-D, the corresponding detection signal was slightly lower in the complex matrices. These results indicated this method had a good specificity and anti-interference ability.

The precision was further analyzed by using the same sample with the same batch of IMBs for parallel five times to obtain the intra-assay variability. The values of intra-assay variability are shown in Table 1 with 4.5 % for H7N9 HA and 3.7 % for H9N2, respectively, and the interassay variability was further tested for the variability using five different batches of IMBs, which was calculated to be 7.3 % for H7N9 and 6.2 % for H9N2 HA. These results indicated that this assay had good precision and reproducibility.

4. Conclusion

In summary, an automated magnetic-based immune assay for multiple influenza HA was established on the magnetism and size mediated microfluidic chip. The automated detection process could reduce the contact of high-risk viruses, protecting the operator. Coupled with magnetism and size mediated multiple physical field design, multiple influenza HA subtypes were detected simultaneously only by different-size magnetic beads. Finally, H7N9 HA could be detected in concentrations as low as 3.4 ng/mL, and H9N2 HA could be detected for 4.5 ng/mL. In addition, the assay exhibited high specificity, anti-interference ability and good reproducibility. In this way, we believe that this method may open up new avenues for simultaneous using different-size magnetic beads to detect multiple pathogens subtypes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2020.127675.
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