An automated microfluidic system for single-stranded DNA preparation and magnetic bead-based microarray analysis

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We present an integrated microfluidic device capable of performing single-stranded DNA (ssDNA) preparation and magnetic bead-based microarray analysis with a white-light detection for detecting mutations that account for hereditary hearing loss. The entire operation process, which includes loading of streptavidin-coated magnetic beads (MBs) and biotin-labeled polymerase chain reaction products, active dispersion of the MBs with DNA for binding, alkaline denaturation of DNA, dynamic hybridization of the bead-labeled ssDNA to a tag array, and white-light detection, can all be automatically accomplished in a single chamber of the microchip, which was operated on a self-contained instrument with all the necessary components for thermal control, fluidic control, and detection. Two novel mixing valves with embedded polydimethylsiloxane membranes, which can alternately generate a 3- μ l pulse flow at a peak rate of around 160 mm/s, were integrated into the chip for thoroughly dispersing magnetic beads in 2 min. The binding efficiency of biotinylated oligonucleotides to beads was measured to be 80.6% of that obtained in a tube with the conventional method. To critically test the performance of this automated microsystem, we employed a commercial microarray-based detection kit for detecting nine mutation loci that account for hereditary hearing loss. The limit of detection of the microsystem was determined as 2.5 ng of input K562 standard genomic DNA using this kit. In addition, four blood samples obtained from persons with mutations were all correctly typed by our system in less than 45 min per run. The fully automated, “amplicon-in-answer-out” operation, together with the white-light detection, makes our system an excellent platform for low-cost, rapid genotyping in clinical diagnosis. © 2015 AIP Publishing LLC.

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I. INTRODUCTION

In modern medicine, molecular diagnosis is a burgeoning field that spans from mutation analysis,^{1,2} single nucleotide polymorphism (SNP) genotyping,³ detection of chromosome abnormalities,^{4,5} as well as infectious disease identification.⁶ Among all the molecular

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diagnostic technologies, such as microarray, capillary electrophoresis, real-time polymerase chain reaction (PCR), Sanger-based DNA sequencing, and more recently, next-generation DNA sequencing, microarray-based analysis is often the best choice for clinical applications due to its combined advantages in throughput, multi-target detection, and cost.^{7,8} However, the conventional microarray has several critical drawbacks: (i) in a conventional hybridization process, samples and spot arrays are covered by a coverslip, and incubated for a long period of time. This static hybridization is time-consuming and inefficient because of the diffusion limit (the typical moving distance of nucleic acids by diffusion is ~ 1 mm in 24 h);^{9,10} (ii) to date, the most common approach for microarray detection is laser-induced fluorescence confocal scanning, which is expensive, delicate, and hard to miniaturize;^{8,11} (iii) the current operation workflow, including nucleic acid extraction, PCR amplification, single-stranded target preparation, hybridization, washing of non-specific binding, detection, and data analysis, relies on several bulky instruments for each individual step. As a result, the process is lengthy, prone to contamination, and unfavorable for the applications in resource-limited settings.^{12,13}

By taking the advantages provided by microfabrication technology, microfluidic-based microsystems have demonstrated a great potential to eliminate the drawbacks associated with the conventional microarray. In the late 1990s, electric fields were first reported to concentrate negatively charged DNA strands to probes on electrodes and the hybridization process was thus accelerated.¹⁴ Since then, a number of mechanisms, such as hydrodynamic flow,^{10,15} acoustic wave,¹⁶ electrokinetic,¹⁷ and magnetic deliveries,^{18,19} have been developed to transform the static microarray into a microfluidic format in order to overcome the diffusion limit of the hybridization. Lee *et al.* developed a recirculating microfluidic device that repeatedly flowed samples to a probe array using a peristaltic pump to reduce the hybridization time from 6 to 2 h.²⁰ Recently, Karsenty *et al.* employed an isotachophoretic method to focus targets to arrays in a straight channel, demonstrating two orders of magnitude improvement in limit of detection.²¹ In parallel with the development of the microfluidic-based microarray, a variety of non-fluorescence-based detection methods, including electrochemical measurement,^{22,23} chemiluminescence,²⁴ gold,²⁵ and magnetic particles,²⁶ have been demonstrated on microchips to further reduce the cost of the systems. For example, Pettersson *et al.* utilized a magnetic field to deliver magnetic bead (MB)-labeled target nucleic acids to complementary probes immobilized on a glass surface in less than 15 s.¹⁹ The detection of the hybridization results was achieved instantly since the beads became visible on the surface. Using the similar method, the hybridization of DNA labeled with MBs can be easily detected with a smartphone camera.²⁷ Unfortunately, all these works were focused on the hybridization and detection steps only, and thus the advantages of the microfluidics were not fully explored.

There is no doubt that the microfluidic-based microarray analysis could be significantly improved, especially for the applications in clinical diagnosis, if the entire operation was fully automated and integrated into a micro total analysis system. In 2004, Liu *et al.* successfully developed an integrated microdevice that is capable of performing magnetic bead-based cell capture, cell lysis, PCR, DNA hybridization, and electrochemical detection.²³ Pathogenic bacteria detection from milliliters of whole blood samples and SNP analysis from blood were demonstrated. Following this pioneering work, PCR amplifications were robustly integrated with microarrays on either a glass microchip²⁸ or a plastic flow cell²⁹ for SNP and infectious agent detections, respectively. More recently, Zhu *et al.* successfully constructed a simple microdevice with a single chamber in which MB-labeled single-stranded DNA (ssDNA) was hybridized to a tag array in about 15 min.³⁰ With the aid of external valves and pumps, the hybridization, washing, and detection processes were fully automated. While these achievements are impressive, ssDNA preparation on a chip has seldom been demonstrated and many fully integrated systems employed asymmetric PCR amplifications instead. In fact, considering the sensitivity and the efficiency demanded by clinical diagnosis, symmetric PCR followed by single-stranded target preparation is still preferred.^{31,32} Magnetic separation of DNA strands with streptavidin-coated magnetic beads is one of the most widely used methods in conventional centrifugal tubes, because of the high yield of ssDNA and the easy handling of MBs.^{33,34} Likewise, the MB-based ssDNA preparation should be suitable to the on-chip microsystem. However, to the

best of our knowledge, the on-chip integration of the ssDNA isolation by MBs and the microfluidic-microarray analysis with a white-light detection has not been reported yet.

Here we present the development of an integrated microfluidic device capable of performing single-stranded DNA preparation and magnetic bead-based microarray analysis automatically. The streptavidin-coated magnetic beads functioning as both strand isolation agents and DNA labels seamlessly combine these two steps in a single microchamber to form an “amplicon-in-answer-out” system. By incorporating two novel mixing valves, as well as using a fluidic control system with an eight-way modular valve positioner (MVP) and a syringe pump, the dispersion and the collection of magnetic beads in the microchip were realized efficiently and reproducibly. Microarray analyses of nine mutation loci associated with hereditary hearing loss were carried out to test the automated operations of this system for mutation detection. This work demonstrated the feasibility of building an automated microfluidic system for the “amplicon-in-answer-out” analysis, which significantly reduces the turnaround time per assay and the risk of contaminations for clinical diagnosis.

II. MATERIALS AND METHODS

A. Reagents

Streptavidin-coated magnetic beads (Dynabeads[®] MyOne[™] Streptavidin C1, 1.05 μm in diameter) were purchased from Life Technologies (Foster City, CA). The binding and washing buffer (B&W buffer) for the magnetic beads was prepared according to the manufacturer’s manual (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, pH 7.5). A nickel-plated neodymium magnet (size: 10 $\times$ 10 $\times$ 5 mm, Link Technology, Beijing, China) was employed for the bead immobilization on the chip. To test the DNA binding efficiency in the microchip, 24-mer oligonucleotides (5′-ATCAGAGCTTAACTGGGAAGCTG-3′) labeled with FAM on the 5′ and biotin on the 3′ end, respectively, were synthesized from Sangon (Shanghai, China). In the test of single-stranded DNA preparation, 19-bp double-stranded DNA (5′-CCCTGGGCTCTGTAAAGAA-3′, Sangon) were labeled with FAM and biotin on one strand and Cy5 on the other. Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO) for the chip coating.

Standard K562 genomic DNA (Promega, Madison, WI) was employed for the limit-of-detection (LOD) test on the microsystem. Four blood samples from persons with known mutations were provided by CapitalBio with informed consents for the on-chip microarray analyses. DNA was extracted from these blood samples using a QIAamp[®] DNA Micro Kit (Qiagen, Germantown, MD). A hybridization buffer (9 $\times$ SSC, 7.5 $\times$ Denhardt’s reagent, 37.5% (v/v) formamide, 0.15% (w/v) SDS) and a washing buffer (0.3 $\times$ SSC, 0.1% (w/v) SDS) were prepared as described previously.

B. Microchip design and fabrication

As presented in Fig. 1, the microfluidic device with dimensions of 25.4 $\times$ 76.2 mm is comprised of three layers (from top to bottom): a PMMA cover, a piece of double-sided adhesive tape (QL-9970-025, Wuxi Bright Technology, Wuxi, China), and an aldehyde-modified glass slide with a pre-spotted tag array (CapitalBio, Beijing, China). The microfluidic structures, which are cut into the adhesive tape and enclosed by the PMMA cover and the glass slide, include a reaction chamber, two mixing chambers, and microchannels. The heights of these structures are determined by the thickness of the adhesive tape (250 μm). The hexagonal reaction chamber, where single-stranded DNA is prepared and hybridized to the tag array, is 10 mm in length and 6 mm in width with a volume of 16 μl . On each side of the reaction chamber, an 8-mm-diameter mixing chamber is designed. To realize the mixing function, a piece of 500- μm -thick PDMS (polydimethylsiloxane) membrane (BISCO[®] HT-6240, Rogers, Woodstock, CT), which can be deformed by an external plunger, is sandwiched between the PMMA cover and the mixing chamber to form a mixing valve. A 10 $\times$ 10 mm square compartment with a depth of 700 μm is machined on the lower surface of the PMMA cover for accommodating the PDMS membrane. In the center of the compartment, an 8-mm-diameter hole is

FIG. 1. Integrated microdevice for single-stranded DNA preparation and magnetic bead-based microarray analysis. (a) Exploded view of the microdevice with three layers: a PMMA cover, a double-sided adhesive tape, and a glass slide with a pre-spotted tag array. (b) Photograph of the microchip with dimensions of 25.4×76.2 mm. (c) Cross-section view of the microchip, showing that one mixing valve is open and the other is deformed by a plunger.

drilled so that the plunger can touch the PDMS. Microchannels with a width of 1.5 mm are designed to connect each structure. The inlet and the outlet of the microdevice are formed by drilling 1-mm-diameter via holes through the PMMA cover in the ends of the microchannels. Finally, a thermocouple groove (6.5 mm in length, 1 mm in width and 1.2 mm in depth) is machined on the upper surface of the PMMA cover next to the reaction chamber for temperature measurement.

The device fabrication was performed as follows: the structures on the PMMA layers were milled by a milling machine (MODEL 5410, Sherline, Vista, CA) and the microfluidic patterns were cut into the adhesive tape by a laser engraving system (Speedy 100, Trotec, Marchtrenk, Austria). To assemble the device, a silicone sealant (HT902, Huitian, Xiangyang, China) was first spread evenly on the compartments of the PMMA cover, and then the PDMS membranes were placed immediately. Once the sealant was fully solidified, the PMMA cover was sequentially cleaned with ethanol, detergent, and deionized water (DI water). After completely dried by N_2 , the PMMA cover, the tape, and the glass slide were assembled and pressed with fingers to squeeze out air bubbles, followed by another one-hour press by a metal block to enhance the adhesion. The coefficient of variation (CV) of the heights of the chip structures between different batches was measured to be 4.3% by a scanning electron microscope.

C. Automated and integrated microfluidic system

The schematic of the self-contained instrument for microchip control and detection is presented in Fig. 2(a). The instrument contains a microchip fixture, a fluid control system, an imaging system, and all the necessary electronics. As shown in Fig. 2(b), a custom-made LabVIEW program (National Instruments, Austin, TX) installed in a laptop is used to send commands through a data acquisition board (DAQ, USB-6259 OEM, National Instruments) and to collect data from the imaging system of the instrument.

As shown in Figs. 2(c) and 2(d), the microchip fixture consists of a chip platform and a manifold. The microdevice is placed into a recessed area on the top of the chip platform and held in place with the manifold using screws. The manifold contains two fluidic port connectors (PK1/16-LSM01, Wenhao, Suzhou, China) to provide leakage-proof connections to the inlet

FIG. 2. Compact, self-contained instrument for microchip control and detection. (a) Schematic diagram of the instrument, including a microchip fixture, a fluid control system, an imaging system, and all the necessary electronics. On the tube rack, total seven reagents are stocked: (1) B&W buffer for sample inlet, (2) B&W buffer for bead inlet, (3) B&W buffer, (4) NaOH, (5) hybridization buffer, (6) washing buffer, and (7) HCl. (b) Photograph of the instrument controlled by a laptop. The analysis system box has dimensions of $40 \times 20 \times 20$ cm. (c) Top view of the microchip fixture. (d) Photograph of the microchip fixture when it is open.

and the outlet of the microchip. To actuate the mixing valves, self-locked solenoids (ZHK-0521, ZONHEN Electric Appliances, Shenzhen, China) installed on the manifold are employed to drive plungers, which can protrude through the PMMA cover to press the PDMS membranes. An ITO (indium tin oxide)-coated glass heater (Sheet resistance: $8 \Omega/\text{sq}$; size: $30 \times 10 \times 1.1$ mm; light transmittance: 80%. Huananxiangcheng, Shenzhen, China) and a thermocouple (TT-K-36-SLE, OMEGA, Stamford, CT) are utilized for temperature control during hybridization. The ITO glass is embedded onto the chip platform underneath the reaction chamber of the microchip and the thermocouple inserted into the thermocouple groove on the microchip is sandwiched between the chip and the manifold while operation. Temperature control is accomplished through a proportion/integrator/differentiator module (PID) within the LabVIEW program. To monitor the process and capture the results, a common digital camera (SJM-133C, Weilin, Shenzhen, China) with a set of magnifying lens (GCO-2303, Daheng Optics, Beijing, China) is mounted above the chip, which is illuminated by a white LED array (G-002, Boshida, Shenzhen, China) from bottom.

The schematic of the fluidic control system in the instrument is also shown in Fig. 2(a). On the tube rack, total seven reagents are stocked, including B&W buffer for sample inlet, B&W buffer for bead inlet, B&W buffer, 100-mM NaOH, hybridization buffer, washing buffer, and 2.5-mM HCl. These tubes are connected to an eight-way MVP (Hamilton, Bonaduz, Switzerland), which can switch each tube to the inlet of the microchip under the control of the LabVIEW program. The seven inlets of the MVP are named as their corresponding reagents. The solutions are aspirated through the chip by a computer-controlled syringe pump (PSD/4, Hamilton), on which a 500- μ l Hamilton glass syringe is installed. Once the syringe is full, it expels the waste to a waste tube for the next round of aspiration.

D. On-chip manipulation of magnetic beads

First, 5 μ l of the magnetic beads in a stock solution (10 μ g/ μ l) was directly added into 75 μ l of the B&W buffer to a concentration of 0.625 μ g/ μ l without any pre-washing steps. To test the capability of bead manipulations of our instrument, the microchip fixture as well as the syringe pump and the MVP was removed from the instrument and mounted onto an inverted microscope (IX71, Olympus, Tokyo, Japan) equipped with a CCD camera (Clara, Andor, Belfast, Northern Ireland) for better visualization. A high speed CCD camera (Motion BLITZ Cube 3, Mikrotrotron, Germany) was also used to observe and estimate the moving speed of the beads. The operation procedure is as follows: the microchip was first filled with a 1% (w/v) solution of BSA and incubated for 30 min to block non-specific bead adhesion to the channels. Next, the entire fluidic system as well as the microchip was primed with the B&W buffer to purge air bubbles out. After that, the prepared MB solution (80 μ l) was loaded into the reaction chamber from the bead inlet of the MVP at a speed of 20 ml/h with the magnet placed on the top of the microchip. Once the bead solution passed the bead inlet, the MVP was switched to the B&W buffer inlet to load extra 200 μ l of the B&W buffer carrying all the beads to the magnet and washing the beads thoroughly. The dispersion of the magnetic beads in the chip was achieved by alternately actuating two mixing valves by the solenoids every 3 s for total 10 min without the magnet. To collect the beads in the reaction chamber, the magnet was placed onto the chip again, and then the solution in the chip was moved 40- μ l backward and then 80- μ l forward at a flow rate of 20 ml/h. This dispersion and collection process was repeated four times to verify the system's performance.

E. Characterization of DNA binding with magnetic beads on the chip

The microchip was first prepared and 80 μ l of the MB solution (0.625 μ g/ μ l) was loaded into the chip as described in Sec. IID. Then, 25 μ l of the B&W buffer containing 25 pmol of the 24-mer biotinylated oligos was aspirated into the reaction chamber from the sample inlet of the MVP followed by the continuous loading of 50 μ l of the B&W buffer from the B&W buffer inlet. This extra B&W buffer will move the sample right into the reaction chamber for the following capture step. After that, the mixing of the beads and the oligos was performed in 6 min, followed by the collection of bead-oligo conjugates by the magnet. Next, another 100- μ l B&W buffer was drawn into the chip from the B&W buffer inlet of the MVP to wash all the unbounded oligos away. Finally, all the beads were dispersed again and then taken out from the chip with a pipette for downstream analysis using a flow cytometer (BD FACSAriaTM III, BD Biosciences, Franklin Lakes, NJ). As a comparison, the same amount of the magnetic beads and the oligos were reacted in a tube following the manufacturer's protocol.

F. Single-stranded DNA preparation on the chip

First, 80 μ l of the magnetic beads (0.625 μ g/ μ l) and 25 μ l of the double-stranded DNA were loaded into the chip and reacted following the previous protocol. After the beads were washed by 100 μ l of the B&W buffer, the 100-mM NaOH solution (350 μ l) was drawn from the NaOH inlet of the MVP into the chip at a rate of 20 ml/h for DNA denaturation. Next, the beads were dispersed completely in 3 min, incubated for 3 min for DNA denaturation, and then collected.

Finally, the beads were washed with 400 μ l of the B&W buffer. Fluorescent images were taken using the microscope to verify the process of the single-stranded DNA preparation.

G. DNA microarray analysis

A commercial microarray-based detection kit (CapitalBio) for detecting nine mutation loci that account for hereditary hearing loss was employed to test the performance of our automated system. The detailed information of this kit can be found in previously published literatures.^{2,27} As shown in Fig. 3, the multiplex allele-specific PCR (ASPCR) was performed in centrifugal tubes. For each locus, allele-specific primers containing coded tag sequences for both wild-type and mutational alleles, as well as a biotinylated common primer, were designed to generate double-stranded PCR products with a biotin on one end and anti-tag sequences on the other. The multiplex reactions were carried out in two separated tubes: tube A includes c.176_191del16, c.235delC, c.299_300delAT, m.1494C > T, and m.1555A > G; tube B includes c.35delG, c.538C > T, c.2168A > G, and IVS7-2A > G.

FIG. 3. Overview of the magnetic bead-based microarray analysis for mutation detection. Allele-specific PCR shown in the upper box is conducted in centrifugal tubes. The single-stranded DNA preparation followed by the magnetic bead-based microarray analysis shown in the lower box is automatically completed in the microfluidic system. W and M stand for wild type and mutation, respectively.

After the amplifications, the rest of the analytical process was performed on the chip. As illustrated in Fig. 3, the biotinylated PCR products were first bound to the streptavidin-coated magnetic beads through biotin-streptavidin interaction. Then, by alkaline denaturation, single-stranded DNA with an anti-tag on one end and a magnetic bead on the other was obtained. To decode the anti-tag sequence of the ssDNA-MB conjugate, a tag array chip containing all the tag probes with the same sequences as those in the allele-specific primers was provided by CapitalBio and directly integrated into our system as a part of the hybrid microchip. Modified with a poly(dT) spacer and a 5' amino group, these tag probes were spotted onto an aldehyde-modified glass slide. The diameter of each tag spot is 150 μm with a center-to-center distance of 300 μm . In the hybridization step, the ssDNA-MB conjugates hybridized to their corresponding tag spots with complementary tag sequences on the chip, leading to the aggregation of the magnetic beads on the spots. The results can be easily detected using a common camera and the genotypes of the samples can be determined.

H. Fully automated microarray-based analysis

K562 standard DNA ranging from 1–25 ng and four 25-ng extracted DNA from four blood samples were used for allele-specific PCR amplifications. Each PCR reaction was performed in a volume of 12.5 μl , containing 2.5- μl DNA template, 5- μl primer mix, and 5- μl PCR master-mix. The thermal cycling performed in a Mastercycler[®] nexus PCR thermal cycler (Eppendorf, Hamburg, Germany) starts with an initial activation of the Taq polymerase at 95 °C for 15 min. In the following 35 PCR cycles, the temperature is held at 94 °C for 30 s denaturing, then ramped to 55 °C at 0.4 °C/s for 30 s annealing, and then to 70 °C at 0.2 °C/s for 45 s extension. Finally, a post extension step is performed at 60 °C for 10 min. After amplification, PCR products in two tubes were mixed together for downstream analysis.

The detailed operation protocol of the automated microarray system is presented in Fig. 4. Briefly, the pretreatment of the microchip, the loading of the 80- μl magnetic beads and the 25- μl PCR samples, the bead dispersion, the bead collection, the DNA denaturation, and the washing were conducted following the exact same protocols listed above. After that, 300 μl of HCl was loaded into the microchip for NaOH neutralization, followed by an extra washing step with 300 μl of the B&W buffer. To start the hybridization, the hybridization buffer (300 μl) was first introduced and then the magnetic beads were completely dispersed in 3 min. Next, the dynamic hybridization was conducted for 15 min at 50 °C. During this process, the buffer flows back and forth with an offset volume of 20 μl at a speed of 20 ml/h. Finally, the microchip was washed with 300 μl of the washing buffer at 40 °C and recorded by the camera. The pictures were converted into grey-scale images for better visualization using the ImageJ. The total analytical time is about 45 min.

III. RESULTS AND DISCUSSION

A. On-chip dispersion and collection of magnetic beads

In this work, we employed magnetic beads functioning as both strand isolation agents and DNA labels to seamlessly integrate the single-stranded DNA preparation and the bead-based microarray analysis together on the chip. Thus, the operations of the magnetic beads, including the bead dispersion and the collection, are crucial to the performance of the entire analysis,³⁵ because aggregated beads would have a very low binding efficiency to targets, and a poor bead collection would lose DNA-bead conjugates during the buffer exchange and the washing steps. Unfortunately, these operations, which are easy to conduct in a tube, could be particularly challenging in a microchip because of the laminar flow nature of microfluidics, the enclosed structure, as well as the automated operation. Here we integrated two mixing valves into the microchip to disperse the beads in the chamber, and then employed a magnet to collect the beads while slowly flowing the solution back and forth with the fluidic control system. To quantitatively evaluate the performance of this method, we defined two characterization

FIG. 4. Operation workflow of the single-stranded DNA preparation and the magnetic bead-based microarray analysis in the microchip. (a) Magnetic bead loading. 80 μ L of the magnetic beads diluted in the B&W buffer was loaded into the reaction chamber at a speed of 20 ml/h with the magnet placed on the microchip, followed by 200 μ L of the B&W buffer to carry all the beads to the magnet and to wash away the impurities in the bead solution. (b) Sample loading. 25 μ L of samples were aspirated into the reaction chamber followed by the continuous loading of 50 μ L of the B&W buffer. (c) Bead dispersion for DNA capture. The magnetic beads were completely dispersed in the chamber by alternately actuating two mixing valves every 3 s for total 6 min. (d) Bead collection and washing. To collect the beads, the magnet was placed onto the chip again, and then the solution in the chip was moved 40- μ L backward and then 80- μ L forward at a flow rate of 20 ml/h to bring all the beads to the magnet. Then, 100 μ L of the B&W buffer was drawn into the chip to remove any unbound DNA. (e) Alkaline denaturation. 350 μ L of NaOH was loaded into the chip while the beads were captured by the magnet. The beads were dispersed completely in 3 min, and then incubated for 3 min for DNA denaturation. (f) Washing and neutralization. The magnetic beads were collected again and then washed with a 400- μ L B&W buffer. After that, 300 μ L of HCl was loaded into the microchip for NaOH neutralization, followed by an extra washing with 300 μ L of the B&W buffer. (g) Loading of hybridization buffer. 300 μ L of the hybridization buffer was introduced and then the magnetic beads were completely dispersed in 3 min. (h) Dynamic hybridization. The hybridization was conducted for 15 min at 50 $^{\circ}$ C, while the buffer flows back and forth with an offset volume of 20 μ L at a speed of 20 ml/h. (i) Washing and detection. The microchip was washed with 300 μ L of the washing buffer at 40 $^{\circ}$ C, and the result was taken by the camera.

parameters. First, the loss of the beads after each dispersion and collection cycle can be measured by the relative density (RD) of the beads shown below

$$RD = \frac{N}{A}, \quad (1)$$

where N is the pixels that the magnetic beads occupy and A is the total pixels in a field of view taken by the CCD camera on the microscopy. Apparently, due to the slight bead aggregation, as well as the bead overlaps in the channel height direction, the relative density can only partially represent the amount of beads in the chamber. But this measurement should be still accurate enough to reflect the change of the bead amounts, as the height-to-width ratio of the microstructure is less than 0.05. Second, to characterize the uniformity of the bead dispersion in the chamber, the field of the microscope view is evenly divided into 4×4 sub-fields as shown in the inserts of Fig. 5(c), and then the RD of each sub-field is measured. The CV of these 16 RD values can be calculated with Eq. (2),

$$CV = \frac{s}{\bar{d}} \times 100\%, \quad (2)$$

where s is the standard deviation of the relative densities of the beads in the 16 sub-fields, and $\bar{d}$ is the average RD. This coefficient could accurately demonstrate how uniform the bead distribution can be achieved in the chamber.

To test the bead manipulations on the microchip, $80 \mu\text{l}$ of the bead solution in a concentration of $0.625 \mu\text{g}/\mu\text{l}$ was loaded into the chamber. The dispersion of the magnetic beads was achieved by alternately actuating two mixing valves in every 3 s for total 10 min. After the dispersion process, the magnet was installed on the top of the microdevice. As the flow carrying the magnetic beads was moved back and forth passing the magnet, all the beads can be efficiently captured. This dispersion and collection cycle was repeated four times in the experiment

FIG. 5. On-chip dispersion and collection of magnetic beads. (a) Photographs of the reaction chamber taken at different time points in a cycle of the bead dispersion and collection using the mixing valves. (b) Photographs of the bead dispersion using the syringe pump without the valves. (c) Evaluation of the on-chip bead dispersion by the mixing valves and by the syringe pump. When the beads were dispersed using the mixing valves, the CV, which reflects the uniformity of the bead distributions, decreased sharply from over 100% down to around 20% in 2 min, and then became stable. In contrast, the CV was almost unchanged throughout the mixing process when only using the syringe pump. The inserts show the images of the bead distributions in the chamber before and after the dispersions. (d) Characterization of the bead loss and the bead distribution in four consecutive cycles. Both the RDs and the CVs were almost unchanged during the process.

to simulate the bead operations in the on-chip microarray analysis. During the dispersing process of each cycle, the images of the bead distributions in the chamber were taken and the CVs were calculated at a series of time points. In the end of each cycle, the RDs were calculated to estimate the bead loss during the process. As a reference, a well-dispersed magnetic bead solution was directly loaded into the chamber without the magnet on the chip and the CV in this situation (reference CV) presented the best dispersion of beads that can be achieved in the chamber. To further prove the function of the mixing valves, bead dispersions on the chip using a syringe pump without the valves were conducted as a comparison. In this experiment, the same number of the magnetic beads were loaded into the chamber by the magnet, and then moved back and forth with an offset volume of $8\ \mu\text{l}$ in a 3-s interval (flow speed: 160 mm/s) using the syringe pump for total 10 min. All the experiments described above were repeated three times independently for a more reliable evaluation.

As demonstrated in Fig. 5, the initial CV at the beginning is above 100% due to the complete bead aggregation under the magnet. When the beads were dispersed using the mixing valves, in the first 2 min of the operation, the CV rapidly reduced to $\sim 20\%$, which is already close to the reference CV (15%). After that, the CV became stable, indicating that our mixing method can disperse the beads completely in only 2 min. In contrast, when the beads were dispersed by the syringe pump, the CV remained around 80% throughout the process. This comparison proved the excellent performance of these two mixing valves for bead dispersions on the chip. Although the offset volume generated by the mixing chamber is only about $3\ \mu\text{l}$, the actuation time of the self-locked solenoid is less than 0.01 s. A maximum flow speed of 160 mm/s in the cross-section of the reaction chamber (see supplementary material video 1 for the measurement of the beads velocity) can be generated by the valves.³⁶ As demonstrated in the supplementary material video 2,³⁶ such high-speed, tiny-volume stroke can efficiently disturb the beads in the chamber while still constraining the beads in the chamber. In contrast, the syringe pump in the instrument can only generate such a high flow rate with a large volume, which will lose the beads completely. Fig. 5(d) demonstrated the relative density and the coefficient of variation measured in the end of each dispersion step. The almost unchanged RD and CV illustrate that the loss of the magnetic beads during four consecutive dispersion and collection cycles is negligible and a uniform distribution of the beads in the chamber can be achieved repeatedly. This result proved that the magnetic beads could be effectively mixed and uniformly distributed without aggregation in the microchip, which will benefit the following binding process dramatically.

B. On-chip binding efficiency of DNA and magnetic beads

The binding of biotinylated DNA with streptavidin-coated magnetic beads is the first and the most important step to prepare single-stranded DNA. To test the binding efficiency in the microchip, the 24-mer oligonucleotides labeled with FAM on the 5' and biotin on the 3' end, respectively, was employed (shown in Fig. 6(a)). First, $80\ \mu\text{l}$ of the magnetic beads ($0.625\ \mu\text{g}/\mu\text{l}$) and $25\ \mu\text{l}$ of the oligo solution ($1\ \mu\text{M}$) were loaded into the reaction chamber. Then the magnetic beads and the oligos were completely mixed using the method described above. After the washing and the dispersing steps, the beads with the bound oligos were collected and tested with a flow cytometer to obtain the histogram statistics of the fluorescent intensity of the beads. As a comparison, equal amount of the magnetic beads and the oligo solution were reacted in a tube using the conventional method. The experiment was repeated three times for a more accurate measurement.

We first gated the populations of single beads in the scatterplots with the forward scatter (FSC) and the sideward scatter (SSC), as shown in Fig. 6(b). This gating step is important because there are debris and impurities present in the solutions, causing scattered noises in the plots. In addition, due to the different washing processes in the on- and off-chip operations, the gates must be slightly adjusted to filter out these noises correctly. Then, based on the FAM fluorescent intensity data, the histograms of these single-bead populations were plotted and the average fluorescent intensities were calculated. As presented in Fig. 6(c), the average

FIG. 6. Comparison between the on-chip and the off-chip binding efficiencies of the biotinylated ssDNA and the streptavidin-coated magnetic beads. (a) Schematic of the experiment design. The 24-mer oligonucleotides labeled with FAM on the 5' and biotin on the 3' end, respectively, was employed to react with the magnetic beads. (b) Scatterplots of the flow cytometry results of the on-chip and the off-chip tests, as well as the negative control. The populations of single beads were indicated by the gates in the scatterplots and the bead numbers are listed on the top left corner of each plot panel. (c) Histograms of the FAM fluorescent intensities plotted from the corresponding single-bead populations in (b). The average fluorescent intensity obtained in the on-chip test (repeated 3 times) is about 80.6% of that of the off-chip control.

fluorescent intensity obtained in the on-chip test (repeated three times) is about 80.6% of that in the off-chip control. The slight drop of the binding efficiency is probably due to the loss of the beads and reagents during the on-chip operation. In addition, the absolute binding capacity of the magnetic beads using the off-chip method was measured to be ~ 500 pmol oligos per 1 mg MBs (data not shown). Consequentially, the binding capacity using our microsystem is estimated as 410 pmol oligos per 1 mg MBs.

C. Verification of single-stranded DNA preparation on the chip

The single-stranded DNA preparation process includes: DNA capture by magnetic beads, washing of unbound DNA, double-stranded DNA denaturation, and washing of denatured DNA strands. To verify this process performed on the microchip, the 19-bp double-stranded oligonucleotides labeled with FAM and biotin on one strand and Cy5 on the other were employed to

test with the streptavidin-coated magnetic beads. The operation parameters for the bead manipulation and the binding reaction were kept the same as those described above. First, following the loading of the magnetic beads, the double-stranded DNA was flowed into the chamber while the beads were captured by the magnet. The images of the chamber in the bright field, the FAM channel, and the Cy5 channel were taken to verify the process. As demonstrated in Fig. 7(a), the dark area in the bright field represents the captured magnetic beads in the chamber. In the views of the FAM and the Cy5 channels, the entire images were bright, indicating the presence of double-stranded DNA in the entire chamber. Next, the beads and the DNA were allowed to react by dispersing the beads completely with the mixing valves for 6 min and then washed to remove unbound DNA in the chamber. As shown in Fig. 7(b), in the views of the FAM and the Cy5 channels, only the areas corresponding to the places where the beads were captured in the bright field showed the green and the red light, respectively, and the background turned to dark, indicating that the double-stranded DNA had been captured by the beads via the streptavidin-biotin interaction and all the free DNA was washed away. Finally, the 100-mM NaOH was drawn into the chamber to release the single-stranded DNA labeled with Cy5. After the bead dispersion and collection cycle, the chamber was washed again with the B&W buffer. Fig. 7(c) illustrates that the Cy5 fluorescence emitted from the magnetic beads decreased dramatically while the FAM signals were about the same as that taken before the NaOH treatment. It proved that the Cy5-labeled strands were successfully released and washed away leaving no signals in the Cy5 channel and the FAM-labeled strands stayed on the beads due to the strong streptavidin-biotin bonding. This experiment validates the feasibility of automatically performing the single-stranded DNA preparation on the chip.

FIG. 7. Validation of the on-chip single-stranded DNA preparation. The 19-bp double-stranded oligonucleotides labeled with FAM and biotin on one strand and Cy5 on the other was employed. (a) Loading. The bright field image shows the captured magnetic beads (black) in the chamber. The FAM and the Cy5 images indicate the presence of double-stranded DNA in the entire chamber. (b) Binding and washing. The beads and the DNA were mixed by dispersing the beads completely for 6 min and then washed to remove unbound DNA. The dark backgrounds in the FAM and the Cy5 images illustrate that the double-stranded DNA had been captured by the beads and all the free DNA was washed away. (c) Denaturation and washing. NaOH was drawn into the chamber to release the single-stranded DNA labeled with Cy5. After the bead dispersion and collection cycle, the chamber was washed again with the B&W buffer. The bright FAM and the dark Cy5 images indicate the Cy5-labeled strands were successfully released and the FAM-labeled strands stayed on the beads.

As demonstrated by a previous study, the NaOH method produces a high yield of single-stranded DNA from PCR products than that using the elevated temperature.³³ Therefore, alkaline denaturation was employed in our experiment. However, due to the etching capability of NaOH to glass, the tag array spotted on the glass surface may be damaged by NaOH in this process. Indeed, we observed lower or even no hybridization signals when using NaOH to prepare single-stranded DNA on the chip. To investigate this issue, three spots of Cy3-labeled tag probes with a 5' amino group were immobilized onto an aldehyde-modified glass. The spots were treated with water, NaOH, or HCl at the room or an elevated temperature (60 °C), and then scanned with a fluorescent microarray scanner. As shown in Fig. 1s of the supplementary material,³⁶ those spots treated with water and 100-mM NaOH at the room temperature, respectively, emitted similar fluorescent signals, indicating that the tag probes can withstand 100-mM NaOH for at least 30 min at room temperature (25 °C). In contrast, when the spots were incubated with NaOH at 60 °C, the spot fluorescence significantly decreased. These results explain why reduced signals were observed in the hybridization process. Fortunately, 2.5-mM HCl did not affect the fluorescent signals at such a temperature (shown in Fig. 1s of the supplementary material).³⁶ Therefore, we employed 300 μ l of HCl to neutralize the excess NaOH in the chamber following the alkaline denaturation.

D. Automated microarray analysis for hereditary hearing loss

To critically test the performance of our automated microsystem, a commercial microarray-based detection kit for detecting nine mutation loci that account for hereditary hearing loss was employed. Due to the consistent performance of the multiplex allele-specific PCR provided by this kit, the single-stranded DNA preparation and the dynamic hybridization conducted on our system can be evaluated specifically without the interferences from sample preparations. In addition, previous study has shown that hearing loss is one of the most common congenital anomalies, affecting one in 1000 live births and at least 60% of these cases are hereditary.^{2,37} The rapid detection of these mutations is highly desired by physicians in order to carry out clinical interventions or consultations timely. Therefore, the successful detection of these mutations on our system will have an immediate impact to the society.

First, the limit of detection of our microsystem was evaluated using the kit with K562 standard genomic DNA, which has no mutations in these loci. ASPCR amplifications with 1, 2.5, 5, 10, and 25 ng of input DNA were carried out following the protocol provided by the manufacturer. After that, PCR products were directly loaded into the microsystem for fully automated single-stranded DNA preparation and hybridization analysis. The layout of the tag array spotted in the reaction chamber was illustrated in Fig. 8(a). Each black box with abbreviated names indicates the location of the spots for each mutation locus. W and M in the end of each name, as well as the blue and brown spot colors, represent wild-type and mutant tag probes, respectively. For quality control, spots for the positive control of magnetic beads (MC) and the negative control of hybridization (BC) were also spotted in the array. The hybridization results of the LOD test were demonstrated in Fig. 8(b). The bright spots in the images represented the attachments of the ssDNA-bead conjugates with an anti-tag and beads to the complementary tag probes on the chip surface. By observing the positions of the bright spots and comparing with the layout in Fig. 8(a), we can easily determine the locus name and its genotype. As shown in Fig. 8(b), all the loci can be accurately typed down to the limit of 2.5-ng input DNA. With only 1-ng DNA template, two loci were completely missed. The negative control with no input DNA only displayed the spots of the magnetic beads control (MC). The LOD obtained on our system is slightly worse than that reported in the previous study using the magnetic bead-assisted microarray method.²⁷ This decrease could be contributed by the lower efficiency of the on-chip single-stranded DNA preparation. However, considering the advantages of automation and the low risk of contaminations, this microfluidic system is still very competitive for clinical diagnosis. Next, four blood samples obtained from persons with known mutations (c.235delC heterozygote, c.299_300delAT homozygote, m.1555A>G heteroplasm, and m.1494C>T homoplasm) were processed and DNA was extracted using the

FIG. 8. Automated single-stranded DNA preparation and magnetic bead-based microarray analysis for mutation detections of inherited hearing loss. (a) Layout of the tag array spotted in the reaction chamber. Each black box with abbreviated names represents each mutation locus. W and M in the end of each name, as well as the blue and brown spot colors, represent wild-type and mutant-type tag probes, respectively. MC is the positive control of magnetic beads, and BC is the negative control for hybridization. (b) Sensitivity test of the single-stranded DNA preparation and microarray analysis using the microsystem. With only 2.5 ng of the K562 standard DNA as template for PCR amplification, all nine mutation loci can be correctly typed in the microsystem. (c) Bead images of four clinical samples (c.235delC heterozygote, c.299_300delAT homozygote, m.1555A>G heteroplasmcy, and m.1494C>T homoplasmcy) genotyped on the microsystem. Yellow boxes indicate the spots with mutations.

Qiagen kit. PCR products amplified from 25-ng DNA of each sample were genotyped on our system. The results in Fig. 8(c) clearly showed that all the samples were detected specifically and the mutations were typed correctly. Three repeated experiments were conducted to confirm the accuracy of the results.

In our system, the magnetic beads and the samples can be loaded into the chip directly without any pre-preparations. This “amplicon-in-answer-out” process is extremely important because the elimination of all the open-tube operations of high-concentration PCR products can dramatically reduce the risk of contamination, which is the top concern in clinical diagnosis. In addition, all the operations on our platform can be executed automatically through the control of computers, thus saving the labor and minimizing human errors. Furthermore, in a conventional microarray analysis, it usually takes about 50 min to complete the single-stranded DNA preparation using the magnetic bead-based method in a tube, and two more hours for the following static microarray process.² With the help of the automated ssDNA preparation and the dynamic hybridization on the chip, the analytical time in our system can be reduced from ~3 h to 45 min: 25 min for the ssDNA preparation and 20 min for the hybridization and detection. Our study clearly proved that this microsystem has a great potential for rapid and automated mutation detection in clinical diagnosis.

IV. CONCLUSIONS

An automated microfluidic system consisting of a low-cost, PMMA-tape-glass hybrid microchip for single-stranded DNA preparation and magnetic bead-based microarray analysis operated on a self-contained instrument has been successfully developed. The entire operation process from the loading of magnetic beads and PCR products, the manipulations of the MBs with DNA, the alkaline denaturation, the dynamic hybridization, to the final white-light detection, can all be conducted on the microchip without any human interventions. The efficient manipulation of magnetic beads on the chip, including dispersion, collection, and hybridization, was achieved by coupling two on-chip mixing valves, a magnet, and the fluidic control system in the instrument. The capability of this microsystem for automated mutation analysis was comprehensively tested using a commercial kit for detecting nine mutation loci that account for hereditary hearing loss. In summary, our microsystem can significantly improve the current mutation detection in the following aspects: (i) the “amplicon-in-answer-out” operation saves the labor and reduces the risk of contaminations; (ii) the shortened analytical time provides

more prompt results in clinical diagnosis, and (iii) the compact instrument with a white light detection reduces the initial investment. As we have demonstrated previously,^{38,39} the sample preparation of DNA extraction and PCR amplification from human whole blood has been integrated into a single chip. Therefore, the most intuitive step in the future is to combine these two microchips together, forming a truly “sample-in-answer-out” system. We believe such a system will promote the wide application of molecular diagnosis in clinical practice as well as the development of microarray technology.

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