

Microfluidics-based extraction of viral RNA from infected mammalian cells for disposable molecular diagnostics

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Abstract

A disposable plastic microfluidic device capable of extracting viral RNA from complex biological mixtures has been developed as a step towards building low-cost, mass produced molecular diagnostic systems for infectious diseases. The device offers a robust, easy-to-use sample preparation platform for rapid processing of clinical samples for nucleic acid-based diagnostics for pathogens. As a platform model, we have successfully isolated viral RNA from mammalian cells infected with influenza A (H1N1) virus. The microfluidic chip was fabricated in cyclic polyolefin by hot-embossing with a nickel–cobalt electroformed master mold. The isolation of total RNA was done on-chip with a solid-phase extraction (SPE) system formed by trapping silica particles in a porous polymer monolith. Separation of RNA is achieved through reversible binding of the nucleic acids to the silica particles in the monolith. The μ SPE system allows isolation of intact total RNA from a mixture of infected whole cell lysates and serum supplemented cell culture supernatant. The system requires minimal user-handling, so the isolated RNA sample has low risk of degradation. The μ SPE system also significantly reduces the required hands-on time for extraction compared to the conventional bench-top extraction procedures. The sample preparation platform presented here uses a very compact design that can be easily coupled with downstream amplification and detection modules to form a fully integrated lab-on-a-chip for nucleic acid analysis.

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1. Introduction

Recent advances in microfluidics and lab-on-a-chip technologies have catalyzed research in the area of miniaturized analytical systems for on-chip diagnostic assays, forensic identification and detection of chemical/biological warfare agents [1–4]. Chip-based assays provide several advantages over bench-top procedures, including reduction in sample/reagent consumption, enabling parallel processing, improving analysis speed, reducing the possibility of sample contamination and the risk of infecting the clinicians [5,6]. The use of high-throughput microfluidic diagnostics would be particularly beneficial for infectious disease detection and surveillance, because they would enable fast diagnosis, treatment and isolation of the infected. Moreover, the device can be very cost-effective if fabri-

cated in thermoplastics instead of silicon or glass, and therefore will be ideal for disposable applications. Microfluidics-based methodologies demonstrated to date are mostly in glass, silicon or PDMS (polydimethylsiloxane). However, microfabricated chips made of glass or silicon are not ideal for disposable applications as they entail high material and manufacturing costs [7], while PDMS lacks dimensional stability and has poor shelf-life. These drawbacks can be averted when working with thermoplastics, while still exploiting the advantages of micro and nanoscale features, while using bulk manufacturing processes using injection or compression molding [8,9].

Molecular diagnostic systems using real-time polymerase chain reaction (PCR) amplification/detection offer high sensitivity and specificity, together with the potential for rapid diagnosis of infectious diseases [10]. The overall sensitivity is dependant on the nucleic acid yield, purity, and the amount of sample equivalents that can be transferred into the amplification reaction [11], which necessitates the development of efficient yet high-throughput nucleic acid extraction protocols.

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A fully integrated PCR-based genetic analysis device should be capable of accepting crude clinical samples and perform sample preparation, various fluidic manipulations and PCR on the same chip [12,13]. Many research groups have shown successful demonstrations of on-chip PCR in a number of different materials sets, including high temperature thermoplastics like polycarbonate and cyclic polyolefins [14–17], but in most of these applications the nucleic acid purification prior to PCR was done on-the-bench. Nucleic acid isolation remains the most technically demanding and labor-intensive procedure performed in molecular diagnostics. Most recently, a device that truly incorporates sample preparation, amplification and detection was reported by Easley et al. [12]. The device was able to detect *Bacillus anthracis* in a mouse blood sample and *Bordetella pertussis* in infected human nasal aspirate. The sample preparation in the device was performed with a silica bead packed column held in place with an etched weir. Application of blood and nasal aspirate directly to the chip makes this demonstration the most advanced to date. However, this multilevel microfluidic device was fabricated in glass using a hydrofluoric acid etch process and a PDMS elastomer valve layer was used to isolate reagents from the different procedures.

Here, we describe a disposable plastic microfluidic device for on-chip isolation of total RNA from complex biomolecular mixtures simulating patient samples, and subsequent amplification and detection of specific sequences from the isolated total RNA. As a platform model, we have tested the isolation of viral RNA from Madin-Darby Canine Kidney (MDCK) cells and cell culture supernatant that were previously infected with influenza A (strain A/PR/8/34) virus. Influenza A was chosen to assess the performance of the device, because it is a persistent threat to public-health. In the United States, flu usually results in approximately 36,000 deaths each year, with the largest numbers of casualties among people over 65 years of age and very young children [18]. The recent emergence of an avian flu strain in Asia that has now been shown to infect humans has brought the need for rapid molecular diagnostics for flu into the popular consciousness. Recently, several groups have focused their efforts on microfluidic devices to detect influenza A. Pal et al. [19] have demonstrated a chip made of silicon and glass that can distinguish between influenza A subtypes using a combination of on-chip PCR, restriction digests and electrophoretic separations. Sample preparation (nucleic acid isolation) is not included in the chip design, and the reported data was collected using cDNA from an off-chip reverse transcription step. Liu et al. [20] have also demonstrated an integrated microfluidic assay that can detect influenza A subtypes. This device integrates a DNA microarray with plastic fluidic components that contain the reagents necessary for sequencing and hybridization. Again, the sample preparation prior to the microarray analysis was performed off-chip.

The RNA purification procedure described in this paper is based on micro solid-phase extraction (μ SPE) of nucleic acids in a plastic microfluidic device. Obtaining high quality, intact RNA is crucial to the molecular detection of viral pathogens. Current laboratory techniques for sample preparation require several bench-top procedures, which often put the integrity of

the RNA sample at risk due to ubiquitous RNase [21]. In case of a microfluidic system, the purification steps are all confined within the microchip, and as a result the chances of sample contamination and RNA degradation are significantly reduced. The microfluidic chip described here is made of cyclic polyolefin (Zeonex[®] 690R, Zeon Chemicals L.P., Louisville, KY) by hot-embossing with a nickel–cobalt electroformed master-mold. The solid-phase for nucleic acid extraction is formed within the microchannels by *in situ* photopolymerization. The total RNA extracted by the μ SPE technology was evaluated through real-time reverse transcription (RT)-PCR assay.

2. Experimental

2.1. Materials

Cyclic polyolefin (Zeonex 690R) was obtained as a gift from Zeon Chemicals L.P. (Louisville, KY). Butyl methacrylate (99%, BuMA), ethylene dimethacrylate (98%, EDMA), ethylene diacrylate (90%, EDA), methyl methacrylate (99%, MMA), 1-dodecanol (98%), cyclohexanol (99%), benzophenone (99%, BP), and 2,2-dimethoxy-2-phenylacetophenone (99%, DMPAP) were purchased from Sigma-Aldrich (St. Louis, MO). Guanidinium thiocyanate (GuSCN) containing lysis buffer (Buffer RLT) was purchased from Qiagen Inc. (Valencia, CA). 0.7 μ m silica microspheres were purchased from Polysciences, Inc. (Warrington, PA). PEEK (Polyetheretherketone) capillaries of 360 μ m o.d. and NanoPort[®] assemblies for chip-based fluidic connections were purchased from Upchurch Scientific (Oak Harbor, WA). MDCK cells (CCL 34) and influenza A (VR-1469, H1N1, A/PR/8/34) were obtained from ATCC (Manassas, VA).

2.2. Chip fabrication

The chips were fabricated in Zeonex[®], which is a medical grade cyclic polyolefin. Zeonex690R has a glass transition temperature (T_g) of 136 °C and exhibits very high UV transmittance and low autofluorescence. The optical properties of Zeonex are essential for achieving *in situ* photopolymerization and the integration of an on-chip fluorescence detection module in the future to read out PCR amplification results. A high T_g is essential for high temperatures needed during PCR. The microchips were fabricated by hot-embossing with a nickel–cobalt alloy electroformed master mold (NiColoyTM mold was fabricated by NiCoForm, Inc., Rochester, NY). Designs from this process flow can be easily transferred to the fabrication of more robust master molds for high cycle processes like injection molding. The hot-embossing method greatly simplifies the fabrication process of plastic microfluidic devices (Fig. 1). It is a cost-effective technique and allows the fabrication of high aspect ratio features. Channels of 400 μ m width, 100 μ m depth and 1.5 cm length were fabricated for this application. The microchannels were formed by hot-embossing with the master at 166 °C (30 °C above the T_g of Zeonex 690R) and 250 psi for 5 min using a hot press (Heated Press 4386, Carver, Wabash, IN). The master and the substrate were manually separated at the de-embossing temperature, 126 °C. Wells of 1.5 mm diameter were drilled at the ends of

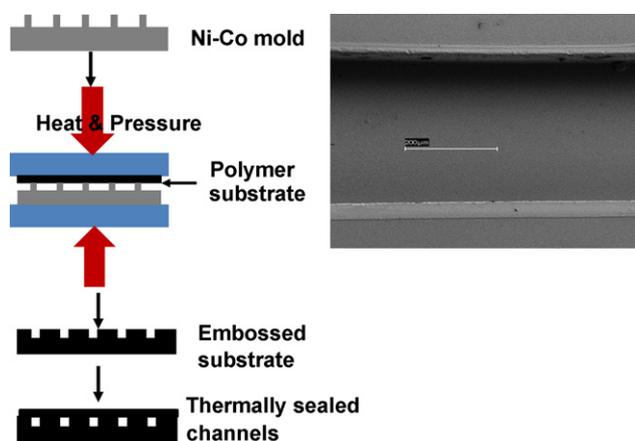


Fig. 1. (a) The fabrication process of the plastic microfluidic chips, (b) SEM micrograph (top view) of a $400\ \mu\text{m}$ (width) \times $100\ \mu\text{m}$ (depth) channel.

the embossed microchannels for sample introduction and collection. To seal the channels, another piece of Zeonex of the same dimensions was thermally bonded ($136\ ^\circ\text{C}$, 250 psi, 2 min) on top in the hot press.

2.3. Formation of the solid-phase

The solid-phase was prepared within the microchannels by means of surface modification via photografting followed by *in situ* photopolymerization through the outside of the chip, as described elsewhere [22]. Briefly, the microchannels were filled with a 1:1 mixture of EDA and MMA with 3% benzophenone, which is a hydrogen abstracting photoinitiator. The chip was then UV-irradiated for 10 min at 254 nm UV wavelength and $100\ \text{mJ}/\text{cm}^2$ energy in an ultraviolet exposure instrument (CL-1000 UV Crosslinker, UPV Inc., Upland, CA). The surface grafting process leaves a number of unreacted double bonds covalently attached to the surface. XPS (X-ray photoelectron spectroscopy) analysis of the unmodified and modified Zeonex surfaces confirmed the success of the grafting reaction, as described elsewhere [22]. The grafting step was followed by the preparation of a porous poly(butyl methacrylate-co-ethylene dimethacrylate) monolith within the grafted channels. The surface-modified channels were filled with a mixture consisting of BuMA (15 wt%), EDMA (10 wt%), 1-dodecanol (52.5 wt%), cyclohexanol (22.5 wt%), DMPAP (1 wt% with respect to monomers) and silica microbeads. The microchips were then irradiated with UV for 1.2 min. The UV-polymerization forms a microporous monolith impregnated with silica particles within the channel (Fig. 2). The excess reagents were rinsed from the channels with methanol prior to use.

2.4. MDCK cell culture and viral culture

MDCK cells were cultured according to the guidelines in the “WHO Manual on Animal Influenza Diagnosis and Surveillance” [23]. Growth medium contained Eagle’s Minimum Essential Medium with Earle’s BSS and 2 mM L-glutamine (EMEM) supplemented with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate and 10%

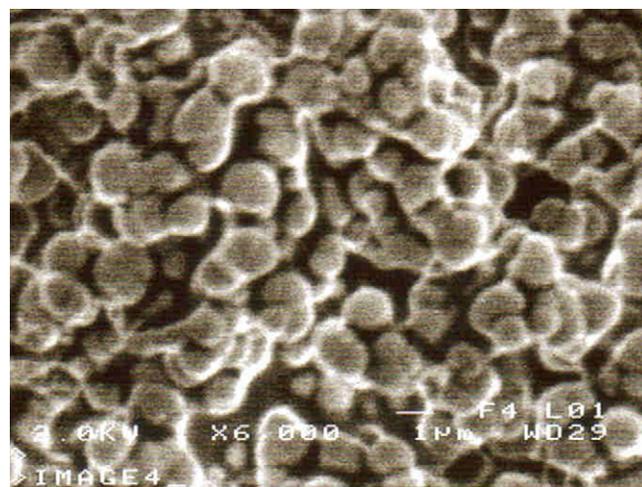


Fig. 2. SEM image of porous monolith at $6000\times$.

fetal bovine serum. Cells were kept at $37\ ^\circ\text{C}$ and 5% CO_2 and medium was changed every 2–3 days. The MDCK cells were inoculated with influenza A when the cells were between 60 and 80% confluent. Before inoculation, MDCK culture medium was changed to contain the virus growth medium. The viral growth medium was EMEM supplemented with $1\ \mu\text{g}/\text{mL}$ TPCK treated trypsin, 0.125% BSA and 1% HEPES buffer. After 5 days in culture following inoculation, cell rounding was observed which indicated the onset of cytopathic effect. A hemagglutination (HA) assay was run to determine the number of virus particles present in the culture. Serial dilutions of the cell culture supernatant were added to a 96 well plate that contained equal numbers of sheep red blood cells (Sigma-Aldrich, St. Louis, MO) in Alsever’s solution and hemagglutination was observed visually. Hemagglutination occurs when influenza virus particles bind to the surface proteins of red blood cells. If the RBCs are in solution, they will remain suspended if the virus is present in large amounts. If the virus is not present, or is present in small dilutions, it cannot bind to the RBCs and they fall out of solution and appear as a button or a ring of red cells at the bottom of the microtiter plate. The highest dilution of the virus that causes complete hemagglutination is the HA titration endpoint. We observed an HA titer of 4 HA units/mL for the assay, which indicated that the virus was present in large amounts in the culture.

2.5. Viral RNA isolation

Both the pelleted cells and cell culture supernatant were collected to test the microfluidic solid phase extraction columns. The sample was mixed with GuSCN (guanidium thiocyanate) containing lysis buffer in 1:1 ratio and flowed over the solid phase extraction columns. Poly A carrier RNA at a concentration $10\ \mu\text{g}/\text{mL}$ was pre-added to the lysis buffer to enhance the recovery of RNA in the subsequent steps. The purification process was carried out at room temperature. The sample, wash solutions and elution buffer were passed through the microchannels at a flow rate of $300\ \mu\text{L}/\text{h}$ with a KDS100 syringe pump (manufactured by KD Scientific, Holliston, MA). $15\ \mu\text{L}$ of the lysed sample

was loaded into a μ SPE channel for 3 min. The channel was then washed with 70% ethanol for 2 min, followed by a wash with 100% ethanol for another 2 min. Finally, 15 μ L of RNase free water was flowed through the channel for 3 min to elute the extracted RNA. The total time required for the entire purification process is 10 min. The eluted total RNA was reverse transcribed into cDNA, and the cDNA was then PCR amplified. The reverse transcription was done off-chip using the SuperScript[®] II Kit (Invitrogen, Carlsbad CA). PCR amplification was performed using an ABI 7500 Real Time PCR machine. The primers and probes specific for influenza matrix protein (M1) gene of influenza A were designed by Applied Biosystems (Assays-on-Demand[™] Lot# 378578, Applied Biosystems, Foster City, CA). 11.25 μ L of the cDNA sample was added to 1.25 μ L of primer/probe mix and 12.5 μ L of TaqMan[®] Universal PCR Master Mix (2 \times) to form a final volume of 25 μ L/sample per well. The thermal cycling protocol used was 95 $^{\circ}$ C for 10 min (for DNA polymerase activation), followed by 50 cycles of 95 $^{\circ}$ C for 0.15 min and 60 $^{\circ}$ C for 1 min. All the samples were run in triplicate to minimize pipetting errors.

We compared the viral RNA extraction results from our μ SPE columns against a commercially available viral RNA extraction kit (QIAamp[®], Qiagen Inc., Valencia, CA), which is considered a “gold standard” method. The Qiagen columns contain a silica-gel membrane which binds the virus RNA while contaminants pass through. The sample volume required for the Qiagen assay is 140 μ L. The columns have to be washed twice with buffers provided with the kit, and the extracted RNA is then eluted in 50 μ L of water or low-salt buffer. The procedure takes up to 40 min and requires either a bench-top centrifuge or a vacuum connection for processing the sample.

3. Results

We have demonstrated the nucleic acid extraction efficiency of the polymer monolith/silica column from several increasingly complex solutions on-chip. We previously showed extraction of phage λ DNA from a buffer solution containing 3% BSA (Bovine Serum Albumin) [22]. The nucleic acid extraction efficiency was tested using spectroscopic measurement (A260/A280) of the eluate and using fluorescence microscopy. The extraction efficiency was found to be as high as high as 70 \pm 3%, which is comparable with the sol-gel methods [24,25]. Here, we demonstrate the ability to obtain PCR amplifiable product from MDCK cells infected with influenza A. The infected cell lysate plus the cell culture supernatant was used to ascertain the viral RNA extraction efficiency. We performed four separate runs with the sample to verify consistent results. For each RNA extraction run, clean unused μ SPE channels were used to verify chip-to-chip consistency. The four separate runs were also performed on different days to verify day-to-day consistency. We then reverse transcribed and PCR amplified all of the extracted RNA samples simultaneously. The fluorescence signal intensity (ΔRn) was plotted as a function of the thermocycle number for the amplification of the M1 gene found in influenza A. Fluorescence intensity (ΔRn) is calculated by using the equation $\Delta Rn = (Rn^+) - (Rn^-)$, where Rn^+ equals the ratio

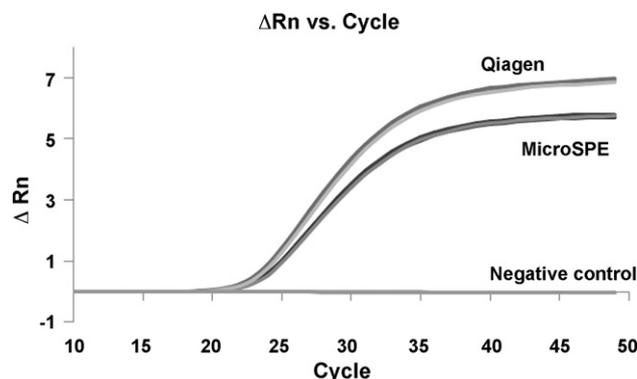


Fig. 3. Real-time PCR amplification of influenza A M1 gene comparing the Qiagen column with our microfluidic isolation method.

of fluorescence emission intensity of the reporter dye and emission intensity of the passive reference dye during a reaction, and Rn^- equals the ratio during the initial PCR cycles where there is little change in fluorescence signal (baseline signal). Fig. 3 shows a representative real-time RT-PCR amplification plot for samples obtained using the Qiagen kit and our μ SPE columns. The PCR results verify that the μ SPE system allowed for successful extraction and elution of viral RNA in the polymeric microchip, and the eluted RNA sample was PCR amplifiable. Fig. 4 shows the threshold cycle value (C_T) for samples obtained using the two different RNA extraction methods (Qiagen versus μ SPE columns). The mean C_T value for the two methods was 26.65 ± 1.11 and 29.58 ± 2.28 for Qiagen and μ SPE column, respectively. There was an average C_T difference of 2.93 between the two methods, which corresponds to a $2^{2.93} = 7.62$ -fold drop in substrate between the two extraction methods. The loss in copy numbers indicated that the RNA yield from the μ chipSPE method was lower than the yield from the Qiagen kit. We were concerned that the wash steps in the channels were not as efficient as the wash steps in the Qiagen method, resulting in the lower yield. We repeated the experiment several times using different wash buffers and washed the channels for longer time periods, but the C_T values did not change significantly, suggesting that the limitation on the amount of DNA and RNA recovered are dependent on the amount of silica surface area available in the polymer/silica monolith and not on the buffers used. We anticipate that increasing the amount of silica in the polymer

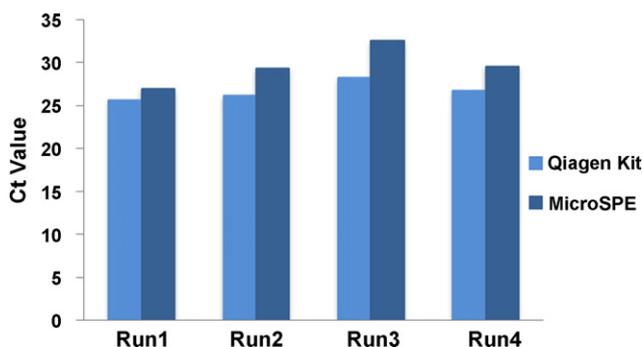


Fig. 4. Threshold cycle (C_T) values obtained with the Qiagen column vs. our μ SPE columns.

monolith and/or increasing the length of the separation channel will increase the capture efficiency of the μ SPE column and raise our nucleic acid yields in future applications. However, the sample purification methodology was able to preconcentrate the RNA samples effectively, and there was sufficient RNA obtained for the PCR amplification of the M1 gene.

Despite the comparatively lower yield from our μ chipSPE system, our data have demonstrated that we can successfully isolate nucleic acids from viruses cultured in mammalian cells. PCR inhibitors such as divalent cations and proteins were efficiently removed in two efficient wash steps, leaving pure nucleic acids to be eluted in water. The PCR amplification results from the different channels were also relatively consistent, which gives us confidence that our on-chip solid-phase system is a robust and a reliable platform for purification of nucleic acids.

4. Discussion

We have developed a plastic lab-on-a-chip platform for purification of viral RNA using microfluidic channels that incorporate silica particles in microporous polymer monoliths to form a μ SPE system. The microfluidic device was fabricated in plastic using micro-hot-embossing method and the μ SPE column was formed inside the plastic microchannel via *in situ* photopolymerization. The low-cost of the fabrication process and the simple procedure for generating the μ SPE column makes this system practicable for one-time use, disposable applications. The μ SPE technique was capable of lysing viral particles and binding, concentrating and eluting nucleic acids from the influenza infected mammalian cell lysate. The sample purification procedure itself is simple and requires minimal user-handling. The entire purification process requires \sim 10 min and can potentially be performed by untrained personnel. The eluted nucleic acids were PCR amplifiable templates as demonstrated by the successful amplification of the M1 gene found in influenza A virus. The reverse transcription and PCR amplification/detection was performed off-chip in this particular application. Our long-term goal is to couple the sample purification process with on-chip RT-PCR to obtain an integrated genetic analyses device for point-of-care clinical diagnostics. The sample purification process developed here is flexible enough for use in a variety of different applications where the preconcentration of nucleic acids is necessary prior to downstream processing.

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Biographies

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