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Virus purification and enrichment by hydroxyapatite chromatography on a chip

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A B S T R A C T
The spread of infectious diseases has become a global health concern. In order to diagnose infectious diseases quickly and accurately, next-generation DNA sequencing techniques for genetic analysis of infectious viruses have been developed rapidly. However, it takes a very long time to pretreat clinical samples for genetic analysis using next-generation sequencers. We have therefore developed a microfluidic chromatography chip that can purify and enrich viruses in a sample using hydroxyapatite particles packed in a micro-column. We demonstrated the purification of virus from a mixture of virus and FBS protein, and enrichment of the virus using this novel microfluidic chip.

1. Introduction
The spread of infectious diseases has become a global health concern. In 2002, an outbreak of atypical pneumonia, referred to as severe acute respiratory syndrome (SARS) and first identified in Guangdong Province, China, occurred and spread to several countries [1,2]. In total, 8273 cases and 775 deaths attributed to SARS were reported in multiple countries according to the World Health Organization (WHO). In April 2009, the outbreak of a novel influenza A H1N1 virus in Mexico spread globally and developed into the first human influenza pandemic in 40 years [3,4]. To diagnose infectious diseases quickly and accurately, DNA sequencers for genetic analysis of infectious viruses have been developed rapidly. Newly developed “next-generation” sequencing technologies for massively parallel DNA sequencing are in fairly widespread use at present [5,6]. However, in order to diagnose infectious diseases using DNA sequences, the virus in the clinical sample must be purified and enriched in concentration before DNA sequencing [7]. These pretreatment processes are labor-intensive, cumbersome and time consuming. There is therefore need to speed up the pretreatment of clinical samples before DNA sequencing to complement the throughput of DNA sequencers. We here describe a novel microfluidic chip for purifying and enriching viruses. The microfluidic chip has many advantages; it requires only a small work place, a small sample volume, an enclosed region, and is comparatively low cost.

Tiselius et al. [8] first described protein purification by hydroxyapatite liquid chromatography in 1956. Since then, hydroxyapatite chromatography has been used extensively for the purification and fractionation of various biochemical substances such as protein [9–11], bacteriophages [12], and viruses [13,14]. Hydroxyapatite (Ca_{10}(PO_4)_6(OH)_2) is a very complex crystalline compound to which a variety of substances adsorb. It has been used extensively as a matrix for the purification and fractionation of an array of biochemical substances, including enzymes, nucleic acids, hormones, and viruses [15]. Many researchers have reported that the interaction between hydroxyapatite and an electrically charged adsorbate is due to ion exchange or static attraction [16,17].

We here describe a microfluidic chip developed for hydroxyapatite chromatography. Hydroxyapatite particles were packed in a micro-column and on-chip chromatography has been conducted to purify and enrich Newcastle disease virus.

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2. Materials and methods

2.1. Design of and theory behind the microfluidic chip

Fig. 1(a) shows the concept of the microfluidic chip for virus purification and enrichment by on-chip hydroxyapatite chromatography. Fig. 1(b) shows the microfluidic chip fabricated using photolithography. The hydroxyapatite used was Ceramic Hydroxyapatite Type II Support (CHT; BIO-RAD, Hercules, CA, USA), with a particle diameter of 40 μm. The microfluidic chip has a column for hydroxyapatite chromatography and a pair of switching valves. Upstream and downstream of the column, 50-μm-diameter cylindrical micropillars placed at 20-μm intervals hold the hydroxyapatite particles in the column, as shown in Fig. 1(b). Hydroxyapatite particles are introduced into the column through inlet 1. The sample and the elution buffers are introduced into the column through inlet 2.

2.2. Fabrication of the microfluidic chip

The microfluidic chip consists of a polydimethylsiloxane (PDMS) microchannel and a PDMS substrate. Fig. 2 shows the processes used to fabricate a PDMS (Silpot 184, Toray-Dow Corning Co., Tokyo, Japan) microchannel and bond it to a PDMS substrate. A PDMS microchannel was produced by replica molding using a master mold fabricated by photolithography [18]. A negative-type photore sist (SU-8 3050, Kayaku MicroChem Co. Ltd., Tokyo, Japan) was spin-coated on a silicon substrate. Spin-coating condition was 1000 rpm for 30 s. After prebaking at 95 °C for 3 h, ultraviolet light was irradiated through a photomask to produce a microchannel pattern using a mask aligner (MJB-3, Suss Microtec, Garching, Germany). After post-exposure baking at 65 °C for 30 s and at 95 °C for 60 s, the substrate was then developed in PM thinner for 8 min and rinsed in ethanol. The channel was 100 μm high. Unpolymerized PDMS (resin:catalyst = 10:1) was poured into the mold and cured in an oven at 90 °C for 20 min. The replica was then peeled from the master and the PDMS microchannel was ultrasonically rinsed in ethanol. The PDMS microchannel with outlet, inlet, drain and sample recovery chamber holes and the PDMS substrate were then treated with oxygen plasma (CUTE-MP, UVOTECH Systems, Concord, CA, USA) to increase its adhesion to the PDMS substrate. After assembling the PDMS microchannel and the PDMS substrate, it was heated in oven at 145 °C for 20 min. Then silicone microtubes with an inside diameter of 1 mm and an outside diameter of 2 mm were assembled into the holes in the PDMS layer to connect the microchannel.

![Fig. 2. Fabrication process of the proposed microfluidic chip. The chip consists of a PDMS microchannel and a PDMS substrate. The PDMS microchannel was produced by replica molding using a master mold fabricated by photolithography.](image-url)
2.3. Switching valve

Impurities in the sample are guided towards the drain port, while the purified viruses are guided towards the sample recovery chamber by a pair of switching valves. Fig. 3 shows the fabrication process for a pair of switching valves. Two 8-mm-diameter holes were punched in the microfluidic chip using a biopsy punch. The PDMS disks in the biopsy punch were reinserted into the holes in the microfluidic chip to provide a pair of switching valves. The valves are opened or closed by rotation of the PDMS disks. This switching valve has the important advantage of being very easy to fabricate.

![Fabrication process of a pair of switching valves.](image)

**Fig. 3.** Fabrication process of a pair of switching valves. Two 8-mm-diameter holes were punched in the microfluidic chip using a biopsy punch. The PDMS disks in the biopsy punch were reinserted into the holes in the microfluidic chip to provide a pair of switching valves. The valves are opened or closed by rotation of the PDMS disks. This switching valve has the important advantage of being very easy to fabricate.

To decrease the risk of leakage from the side of the valve, parylene thin film about 800 nm thick was coated on the side surface of each switching valve by using a dix coating apparatus (DACS-LAB, KISCO, Osaka, Japan). Pressure tests of the valves uncoated and coated with parylene were conducted to demonstrate the utility of the parylene thin film by using a pressure sensor (Pressure Transducers with Amp, Copal Electronics, Tokyo, Japan).

**Fig. 4.** Fabricated switching valve. The proposed valves enable the microchannels to be switched by rotation of the valves. The switching valve can be easily rotated by hand.

2.4. Virus purification

A Newcastle disease virus (NDV) [19,20] suspension was used to demonstrate the purification and enrichment of viruses. To purify the virus, the right valve was closed and the left valve was opened, and 100 μL of a 2.5 × 10^5 pfu/mL NDV suspension which included FBS proteins was introduced into the column through inlet 2. Next, 100 μL of a 500 mM KCl solution was introduced into the column through inlet 2 to elute the FBS proteins. Finally, the left valve was closed and the right valve was opened, and 100 μL of a 1 M phosphate buffer was introduced into the column through inlet 2 to elute the viruses. The phosphate buffer was collected at the sample recovery chamber to detect the viruses by hemagglutination reaction [21]. PBS was used as a control for a negative result, and a pure NDV suspension was used as a control for a positive result. For the hemagglutination reaction, blood for the hemagglutination reaction was collected from laboratory volunteers, after obtaining informed consent according to the protocol approved by the Research Ethics Committee of Nagoya University. First, blood was pipetted into a 1.5-ml Eppendorf tube and washed with PBS three times. After the final wash the supernatant was aspirated and enough PBS was added to make a working solution of 2% RBCs in PBS. Next, 50 μL of the working solution was added to each well of a round-bottomed 96-well dish, then 50 μL of the sample was added to each well and mixed with the working solution. The mixture was left at room temperature for 30 min. As shown in Fig. 5(a), the negative result appears as a red dot in the center of the round-bottomed plate because the red blood cells settle out, and as shown in Fig. 5(b), the positive result forms a uniform reddish color across the well because each agglutinating molecule on the virus coat binds to multiple red blood cells to form a bridged structure.

![Virus purification process.](image)

**Fig. 5.** Negative control (a) and positive control (b) for the hemagglutination reaction. The negative result appears as a red dot in the center of the round-bottomed plate because the red blood cells settle out. The positive result forms a uniform reddish color across the well because each agglutinating molecule on the virus coat binds to multiple red blood cells to form a bridged structure.

2.5. Virus enrichment

To demonstrate enrichment of the virus, 1 mL of a 1.0 × 10^7 pfu/mL NDV suspension was introduced into the
column through inlet 2. Five microfluidic chips with different shapes of microcolumn were prepared, as shown in Fig. 6. The width and the length of the microcolumns were (A) 2 mm and 10 mm, (B) 1 mm and 10 mm, (C) 1 mm and 20 mm, (D) 1 mm and 40 mm, and (E) 0.5 mm and 40 mm.

Extension at 60°C for 20 s. Real-time RT-PCR was conducted using an Eco<sup>TM</sup> Real-Time PCR System (Illumina, San Diego, CA, USA).

2.6. Statistical analysis

Data represent mean values ± standard errors. One-way analysis of variance (ANOVA) was used to compare the mean values among groups. P values < 0.05 were considered to indicate statistical significance. The number of test samples (N) for each experiment was four.

3. Results

3.1. Virus purification

Fig. 7 shows the results of the virus purification process from virus suspensions which included 5%, 10%, 15%, 20% FBS protein. Before chromatography, the hemagglutination reaction gave negative results even though the suspensions contained virus because FBS protein inhibits hemagglutination [23]. After chromatography,
the hemagglutination reaction gave positive results because the FBS had been removed by chromatography. These results show that hydroxyapatite chromatography on the microfluidic chip allowed the sensitive detection of viruses.

3.2. Virus enrichment

To evaluate virus enrichment efficiency of the microfluidic chip, the amount of RNA was investigated by real-time RT-PCR. Using RNA templates of NDV from $10^4$ to $10^3$ pfu/mL, a standard curve was obtained and the linear regression between the Ct value and the logarithm of the virus concentration was determined. Based on the equation provided by the standard curve, the efficiencies of virus enrichment were quantified once the Ct value of the enriched NDV sample obtained from our microfluidic chip was measured by real-time RT-PCR.

Fig. 8 shows the results of virus enrichment. Five microfluidic chips with different shapes of microcolumn were prepared, as described above. The most effective enrichment was achieved using the 1-mm-wide, 20-mm-long microcolumn. Its enrichment efficiency was 7.26.

4. Discussion

In the virus purification process, the results of the hemagglutination reaction showed that the viruses were successfully isolated from the mixture of virus and FBS protein. A clinical sample such as an oral swab contains many impurities such as protein which can inhibit virus detection. To improve the sensitivity of virus detection, these impurities must be removed and the viruses purified. In this study, FBS protein in the sample was removed and NDV was successfully purified by on-chip hydroxyapatite chromatography.

In the virus enrichment process, the most effective enrichment was achieved with a microcolumn 1 mm wide and 20 mm long, providing an enrichment efficiency of 7.26. Microcolumns B, C and D in Fig. 8(a) are 1 mm wide. Comparison of microcolumns B and C shows more effective enrichment with microcolumn C due to its longer length, allowing more hydroxyapatite to be packed in it. Comparison of microcolumns C and D shows more effective enrichment with microcolumn C, even though microcolumn C is half the length of D. However, the path in microcolumn D is tortuous while in microcolumn C it is straight. The height equivalence to theoretical plate (HETP) in chromatography increases if the column is tortuous. According to Giddings [24,25], HETP is inversely proportional to the square of curvature of the column [24–26]. Therefore the curvature of the column should be infinity for small HETP, so a column should have a straight structure. Microcolumns A, C and E in Fig. 8(b) are equal in volume. Comparison of microcolumn A and C shows more effective enrichment with microcolumn C because it is thinner than microcolumn A. According to Taylor's experiment, the chromatogram has a peak due to diffusion in the column [27,28]. For more effective elution, the chromatographic peak should be sharp. The width (W) of the chromatographic peak is proportional to the column radius, and the height (H) of the chromatographic peak is inversely proportional to the cube of the column radius. To obtain a sharp peak, W should be small and H should be large. Therefore the column radius should be smaller for more effective chromatography. In our virus enrichment experiments, microcolumn C is thinner than microcolumn A, and thus provides more effective enrichment. More effective enrichment was achieved with microcolumn C than with microcolumn E, even though E is thinner than C, because E has a tortuous shape while microcolumn C is straight.

We here demonstrated purification and enrichment of viruses on a microfluidic chip. Such a simple sample processing unit can be integrated with other units such as extraction of viral RNA and detection of viral subtype on a single microfluidic chip. Viral RNA is specifically adsorbed to silica when the solution contains chaotropic agent such as guanidine salt. The adsorbed RNA can be eluted by low-salt buffer such as nuclease-free water. A silica-packed microcolumn for viral RNA extraction is producible like hydroxyapatite microcolumn of this study. Furthermore, a viral detection could be achieved by immobilizing peptide nucleic acids (PNA) for the viral genome capture to a sample recovery chamber in this microfluidic chip. PNA is an RNA/DNA mimic in which the phosphate deoxyribose backbone is replaced by a neutral amide backbone composed of N-(2-aminoethyl) glycinic linkage [29,30]. Base pairing by PNAs is not affected by intrasound electrostatic repulsion and occurs with high affinity and enhanced rates of association with strict sequence specificity such as virus subtype. Integration of such units on a single microfluidic chip will allow the continuous treatment of the clinical samples in an enclosed region with low risk of contamination and infection to the operators. Therefore, we believe the microfluidic chip presented here has a significant potential for easy and rapid diagnosis of the infectious diseases.

5. Conclusions

In this paper we proposed a microfluidic chip with a microcolumn for hydroxyapatite chromatography. Clinical samples from patients contain many impurities which can inhibit the detection of viruses. Purification of viruses in the clinical sample allows the sensitive detection of virus. And in order to detect a virus infection at an early stage, it is very important to enrich the low-concentration virus suspension. Using our microfluidic chip, we succeeded in purifying and enriching NDV, showing that the microfluidic chip allows pretreatment of samples for accurate diagnosis of viral infectious diseases. Furthermore, we proposed a novel and simple switching valve which can switch the microchannels by hand. The switching valve has the important advantage of being very easy to fabricate and it does not need any driving source. Therefore our microfluidic
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.04.011.

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