On-chip isolation and enrichment of circulating cell-free DNA using microfluidic device

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ABSTRACT

Circulating cell-free DNA (cfDNA), containing cancer-specific DNAs derived from tumor cells, plays an important role in real-time monitoring of disease progression. Due to the abnormal growth of cancer and the promotion of cancer cell apoptosis by chemotherapy, the higher cfDNA concentration than healthy individuals is closely correlated with the diagnosis and treatment of cancer. Also, the mutation detection in tumor cell-derived cfDNA can be used to predict tumor progression. Human blood contains many blood cells (red blood cells, white blood cells, and platelets), proteins, extracellular vesicles, and so on. These blood components act as the inhibitors when the cfDNA is analyzed using polymerase chain reaction. So, analysis of cfDNA using whole blood directly may affect the sensitivity of the analysis or result in false-negative. The conventional methods of cfDNA isolation, such as silica absorption and polymer-mediated enrichment, are labor-intensive and time-consuming processes that can also lead to the loss of cfDNA in cumbersome procedures. Here, we designed an integrated microfluidic chip capable of on-chip cfDNA extracting to reduce sample loss and processing time. Our proposed device minimizes the number of experimental steps from 5 to 1, the total processing time from 42 to 19 min, and the required volume of washing reagents from 2 to 0.4 ml for cfDNA enrichment compared to the conventional method.

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I. INTRODUCTION

Liquid biopsy is a method of sampling body fluids such as blood, urine, saliva, and cerebrospinal fluid. It is more practical to monitor disease progress in real time because patient-derived specimens can be obtained more safely and easily with minimally invasive methods than tissue biopsy.¹ Blood is the most widely used liquid sample, which includes cells, cell-derived vesicles, circulating cell-free DNA (cfDNA), and proteins.^{2,3} Among these blood components, because the cfDNA, which is a type of cell-free nucleic acid that is derived from apoptosis or necrosis of cells, provides information of origin cells, cfDNA analysis is a key factor in the medical field. So, studies on cfDNA have increased since the past several decades.^{2,4} The apoptotic and necrotic residues are removed

by infiltrating phagocytes under normal physiological conditions; however, the cfDNA concentration of cancer patients is higher than that of normal individuals because the tumor growth rate is faster than eliminating DNA fragments by phagocytes.⁵ In addition, anticancer drugs and radiation for cancer treatment induce cell apoptosis and necrosis so that the level of cfDNA in the body increases.⁶ Although the changes in location, size, and vascular distribution of the tumor cells result in a different size distribution of cfDNA, migrating from either apoptosis or necrosis to bloodstream, the length of cfDNA fragments is mainly between 180 and 200 base pairs (bp).⁷

In general, the extraction of cfDNA from blood involves silica column-based techniques, precipitation, and centrifugation.⁸

Conventional methods for cfDNA extraction require several experimental steps and equipment (pipettes, tips, vortex mixers, tubes, and centrifuges). Thus, the time-consuming process requires skilled technicians and expensive machinery. To overcome these shortcomings, microfluidic technology has been studied to perform batch experiments on the on-chip because the fluid and particle behavior can be controlled with minimal reagent use.^{9–11} Karle *et al.* developed a continuous microfluidic device to extract DNA from lysed bacteria.¹⁰ Target DNA was captured to superparamagnetic beads in the first channel. The permanent magnet is then rotated to move the DNA carrying magnetic beads to the second channel to wash off the DNA that has not bound to the beads. In the final step, the magnetic beads travel to the third channel, including elution buffer, for DNA elution. This method can extract DNA continuously but requires additional setup to rotate the permanent magnet. Due to the nature of the laminar microfluidic flow, it is also difficult to increase the coupling efficiency between the magnetic particles and the target DNA. Recently, Kim *et al.* developed a lab-on-a-disc for cfDNA isolation.¹² The total process from depletion of blood cells to elution of cfDNA was carried out within 30 min. However, the capacity of the sample to be treated is limited to the volume of the disk chamber. The level of cfDNA concentration is dependent on patients' condition, and the ctDNA is only a small portion of the amount of cfDNA.¹³ Therefore, a large amount of blood samples may be required to extract extremely rare ctDNA according to the characteristics of the patient.

Here, we developed a microfluidic vortex along with a gradient magnetic-activated cfDNA sorter (vortex-GMACS) for the on-chip separation of cfDNA found in blood. The vortex-GMACS chip consists of a microfluidic multivortex mixing module to increase the binding probability between cfDNAs and silica magnetic particles (MPs) and a magnetophoresis module for cfDNA enrichment by capturing MPs. Continuous injection of samples into the vortex-GMACS chip enables one to handle samples regardless of sample volume.

II. MATERIALS AND METHODS

A. Fabrication of vortex-GMACS

Microfluidic devices were fabricated using polydimethylsiloxane (PDMS) replicas. The micro-vortexer design was patterned on a film combine glass (FCG) mask. Negative photoresists (SU-8 3035, Microchem, USA) was deposited on a 4-in. silicon wafer. The wafer was spun at 2000 rpm for 40 s to attain a 50 μm thickness. The spin-coated wafer was exposed to UV light through the FCG mask to cure the photoresists. The wafer was baked for 1 min at 65 °C and for 15 min at 95 °C. The unexposed photoresist was removed by the developer (SU-8 developer, Microchem, USA). The process of fabricating the mold was completed after washing it with isopropyl alcohol and deionized water. After mixing PDMS with a curing agent (Sylgard® 184 Silicone Elastomer Kit, Dow Corning, USA) in the ratio 10:1, the mixed solution was sequentially poured on the mold, cured for 4 h at 80 °C, and peeled off from the mold. The removed PDMS was cut along the shape of the chip and punched to fabricate for inlets and outlets. The PDMS and a slide glass (Paul Marienfeld GmbH & Co. KG, Germany) were bonded by using an oxygen plasma generator (Cute-B Plasma, FEMTO Science, Korea)

and were put in an oven for 1 h at 70 °C.¹⁴ The bonded PDMS-slide glass was subsequently placed on a magnet holder, which was designed and printed by a CAD program (SolidWorks, Dassault Systèmes, France) and a 3D printer (3DISON MULTI, Rokit, Korea).

B. Sample preparation

The 100 and 500 nm fluorescent particles (Invitrogen, USA) were used to test the mixing performance between cfDNAs and MPs in the vortex channel. Both the fluorescent particles were suspended at 0.002 g/ml in a 0.2% phosphate buffered saline (PBS).

The standard size of complementary DNA (cDNA) was precisely obtained by using a reverse transcription polymerase chain reaction (PCR). After the extraction of total RNA from Jurkat cells using TRIzol reagent (Invitrogen, USA), 5 μg of total RNA was added to synthesize the first strand of cDNA using the AccuScript first strand cDNA synthesis kit (Stratagene, USA). The cDNA of 202 base pairs (bp) was synthesized with the primers specific to interleukin 2 (IL2, Gene Bank access No: NM_008366.3): Forward (sense) 5'-AGCCATCCT GTGTCACATT-3', Reverse (antisense) 5'-TGGGGAGTTTCAGGTTCTCTGTA-3'. The PCR products were analyzed with 1% agarose gel and purified with QIAquick Gel Extraction Kit (QIAGEN, Germany).

C. Operation procedure

Chip-based DNA extraction steps were performed using the vortex-GMACS chip. To utilize this chip, PBS with 3% bovine serum albumin was filled in the chip for 5 min to prevent the non-specific binding of cfDNAs. The PBS with cfDNA sample and binding buffer with MPs were injected into the inlets of each channel by a syringe pump (KDS210, KDS Scientific Inc., USA) at various flow rates ranging from 50 to 200 μl . To evaluate the mixing performance between MPs and DNA in the vortex channel, an inverted optical microscope (IX-70, Olympus, Japan) and a camera (Progress Gryphax® Arktur, Jenopick, Germany) were used to capture fluorescent images, which were subsequently analyzed by ImageJ (NIH, USA). The complex of MPs and cfDNA in the channel were captured by magnets. MPs were washed two times by a washing buffer and 80% ethanol. To completely remove the supernatants, the inside channels were dried by airflow for 5 min. The cfDNA-contained elution buffer was subsequently collected in a tube after loading the elution buffer in the GMACS channel and incubating it for 5 min to elute cfDNAs from MPs.

D. DNA quantification

The isolated DNA was quantitated to evaluate the performance of the vortex-GMACS. A double-stranded DNA sample was measured using a fluorometer (Qubit 3.0 Fluorometer, Invitrogen, USA), which can accurately measure either the quantity or the quality of DNA using the Qubit dsDNA HS Assay Kit (Invitrogen, USA). The Qubit dsDNA HS reagent and buffer were mixed in the ratio of 1:200 for the preparation of the Qubit working solution. The extra 190 μl of working solution was added to 10 μl of DNA sample in each assay, thereby reaching to 200 μl of the final volume. The quantities of DNA samples in the assay tubes can be measured by the fluorometer. After the extraction of cfDNA from the

vortex-GMACS, a real-time PCR (CFX96 System, Bio-Rad, USA) was performed with a 10 μ l of the reaction buffer (TaKaRa SYBR® Fast qPCR Mix, Takara Holdings, Japan) to validate the existence of the cfDNA contents.

E. Clinical samples

This study was approved by the Institutional Review Board (IRB) of Severance Hospital, Yonsei University (4-2015-1151), Republic of Korea. The following experiments were conducted through the IRB consent process. The blood samples were subjected to clot activator tubes (VacuLab® SSGT tubes) and left undisturbed for 30 min. The clotted blood in the tube was centrifuged at 2000g for 10 min and the serum was collected from the tube.

III. RESULTS AND DISCUSSION

A. Microfluidic vortexer facilitating the collision of cfDNAs with MPs

Vortex-GMACS consists of microfluidic vortexer for the binding of cfDNAs with MPs and a GMACS for the enrichment of cfDNA. Both cfDNAs and MPs have negative charges on their surfaces to prevent from attracting each other. However, the binding buffer, containing a high concentration of salt, allows DNA and MPs to move together. The injection of cfDNAs and MPs through the two inlets of the vortex-GMACS triggered the cfDNAs to conjugate with the MPs in Fig. 1(a). Subsequently, the conjugated cfDNAs were transported into the GMACS channel, where they were captured by the magnetic force. The introduction of the washing buffer to the channel removed the nontarget molecules (residues), and the binding buffer, except the MP-conjugated cfDNAs, got attached to the magnets. The cfDNAs were finally released by inserting the elution buffer into the channel in Fig. 1(b).

The transverse dispersion in the fluidic flow is related to the dimensionless dean number (k), which denotes the magnitude of centrifugal force and ratio of inertial to viscous force. A 180° rotation caused two fluid streamlines to create a transverse flow.¹⁵

The occurrence of dean vortices led to the rotation of two fluids in opposite directions from the vertical plane. When the microfluidic channel suddenly expanded, expansion vortices were generated on both sides of the channel in the horizontal plane. These two vortices enabled to cross and sufficiently mixed the two fluids in the microfluidic channel. The expansion phenomenon in the horizontal plane was combined with the dean effect on the vertical plane. The magnitude of the dean effect can be defined by the dean number $k = \delta^{0.5} Re$, where $\delta = d/R$, where R is the radius of curvature of the channel, and d is the channel hydraulic diameter defined as $d = 4A_C/P$, where P is the wetted perimeter and A_C is the cross-sectional area. Reynolds number (Re) is a dimensionless quantity in fluid mechanics and represents the ratio of inertial to viscous force within a fluid. It is calculated by the equation $Re = \rho Vd/\mu$, where ρ is the density of the fluid, μ is the viscosity of the fluid, V is the maximum flow velocity in the channel, and d is the channel hydraulic diameter. A transverse flow appears when k exceeds one. The two fluids were sufficiently mixed when k was more than 10. The microfluidic vortexer was designed based on the above notion. The dimensions of the microfluidic vortexer were $R = 600 \mu m$, $P = 300 \mu m$, and $A_C = 5000 \mu m^2$ in the curved microchannel, and $R = 600 \mu m$, $P = 1100 \mu m$, and $A_C = 25\,000 \mu m^2$ in the curved expansion microchannel. The value of k was from 2.35 ($Re = 6.05$) to 9.42 ($Re = 24.19$) in the curved expansion microchannel at a flow rate from 50 to 200 $\mu l/min$.

The results show that the mixing efficiency is affected by dean flow. The mixing performance in the microfluidic vortexer was tested by injecting blue fluorescent particles (100 nm) of sizes similar to those of cfDNA fragments (200 bp), and red fluorescent particles (500 nm) of the same size as the MPs used in the experiment. Fluorescent images of the behavior of the two particles injected into the channel were captured by the camera, and the intensity of pixels was analyzed using the ImageJ program. The two fluorescent particles were injected through the two inlets at flow rates of 50, 100, 150, and 200 $\mu l/min$. The curvature direction of the curved expansion channel changes the flow direction once during each cycle. The color of the fluorescent images changed

FIG. 1. Schematic diagram of vortex-GMACS chip. (a) Mixing channels, which can mix up liquids, consist of a curved microchannel (100 μm wide; 50 μm long; and 600 μm radius of curvature) and the curved expansion microchannel (500 μm wide; 50 μm long; and 600 μm radius of curvature). MPs and cfDNAs intersect in the mixing channel and expansion and dean vortices occur with an increasing dean number. cfDNAs and MPs are bound by a binding buffer in the channel. (b) The MPs bind with cfDNAs, move into the GMACS channel, and they are captured by neodymium magnets. Washing buffers are passed through the chip and the residues are washed. After the elution, cfDNAs are extracted from the outlet.

according to the flow rates and number of cycles in Fig. 2(a). The images were categorized into blue and red, and each color intensity in the A–B zone in Fig. 2(b) was analyzed by the ImageJ program. M is the maximum intensity of the pixels of the expanded zone, and E is the total intensity of the pixels of the expanded zone

in the curved expansion channel. R is the mixing rate defined as $R = (E / M)$. Although the number of cycles increased at a flow rate of $50\text{ }\mu\text{l/min}$, the mixing rate of the red fluorescent particles was less than 0.16 in Fig. 2(c). Because k is less than 10, it is insufficient to mix two fluids. As the number of cycles increased at the flow rates of

FIG. 2. The evaluation of mixing performance in the vortexer chip. (a) Schematic drawing of the vortexer chip. (b) Fluorescent images of the mixing between the 100 nm fluorescent particles (blue) and 500 nm fluorescent particles (red) in the curved expansion channel. (c) Mixing rate increases when the number of cycles increases in red fluorescent particles (MPs). (d) Mixing rate saturates after passing through cycle 6 at a flow rate of $100\text{ }\mu\text{l/min}$ in blue fluorescent particles (cfDNAs).

FIG. 3. The binding efficiency of cfDNAs with MPs based on the number of cycles and the concentration of MPs in the microfluidic vortexer. (a) The binding efficiency is 90.6% from cycle 5 to 20. (b) The binding efficiency is the highest at a concentration of 10⁶/ml of MPs.

100 and 150 $\mu\text{L}/\text{min}$, the mixing rate continuously increased and had a mixing rate of 0.4. The particles were focused along a streamline at high flow rates; hence, the mixing rate was uneven at the flow rate of 200 $\mu\text{L}/\text{min}$. The mixing rate of the 100 nm fluorescent particles (blue) increased at a flow rate of 50 $\mu\text{L}/\text{min}$; however, it was lower than those at other flow rates in Fig. 2(d). The mixing rate was more than 0.4 and saturated at the flow rates of 100 and 150 $\mu\text{L}/\text{min}$ after cycle 4. The mixing rate was more than 0.2 and 0.4 for the red and blue fluorescent particles, respectively, at the flow rate of 100–150 $\mu\text{L}/\text{min}$. The results show that increasing the number of cycles has an effect of improving the dean and expansion vortexes at the flow rate of 100–150 $\mu\text{L}/\text{min}$. The high flow rate reduced the mixing time and increased the mixing rate; however, chip breakage due to high pressure and the incubation time for binding cfDNAs with MPs should be considered. The flow rate of 100 $\mu\text{L}/\text{min}$ was selected to increase the incubation time in the mixing channel and reduce the pressure of the microfluidic channels.

The binding efficiency between the MPs and cfDNAs was tested. They were injected through two inlets and passed through the mixing channels. The MPs with the cfDNAs were collected in a 2 ml microcentrifuge tube, and the tube was placed on a magnetic stand. Subsequently, we removed the residue and measured the cfDNA concentration by the Qubit 3.0 fluorometer. The concentration of the MPs was fixed at 10⁵/ml and tests were conducted from cycle 5 to 20. The binding efficiency between cfDNAs and MPs was 90.87% in cycle 5, 90.83% in cycle 10, 90.85% in cycle 15, and 89.80% in cycle 20, and the standard error was under 0.006% in Fig. 3(a). The results show that the maximum error range of the binding efficiency is within 2% for all the cycles. When the number of cycles increases, the binding efficiency between MPs and cfDNAs also increases because nonspecific binding is created on the surface of the microfluidic channel. Hence, the mixing channel was set at cycle 15 to increase the time for the stable binding of cfDNAs with MPs. The binding efficiency, depending on the concentration of MPs, was 84.72% at 1 $\times$ 10³/ml, 87.71% at 1 $\times$ 10⁴/ml, 89.57% at 1 $\times$ 10⁵/ml, and 94.57% at 1 $\times$ 10⁶/ml, and the standard error of the binding efficiency was less than 0.02% in Fig. 3(b). We chose the concentration of 1 $\times$ 10⁵/ml because excessive MPs interfere with channel clogging and elution.

B. GMACS for cfDNA enrichment

The GMACS was designed as stage 1 GMACS (width: 5 mm, length: 35 mm, height: 0.05 mm), and the volume of the stage 1 MACS was 10 μL in Fig. 4(a). Circular disk magnets (EP303; e-Magnets UK, UK), which had a maximum energy product of 42 MGOe (grade N42) were arranged under the GMACS channel in a row and piled up from one to nine. The magnetic field strength was in the range of 98.34 to 151.16 mT in Fig. 4(b). The magnetic field strength increases as the number of magnets increases, and the slope of the magnetic field strength was 22.8 in stage 1, 19.8 in stage 2, 18.6 in stage 3, and 18.0 in stage 4 GMACS in Fig. 4(c).

This enabled a gradual increase in the strength of the magnetic forces and this arrangement prevents clogging caused by magnetic particles. If magnetic forces are very strong, most of the MPs will be captured at the front of the channel. To use a GMACS chip, the magnet must be fixed under the channel. The magnet holder component was designed using a 3D printer, and the holder and vortex-GMACS chip were combined in Fig. S1 of the supplementary material. The elution volume in commercialized kits is usually from 10 to 50 μL . The GMACS was designed with a volume of 10 μL at stage 1 and was tested from stage 1 to 4 in Fig. 4(a).

The absorbance of the MPs at the inlet and outlet was measured at a wavelength of 650 nm using a UV spectrophotometer (V-650 UV/VIS spectrophotometer, JASCO Corporation, Germany). As the absorbance of the MPs decreases, the concentration of MPs reduces. The MPs (1 $\times$ 10⁵/ml) were injected into the gradient GMACS at a flow rate of 100 $\mu\text{L}/\text{min}$. The capture efficiency was 84.33 $\pm$ 3.25%, 87.69 $\pm$ 4.16%, 91.93 $\pm$ 3.87%, and 99.02 $\pm$ 0.45% for stages 1, 2, 3, and 4, respectively, in Fig. 4(d). The channel length is insufficient to capture the MPs in stage 1 GMACS and some MPs follow flow direction. Hence, the capture efficiency was the highest in stage 4, and stage 4 GMACS was chosen for the enrichment of cfDNAs.

C. Evaluation of the vortex-GMACS using serum samples from breast cancer patients

A 15-cycle microfluidic vortexer and 4-stage GMACS were integrated in the vortex-GMACS to extract cfDNAs in one step.

FIG. 4. (a) Image of vortex-GMACS. The microfluidic chip positioned on the 3D printed magnet holder during experiments. Dimensions of stage 1 GMACS (width: 5 mm, length: 35 mm, height: 0.05 mm). (b) The analysis of MP trajectory in the GMACS channel. The magnetic force gradient increases following the fluid flow direction and MPs move by fluid force and magnetic force. (c) Magnetic field strength. The slope gradient of magnetic field strength decreases from stage 1 to stage 4 in the GMACS chip. (d) Capture efficiency of MPs of the GMACS chip. Concentration of MPs in the outlet decreases as the number of stages increases. MPs (1.0×10^5 /ml) were injected into the GMACS channel and were captured by magnets.

MPs-added binding buffer (500 μL) and cfDNA-spiked PBS (500 μL) were injected through the two inlets at a flow rate of 100 $\mu\text{L}/\text{min}$. The MPs and cfDNAs were bound by passing them through the microfluidic vortexer and subsequently into the GMACS. In the GMACS channel, the MPs with the cfDNAs were captured by magnets. Other biomarkers and residue buffers were washed away two times by a washing buffer (200 μL) and 80% ethanol (200 μL) at the flow rate of 100 $\mu\text{L}/\text{min}$. It was dried by airflow at the same flow rate for 2 min. An elution buffer (50 μL) was injected into the chip and incubated for 5 min. With MPs attached, the extracted cfDNAs with the elution buffer were collected in the outlet. The experiments were conducted at DNA concentrations of 500, 100, 50, and 1 ng/ml. Real-time PCR cycle threshold (Ct) values of the extracted cfDNAs were 7.56 and 12.64 at the DNA concentrations of 500 and 1 ng/ml, respectively. To compare the vortex-GMACS with a commercialized kit (MagMAX^{TM} Cell-Free DNA Isolation Kit, Applied Biosystems, USA), we followed the manual protocol mentioned in the kit for the cfDNA extraction. Real-time PCR Ct values of the extracted cfDNAs were 12.75 using the cfDNA extraction kit and 12.63 using the vortex-GMACS at the DNA concentration of 1 ng/ml in Fig. 5. The recovery was 51.8% in the vortex-GMACS and 48% in the

FIG. 5. Ct values of cfDNAs extracted using kit and vortex-GMACS as measured by real-time PCR.

FIG. 6. Comparison between the concentrations of cfDNAs extracted from healthy controls and breast cancer patients.

cfDNA extraction kit by calculating the Ct values obtained from each of the cfDNA concentrations.

Further, the performance of the vortex-GMACS was evaluated by measuring the concentration of cfDNAs extracted from serum samples of healthy individuals and breast cancer patients. The concentrations of cfDNAs extracted using both AccuCirculating DNA Preparation kit and the vortex-GMACS were directly analyzed by quantifying double-stranded DNA (dsDNA) using the Qubit 3.0 fluorometer in Fig. 6. The test results of extracting cfDNAs were similar for both the vortex-GMACS and commercialized kit. However, the vortex-GMACS continuously operated in one step and reduced the concentration of the MPs by 5 times, processing time from 42 to 19 min, and amount of washing reagents from 2 to 0.4 ml for extracting cfDNAs.

IV. CONCLUSION

In this study, we designed a vortex-GMACS for the isolation and enrichment of cfDNA, which was performed in one step at 100 μ l/min. The device consists of a microfluidic vortexer to mix two fluids and GMACS to capture MPs. The curved expansion channel of the microfluidic vortexer enhances the binding of cfDNAs with MPs. The experimental result shows that 94.57% of MPs and cfDNAs were successfully bound in the vortex channel. The magnets of GMACS efficiently enabled to enrich MP-conjugated cfDNAs by trapping 99.02% of MPs by gradually increasing magnetic forces. The introduction of washing buffer to the channel removed all of the other residues except MP-conjugated cfDNAs, whereas the elution

buffer resulted in the release of cfDNAs. In summary, the vortex-GMACS enabled to extract high purity cfDNAs from the serum within 19 min using a small volume of washing buffer and MPs without incubation, and reduce the chance for a sample loss compared to the loss in commercialized kits. Thus, the vortex-GMACS will be applied to the early stage of cancer diagnostics and to an optimal personalized therapy by cfDNA analysis.

SUPPLEMENTARY MATERIAL

See the [supplementary material](#) for a detailed description of Assembly of Vortex-GMACS chip, magnets, and a magnet holder (Fig. S1).

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