Automated selection of aptamers against cholangiocarcinoma cells on an integrated microfluidic platform

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(Received 5 April 2017; accepted 19 June 2017; published online 5 July 2017)

An integrated microfluidic system capable of automatically identifying aptamers specific to cholangiocarcinoma (CCA) cells was developed herein. The developed system was capable of performing cell-based systematic evolution of ligands via an exponential enrichment (Cell-SELEX) process on-chip, and only six rounds of Cell-SELEX were required to identify high specificity aptamers; this represents a significant improvement in speed over conventional SELEX, in which 15–20 rounds are typically required. Using the microfluidic chip developed, three aptamers specific to CCA cells (one for SNU-478 cells and two for HuCCT-1 cells) were successfully screened. This automated system could be modified to uncover aptamer probes against other cancer cells, thereby allowing for earlier diagnosis and consequently a potentially improved prognosis. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4991005>]

NOMENCLATURE AND ABBREVIATIONS

CCA	Cholangiocarcinoma
DPBS	Dulbecco's phosphate-buffered saline
EMV	Electromagnetic valve
FAM	Carboxyfluorescein
FBS	Fetal bovine serum
K _d	Dissociation constant
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
SELEX	Systemic evolution of ligands by exponential enrichment
ssDNA	Single stranded deoxyribonucleic acid
WBC	White blood cell

I. INTRODUCTION

Cholangiocarcinoma (CCA) is a malignancy arising from the epithelial cells of the intra- and extra-hepatic bile ducts.¹ The incidence of CCA has been rising over the past two decades

Note: The preliminary results of this study have been presented at the MicroTAS 2016, Dublin, 2016.

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worldwide, though it is still somewhat uncommon. Despite the relative rarity, these tumors generally pose a significant therapeutic challenge since they are often diagnosed at a late stage when surgical resection is infeasible and treatment options are limited.² Unfortunately, the 5-year survival rate for patients with biliary duct cancers is only ~15%. CCA is often misdiagnosed as simply hepatic inflammation or cholestasis; even modern ultra-sonographic scanning and computerized tomography instruments can yield false negative diagnoses.

Tumor biomarkers for CCA could aid in clinical diagnostics, and several do currently exist. However, carbohydrate antigen (CA 19-9), a widely studied serum biomarker for CCA, is characterized by a wide variation in sensitivity (50%–90%) and specificity (54%–98%); furthermore, it is often produced at elevated levels in benign biliary diseases and cholangitis, with levels falling after relief of biliary obstruction and sepsis. Another widely studied serum cytokeratin biomarker, CK-19, has been associated with CCA, but the corresponding assay lacks sensitivity.^{3,4} As such, there is still a great need for highly specific and sensitive CCA-diagnostic biomarkers.^{5,6}

Over the past decade, short, single-stranded oligonucleotides called aptamers, which are typically selected from a random combinatorial library *in vitro* by a process called SELEX (systemic evolution of ligands by exponential enrichment), have been increasingly utilized as bio-recognition tools. Specifically, they have seen widespread use in biomedical research, and especially cancer and pathogen diagnostics, given their (1) high specificity and binding affinity, (2) ease of synthesis and chemical modification, (3) relatively low cost, (4) non-toxic nature, and (5) good stability.^{7,8} Briefly, SELEX works by enriching candidate aptamers from a large initial library of 10^{14} – 10^{15} random sequences after many iterative rounds of selection.⁹

Aptamers have been generated to bind to a variety of targets, ranging from small molecules, to purified proteins and peptides, and even to red blood cell (RBC) membranes and whole cells.^{10–13} Indeed, whole-cell-SELEX is important for molecular biomarker-based cancer diagnostics, as it exploits molecular differences between cancer cells and healthy ones.^{14,15} However, the isolation of aptamer sequences from randomized libraries by SELEX is time-consuming (e.g., months may be required), resource-intensive (e.g., liters of expensive reagents may be needed), and requires well-trained personnel. Recently, microfluidic technology has been demonstrated to have the potential to increase the efficiency of conventional biological and medical protocols by reducing hands-on time and resource usage. Automation and small reaction volumes, which are associated with enhanced reaction efficiencies, are additional advantages of microfluidic technologies. Micro-scale SELEX has been used to identify and isolate aptamers on-chip in a manner that allows for the removal of weakly binding sequences;^{16,17} reagent volumes were reduced by at least an order of magnitude in these studies.

Capillary electrophoresis (CE)-based SELEX,¹⁸ sol-gel-based SELEX,¹⁹ and magnetic bead-based SELEX^{20–22} have all been integrated into microfluidic systems, resulting in the rapid and highly efficient isolation of aptamers.²³ Among them, whole-cell magnetic bead-based systems have gained considerable attention due to their simplicity, short processing times, use of small reagent quantities, rapid and efficient removal of unbound molecules, and automated control features.²⁴ Given the aforementioned need to uncover superior biomarkers for CCA diagnosis, a magnetic bead-based system automated entirely on an integrated microfluidic chip was used herein with two CCA cell lines to attempt to identify CCA-specific aptamer probes.

II. MATERIALS AND METHODS

A. Cell lines and cell culture

SNU-478 (an inter-hepatic CCA cell line) and HuCCT-1 (an intra-hepatic CCA cell line) were used as the target cell lines, and MMNK-1 (non-tumorous, human CCA cell line) was used as the negative control cell line. All human-derived cell lines used in this study were obtained from the Institute of Clinical Medicine of Taiwan's National Cheng Kung University Hospital. SNU-478 and HuCCT-1 cells were cultured in RPMI-1640 media (Invitrogen, USA), and MMNK-1 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen). These

media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine, and 100 U ml⁻¹ penicillin–streptomycin (Invitrogen), and the cells were incubated at 37 °C and 5% CO₂.

B. Immuno-magnetic beads

Dynabeads[®] Epithelial Enrich immuno-magnetic beads (EpiEnrich, 4 × 10⁸ beads ml⁻¹, 4.5 μm, Invitrogen) were used for cell capture. Briefly, 100 μl of the beads were washed twice with 1 ml of 1× phosphate-buffered saline (PBS; Merck, Germany) and suspended in 1× PBS in a final volume of 1 ml. The cell lines were removed from the culture dish and suspended in 1 ml of binding buffer (0.02 M glucose, 1 g BSA, and 5 ml of 1 M MgCl₂ in 1 l of Dulbecco's phosphate-buffered saline (DPBS, Invitrogen) at a concentration of 10⁵ cells per ml. Then, 10 μl of the EpiEnrich immuno-magnetic beads were mixed with the cells in a micro-centrifuge tube on a rotary shaker (Elmi Intelli-Mixer RM-2, Rose Scientific, Canada) for 1 h. The cell-bead complexes were then washed thrice with washing buffer (0.02 M of glucose and 5 mM MgCl₂ in 1 l of DPBS) and suspended in 1× PBS.

C. Single stranded deoxyribonucleic acid (ssDNA) library

The ssDNA library was synthesized at a final concentration of 1 μM by Medclub Scientific Co., Ltd. (Taiwan). Each ssDNA sequence contained a central region of 40 random nucleotide bases ("N40") flanked by two specific 16-bp sequences that functioned as polymerase chain reaction (PCR) primer binding sites (72 bp = 5'-GGCAGGAAGACAAACA-N40-TGGTCTGTGGTGTCTGT-3').

D. Chip design and fabrication

In order to fabricate the microfluidic chip, a computer-numerical-control (CNC) machine (EGX-400, Roland Inc., Japan) equipped with a 500-μm drill bit was used to generate two poly-methylmethacrylate master molds.²⁵ Polydimethylsiloxane (PDMS) (Sylgard 184A/B, Dow Corning, USA) was then poured over the master molds, and a replica was created. PDMS was prepared by mixing the PDMS pre-polymer with the curing agent in a 10:0.9 ratio (w/w). The polymer mixture was then placed under vacuum in order to release the air trapped during mixing. The mixture was then poured into the mold and cured at 70 °C for 3–4 h. The cured PDMS structures were de-molded mechanically. Oxygen plasma treatment (FC-12064, FEMTO Science, USA) was used to bond the two PDMS layers onto a boro-silicate glass substrate (G-Tech Optoelectronics Corp., Taiwan) to form the complete chip.

The microfluidic chip was equipped with three suction-type transportation units designed such that a vacuum generated from the deflection of the PDMS membranes could provide a suction force that would force the samples and reagents throughout the chip. In addition to the pumping devices, three reagent loading chambers (for fetal bovine serum, the washing buffer, and the binding buffer), a PCR chamber for storing PCR reagents and performing ssDNA amplification, normally closed micro-valves, one open-chamber micromixer/micropump unit located near the target cell chamber (for SNU-478 and HuCCT-1 cells), and one open-chamber micromixer/micropump unit located near the negative control cell chamber (for the MMNK-1 cells) were integrated into a single chip such that the entire Cell-SELEX process could be automated. The positive selection open-chamber micromixer/micropump unit was used to mix cells with the ssDNA library. The working principle of the open-chamber micromixer/micro-pump has been described previously. Briefly, it was activated by the sequential uplifting and deflecting of the PDMS membranes such that a mixing effect was generated within the open-chamber by connecting it to an electromagnetic valve (EMV) that was driven by a custom-made digital controller attached to a vacuum pump. Furthermore, this micro-device could also be used as a micropump when the membranes were uplifted sequentially; this would cause the sample to flow into the microfluidic chambers due to the lower air pressure in the air chamber (as generated by the suction force from the vacuum pump).

E. Mixing and pumping devices

Experiments were conducted to determine the optimum pressures that (1) maximized transport and mixing and (2) minimized cell damage. In order to determine the mixing efficiency of the micromixer/micropump in the target cell chamber, the mixing chamber was loaded with red ink ($10\ \mu\text{l}$) and $30\ \mu\text{l}$ of $1\times$ PBS. The PDMS membranes were activated at an applied air pressure of 6.8 kPa and a vacuum ranging from -13.3 to -80 kPa (-13.3 , -26.6 , -39.9 , -53.3 , -66.6 , and 79.9 kPa) at pulsation frequencies of 0.5, 1, 2, or 4 Hz. Shear force and pumping rates of the micromixer/micropump were then calculated. The flow velocity was measured by using polystyrene beads ($20\text{-}\mu\text{m}$ diameter, the approximate size of a single cancer cell) and a high-speed charge-coupled-device camera (Mikrotron/MC1311, Germany) by an image acquisition method. Briefly, the frame rate of the image acquisition was set at 500 frames/s to capture the movement of the membrane pulsations. The flow measurement was made at the mid-height of the chamber and shear force was calculated using the following equation:

$$\text{shear force} \sim \tau A = \mu \frac{du}{dy} A \sim \mu \frac{\Delta u}{\Delta y} \times A,$$

where τ is the shear stress, μ is dynamic viscosity (μ_{PBS} , $20^\circ\text{C} = 1.05 \times 10^{-3} \text{ N s/m}^2$), u is the flow velocity, y is the height of the chamber, and A is the area of the cross-section ($A = 0.0172 \text{ cm}^2$). The details of this analysis have been described elsewhere.²³

F. Working conditions and PCR

The target CCA cell chamber was used for the denaturation of 72-bp ssDNA at 95°C for 10 min. It was then immediately cooled down to maintain the DNA in a single-stranded form. The target cell-magnetic bead complexes and negative control cell-magnetic bead complexes were preloaded into the designated chambers. The target cell-magnetic bead complexes were allowed to mix with the ssDNA by activating the designated micromixer/micropump at 6.8 kPa of positive pressure and -40 kPa of vacuum at a frequency of 1 Hz for 10 min. The operational frequency and positive pressure were kept unchanged during the whole process, and alterations were made accordingly for the negative pressure. It is to be noted that the magnet was moved to the negative control cell chamber during the positive selection step (i.e., with the target CCA cell lines) and to the target cell chamber for the collection of target cell-magnetic bead complexes. Similarly, the magnet was moved to the target cell chamber during the negative selection step (i.e., with the negative control cell line). The negative control cell-magnetic bead complexes were collected by moving the magnet to the negative selection chamber, and the supernatant was transferred to the PCR chamber for amplification. All throughout the process, the microfluidic chip was placed on a custom-designed, dual-temperature control module composed of two Peltier cooling/heating units capable of regulating temperatures to as low as 7.7°C and as high as 104°C .²⁴ Target cell-magnetic bead complexes and negative control cell-magnetic bead complexes were loaded into the respective micromixer/micropump chambers. Binding buffer, washing buffer, and FBS were loaded into their respective chambers. The reaction cocktail for positive selection ($50\ \mu\text{l}$) comprised the denatured ssDNA library, target cell-magnetic bead complexes in binding buffer, and FBS. FBS has been shown to increase the stringency of aptamer selection.⁹ Washing steps were carried out using $50\ \mu\text{l}$ of the washing buffer. Six rounds of selection were performed with the SNU-478 and HuCCT-1 cell lines, and each round comprised a positive selection step with the target cell line and a negative selection step with the negative control cell line. The applied negative gauge pressure was increased with each round, and the number of washings was also increased stepwise from 2 to 5 with each passing round of selection to increase the stringency of aptamer selection. With the aim of reducing the non-specific binding of the aptamers, one additional round of negative selection with MMNK-1 cell lines was performed with the 6th SELEX round PCR product for the SNU-478 and HuCCT-1 cell lines. The product of this final round was then used in a negative

selection step with White blood cells (WBCs) isolated from whole blood (described in more detail below).

After each round of Cell-SELEX, the magnetic field was activated such that the negative control cell-magnetic bead complexes could be trapped in the negative selection micropump/micromixer chamber. The gauge pressure and frequency were then regulated such that an aliquot of the supernatant was transferred to the PCR chamber. The PCR chamber was preloaded with 0.5 μ l of the forward primer (5'-GGCAGGAAGACAAACA-3', 0.5 μ M), 0.5 μ l of the reverse primer (5'-ACAGCACCACAGACCA-3', 0.5 μ M), 0.5 μ l of deoxynucleotide triphosphates (0.2 mM), 2 μ l of ssDNA (0.1 μ M) or the previous amplified product from the last round of Cell-SELEX, 0.125 μ l of Taq DNA polymerase (New England Biolabs, USA), and double-distilled water (ddH₂O) to a final volume of 25 μ l. The PCR was initiated at 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 63 °C for 15 s, and 72 °C for 30 s. A final extension step at 72 °C for 10 min was also incorporated into the protocol. The amplified products were then transferred to the positive selection chamber, and the gauge pressure and frequency were fine-tuned such that an aliquot of the amplified product was used for the next round of SELEX, with the remainder collected via the waste collection chamber. The amplified product was then heated to 95 °C for 10 min to denature the double-stranded DNA and then cooled rapidly to maintain the denatured DNA in a single stranded form. Consequent rounds of SELEX were performed similarly.

G. Negative selection and WBC isolation

In order to reduce the non-specific binding of aptamers, an additional round of negative SELEX was performed with MMNK-1 cell lines and the 10 μ l of the PCR product from the 6th round of Cell-SELEX. The PCR product was incubated with MMNK-1-magnetic bead complexes in binding buffer for 10 min. Then, a magnet was used to collect the bead-cell complexes, and the supernatant was transferred to the PCR chamber for consequent amplification. The PCR product of this round was then used for another round of negative selection with WBCs isolated from whole blood in the following manner. Whole blood (1 ml), stored in EDTA tubes (BD Vacutainer, BD, USA), was added to 5 ml of 1 $\times$ red blood cell (RBC) lysis buffer (BioLegend, USA) and gently mixed on a rocker panel (Rocker RS01, TKS Instruments, Taiwan) at 100 rpm, 4 °C for 5 min. The lysis reaction was then stopped by adding 20 ml of 1 $\times$ PBS. The collected WBCs were then pelleted, washed with 1 $\times$ PBS, and suspended in 1 $\times$ binding buffer. Then, 10 μ l of the WBC solution was incubated with the PCR product from the 6th round of SELEX for both SNU-478 and HuCCT-1 cell lines for 10 min. Samples were then centrifuged at 4000 rpm, and the supernatant was collected. PCR was performed using an aliquot of this supernatant.

H. Monitoring the enrichment of selected DNA pool

In order to determine the enrichment of the ssDNA library pool, real-time PCR analysis was performed with melting curve analysis. The concentrations of ssDNA in the different rounds of enriched pools were determined by using an ultraviolet-visible spectrophotometer (NanoPhotometer[®] P-Class, Impln GmbH, Germany) and then adjusted to an amount of 50 ng per 2 μ l for further PCR amplification. Real-time PCR was carried out using KAPA SYBR FAST master mix (Kapa Biosystems, Roche, Switzerland) in an Applied Biosystems StepOne Plus detection system (Applied Biosystems, Foster City, USA). Each PCR was performed in a 20- μ l reaction mixture and was run in triplicates. Initial DNA denaturation was performed at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 15 s, extension at 72 °C for 15 s, and final extension at 72 °C for 5 min. At the end of the amplification, the protocol started at 70 °C with increments of 0.3 °C per cycle. The melting curve was obtained from StepOne Plus software v2.3 and used as the indicator of the enrichment of the aptamer pools.

I. Cloning, sequencing, and synthesis of selected aptamers

The amplified products obtained after negative selection with WBCs were purified, cloned using the TOPOTM TA cloning kit (Invitrogen), and the plasmids were replicated in *Escherichia coli*. Thirty *E. coli* colonies were randomly selected for plasmid purification (Plasmid DNA extraction mini kit, Favorgen, Taiwan). Competitive assays were then performed to verify the selectivity of the aptamers. First, a negative cocktail tube with 3 μl of the aptamers, 100 μl of binding buffer, and negative control cells coated with magnetic beads (10^5 cell ml^{-1}) was prepared. The positive cocktail tube comprised 3 μl of selected aptamers and 100 μl of target cells coated with magnetic beads (10^5 cells ml^{-1}). A competitive cocktail tube comprising free (not bound to magnetic beads) target cells (10^6 cells ml^{-1}) in addition to the components of the positive cocktail tube was also prepared. The three tubes were mixed on a rotary mixer (Elmi Intelli-Mixer RM-2, Rose Scientific, California, USA) for 10 min. They were then placed on a magnetic separation rack, and the magnetic beads were attracted to an external magnetic field. The suspension was removed, and the washing buffer was used to remove the unbound ssDNA. Finally, the aptamers were PCR amplified and sent to be sequenced by Protech Technology Enterprise Co., Ltd. (Taipei, Taiwan) with the common plasmid sequencing primer M13. Three-dimensional structures of the sequences were analyzed at 25 °C with Na^+ and Mg^{2+} concentrations of 137 and 0 mM, respectively, using MFOLD version 3.5 (<http://mfold.rna.albany.edu/>). The single-stranded aptamer sequences were then synthesized and labeled with 5'FAM by Protech and used for the cell staining experiments discussed below.

J. Determination of dissociation constants by flow cytometry

To determine the binding affinities of the synthesized aptamers towards the target and negative control cell lines, carboxyfluorescein (FAM)-labeled aptamers (concentrations ranging from 0.1 to 150 nM) were incubated with 10^5 SNU-478 cells, 10^5 HuCCT-1 cells, and 10^5 MMNK-1 cells for 20 min in 50 μl of the binding buffer. Cells were then washed thrice with 50 μl of the washing buffer and suspended in 50 μl of binding buffer for analysis via flow cytometry (BD AccuriTM C6, Becton-Dickinson, USA). Note that the cells were removed from the culture dish with the Enzyme-free Cell Dissociation Buffer (Thermo-Fisher Scientific, USA) as recommended by the manufacturer to limit damage caused to the cell surface proteins by trypsin. Cells (10^5) without the labeled aptamers served as the background control. The K_d of each fluorescent aptamer was calculated from the mean fluorescence intensity and the aptamer concentration using the “one binding site” equation: $Y = B_{\text{max}}X/(K_d + X)$, where Y represents the bound fraction, B_{max} is the saturated binding concentration, and X is the ligand concentration. The analysis was conducted in triplicates.

K. Fluorescent imaging of FAM-labeled aptamers bound to CCA cells and negative control cells

Target CCA and MMNK-1 cells were incubated with FAM-labeled aptamers at a concentration of 500 nM in 200 μl of binding buffer with 10% FBS for 20 min at 4 °C. The cells were then washed thrice with washing buffer and stained with Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate, Thermo-Fisher Scientific) for 5 min. They were then washed twice with washing buffer and once with 1 $\times$ PBS. The cells were then fixed with 4% paraformaldehyde for 10 min and air dried. The cells were embedded on microscope slides with ProLong[®] Gold Antifade Reagent (Invitrogen) and analyzed under a BX43, Olympus, Japan microscope equipped with a DS-Qi1Mc camera.

K. Testing the binding selectivity of CCA aptamers to different cancer cell lines

Binding selectivity tests were conducted to determine each aptamer's selectivity and specificity towards the target cell lines and other cancer cells.

- (i) Coating magnetic beads with the aptamer
 Dynabeads MyOne[®] carboxylic acid beads (100 μ l; $9\text{--}12 \times 10^9$ beads ml^{-1} 1.0 μm ; Invitrogen) were transferred to a micro-centrifuge tube and washed twice with 900 μ l of ddH₂O. They were then suspended in a solution comprised of 950 μ l of ddH₂O, 30 μ l of amine-modified aptamer (100 μM), and 20 μ l of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC, 120 mg ml^{-1} Sigma, USA). Incubation was carried out for 18 h at room temperature in the dark. The micro-centrifuge tubes were then placed on the magnetic separation rack, and the supernatant was discarded. After repeating the washing step twice, 1 ml of a solution containing 0.02% Tween 20 (Sigma), 0.1% sodium dodecyl sulfate (SDS, Sigma), and 0.1 M ethanol amine (Sigma) was used to block the free uncoupled sites on the surface of the aptamer-magnetic beads at room temperature for 1 h in the dark. After blocking, the aptamer-magnetic bead complexes were washed twice with 1 ml of ddH₂O, suspended in 1 ml of ddH₂O, and stored at 4 °C.
- (ii) Binding selectivity test with various cancer cell lines and Ber-Ep4
 Cells (10^5 in 100 μ l) of the following cancer cell lines were incubated with 10 μ l of aptamer-coated MyOne beads in 40 μ l of binding buffer: BxPC-3 (pancreatic adenocarcinoma cell line), HepG2 (human liver cancer cell line), Huh-28 (cholangiocarcinoma cell line), Kku-100 (cholangiocarcinoma cell line), Huh-7 (hepatocyte-derived cellular carcinoma cell line), Hep3B (human liver cancer cell line), SNU-478, and HuCCT-1. The open-chamber micromixers/micropumps of the positive selection chamber were used to perform the binding selectivity test, and they were operated at a driving frequency of 1 Hz under a gauge pressure of -60 kPa and 6.8 kPa of positive pressure for 10 min. The cells were then washed thrice with 50 μ l of washing buffer and suspended in 50 μ l of binding buffer. The cells were then removed from the chip via the waste chamber, collected, and counted using a hemocytometer under a microscope. As a comparison to the binding selectivity test, a monoclonal antibody raised against BerEp4, a known cancer biomarker, was coated on magnetic beads (Dynabeads Epithelial Enrich) to serve as a reference marker.²⁶ Cells of the eight target cell lines (10^5 cells in 100 μ l) were incubated with 10 μ l of the antibody coated beads for 10 min under the same operational conditions of the chip as mentioned above and the bead + cell complexes were separated with a magnet. The incubation timings and conditions were the same as used in the aptamer + target cell binding assays described above. Each experiment was conducted in triplicate.

III. RESULTS AND DISCUSSION

A. Chip characterization

The performance of the microfluidic platform (Fig. 1), which was fabricated from PDMS, was characterized in order to determine the optimal conditions for chip operation. The optimal conditions for operating the normally closed valves were found to be 7 kPa pressure, 1 Hz pulsation frequency, and -40 kPa negative gauge pressure. Pumping rate, mixing efficiency, and shear stress were calculated for the micropumps/micromixers. The pneumatic micropump/micromixer featured a normally closed microvalve that not only pumped effectively but also was characterized by a high back pressure. It is worth noting that such back pressure prevented cross-contamination.

The relationship between the liquid pumping rate and the EMV driving frequency at different vacuum pressures was first tested [Fig. 2(a)]. The maximum pumping rate obtained was found to be $1999 \mu\text{l min}^{-1}$ when operated at a pulsation frequency of 1 Hz, a pneumatic pressure of 6.8 kPa, and a -40 kPa vacuum. The maximum pumping rate was limited by the release time of the compressed air via membrane actuation. The reduction in the pumping rate at higher frequencies and air pressures could be attributed to the fact that compressed air may not have been completely released from the air chamber between cycles.²⁷

Having first calculated the pumping rate, we then determined the mixing efficiency of the micropump/micromixer unit, as precise mixing and washing are important for successful Cell-SELEX.²⁴ Furthermore, a higher mixing efficiency is associated with a shorter reaction time,

FIG. 1. Integrated microfluidic chip: (a) The chip was equipped with multiple micro-devices, including three reagent loading chambers (for the washing buffer, binding buffer, and fetal bovine serum (FBS)), three transportation units, normally closed micro-valves, two open-chamber micromixers/micropumps, and a PCR chamber. (b) A photograph depicting the three layers of the microfluidic chip. The thick and thin PDMS layers served as the air control and liquid control layers, respectively. These two were bound together by O_2 plasma treatment before adhesion onto a glass substrate.

meaning that the incubation time can be shortened. The relationship between the mixing index and the total mixing time at different driving frequencies of the electro-magnetic valves (EMV) was investigated [Fig. 2(b)], and complete mixing could be achieved at a pulsation frequency of 1 Hz in about 1.75 s ($\sigma > 95\%$) at an applied pressure of 6.8 kPa and a negative gauge pressure (vacuum) of -40 kPa. The mixing index in the initial stages of mixing when the two fluids were separated and minimum diffusion existed between the two was found to be as low as 34%; this value increased to 97% when complete mixing was achieved (less than 2 s).

Different gauge pressures correspond to different shear stress levels during mixing, and proper regulation of shear stress leads to better control of stringency in the aptamer selection process.²⁴ It has been well documented that higher shear forces generated by the pneumatic membranes driven by compressed air may damage cells during incubation.¹⁶ High shear forces also have a significant effect on receptor-mediated apoptosis of cancer cells.²⁸

Herein shear force as calculated from the experiments was 5.86 nN at a frequency of 1 Hz and a compressed air pressure of 6.8 kPa at a negative gauge pressure of -80 kPa [Fig. 2(c)]. The shearing force was increased with each increasing round of SELEX by varying the applied pressure, resulting in an increase in the specificity of the selected candidate pool. It is to be noted that the operating conditions for the first round of SELEX were 6.8 kPa of positive pressure, -40 kPa vacuum, and 1 Hz frequency. Negative pressure was increased with every passing round of SELEX in order to increase the shear force; this improves the stringency of selection,

Working pressure (kPa)	Shear force (nN)
-27	2.72
-53	3.98
-80	5.86

FIG. 2. Characterization of the integrated microfluidic chip: (a) pumping rate, (b) mixing index, and (c) shear force measurements. Error bars represent standard deviation ($n = 3$ independent experiments).

as demonstrated in our earlier works.²⁹ As a result of such precise control over the operating conditions, our novel microfluidic platform was capable of reducing the analysis time to 1/6th that of prior works; aptamer candidates were successfully selected in just six cycles, and at least 15 such cycles are required for traditional protocols.³⁰

B. CCA Cell-SELEX screening

A magnetic bead-based microfluidic platform was developed herein for CCA aptamer isolation. Specifically, an integrated microfluidic chip was designed in order to perform the entire process of CCA Cell-SELEX. It successfully selected two aptamers against an intra-hepatic cell line (HuCC-T1) and one aptamer against an inter-hepatic cell line (SNU-478) on-chip. A non-tumorous cholangiocyte cell line (MMNK-1) was used as a negative control. Briefly, each

round of Cell-SELEX involved a positive selection step (for targeting the CCA cells), a negative selection step (to eliminate non-specific sequences), and a polymerase chain reaction (PCR) step. A small volume ($2\ \mu\text{l}$) of the PCR products from the previous was denatured, cooled rapidly to 4°C , and immediately used for the next round of selection. This method has already been documented earlier.^{31,32} Each of these steps is detailed in the Materials and Methods, and a schematic is presented in Fig. 3. In order to reduce the non-specific binding of aptamers, an additional negative selection round was carried out with MMNK-1 cell lines. With the future perspective of using the selected aptamers for isolation of circulating tumor cells from whole blood, an additional round of negative selection with white blood cells (WBCs) was also incorporated.

FIG. 3. The Cell-SELEX process performed on a microfluidic chip. (a) Incubation and mixing of the single-stranded DNA (ssDNA) library with target cell-magnetic bead complexes in binding buffer and fetal bovine serum (FBS) in the positive selection chamber. (b) Washing and removal of unbound ssDNA using the washing buffer and removal of wastes through the waste collection chamber. (c) Thermal release of bound ssDNA and application of a magnetic field to isolate the cells and transfer the bound ssDNA to the negative selection chamber. (d) Negative selection in the negative selection chamber with negative control cell-magnetic bead complexes in binding buffer. (e) Magnetic separation of negative control cell-magnetic bead complexes, and transfer of supernatant to the PCR chamber. (f) PCR amplification of selected sequences. The amplified product was then transferred to the positive selection chamber for the next round of SELEX. Blue arrows indicate the micro-chamber in which the respective reaction step was carried out.

Cell-SELEX was performed on-chip for 1–6 cycles with each target cell line followed by negative selection with the negative control cell line. After each cycle, aliquots (5 μ l) were removed from the PCR chamber manually for loading into an agarose gel (2%) to confirm the success of the screening. Figures 4(a) and 4(b) show the gel results of the HuCCT-1 and SNU-478 cell lines,

FIG. 4. Agarose gel electrophoresis: (a) PCR products were electrophoresed on an agarose gel (2%) and stained with ethidium bromide. For panels (a) and (b), lane L = 50-bp DNA ladder, lane N = ddH₂O (negative control), lane P = 1 μ M of ssDNA library, and lanes S1 to S6 = PCR products (30 cycles) obtained after 1 to 6 rounds of SELEX, respectively, with HuCCT-1 (a) and SNU-478 (b) cells. (c) Gel electrophoresis analysis after a second round of negative selection performed with MMNK-1 cells and WBCs. Lanes S and H depict PCR products after negative selection with SNU-478 and HuCCT-1 cells, respectively.

respectively. Note that ethidium bromide was used to stain the gels. A clear band indicating the amplification of the selected sequence was documented after every round of SELEX. The occurrence of a faint band of about 150 bp in lane S1 may be due to non-specific amplification. This fragment was not produced in subsequent rounds of SELEX, likely due to the increase in stringency of the operating conditions. A gel image depicting results of the final round of negative selection with WBCs (described in the Materials and Methods) is shown in Fig. 4(c). The bands depicted on this gel are faint, which is indicative of the removal of non-specific sequences. It is to be noted that negative selection with WBCs was carried out with the amplified product obtained after the second round of negative selection with the negative control cell lines. Traditionally, 15–20 rounds of selection are required, but automation of the screening process on an integrated microfluidic system is capable of reducing the number of rounds from over 22 to 5 or 6 rounds as the selection stringency could be gradually increased from one selection round to another. Furthermore, the washing process performed by activating micropumps could alleviate the “dead volume” issue, which refers to the fact that the un-bound ssDNA (with no or weak affinity toward target cells) could be amplified in the following PCR process if they were retained during the washing process.

C. Monitoring the enrichment of selected DNA pool

A successful PCR forms only one PCR product, and this in turn produces only a single melt curve. Therefore, purity of the PCR reactions can be monitored accordingly. Real-time PCR was carried out to confirm the enrichment of the aptamer pool. The melting curves of the products from the ssDNA pools, as shown in Fig. 5, demonstrate that for both SNU478 and HuCCT-1 cell lines, the melting temperature rose as selection rounds increased until the 5th round and there was no further increase in the 6th round. Figures 5(a) and 5(b) depict the melting curve of ssDNA pool for SELEX with SNU-478 cell line and HuCCT-1 respectively. The RT-PCR products of the 5th and 6th cycles depicted a melting temperature of 84.88 °C and 85.02 °C, respectively. These data suggested that the enriched aptamer pool was finally achieved after 6 rounds of selection.^{33,34}

D. Selection of aptamers

Prior to sequencing and synthesizing the aptamers, their specificity was verified. During a competitive assay, the magnetic bead-target cell complexes and free target cells were allowed to compete for the limited number of available, pre-screened ssDNA.¹⁶ As a result, only cell-specific aptamers bound to the magnetic beads would ultimately be amplified by PCR. Based on this test (data not shown), one sequence revealed significant binding to SNU-478 cells, and two sequences displayed affinity towards HuCCT-1 cells. Binding affinity curves of the selected aptamers with their target cell line and the negative control cell lines have been shown in Fig. 6.

FIG. 5. Melting curves analysis of the ssDNA pools after each round of SELEX. (a) Melting curve of SELEX rounds 1 to 6 for SNU-478 cell line. (b) Melting curve of SELEX rounds 1 to 6 for HuCCT-1 cell lines. The RT-PCR products of the 5th and 6th cycles depicted a melting temperature of 84.88 °C and 85.02 °C.

The selected aptamers were incubated with negative control cell lines in order to determine their affinity if any towards them. As depicted in Figs. 6(b), 6(d), and 6(f), the aptamers were independent of the aptamer concentration when incubated with the negative control cell lines but showed a definite binding curve and concentration dependency when incubated with their respective target cell lines, as shown in Figs. 6(a), 6(c), and 6(e). The aptamer sequences and their equilibrium dissociation constants (K_d) can be found in Table I, and the K_d for the CCA cell-specific aptamers ranged from 20 to 41 nM.

The 3D structures of the aptamers with the respective free energies of various structures (as determined by MFOLD) have been shown in Fig. 7. The aptamers showed complex secondary structures including protruding stems and loops. The Gibbs' free energies of the structures were low suggesting that the aptamers might maintain a rather stable structure.

E. Cell staining and fluorescent imaging

CCA-specific aptamers labeled with 5' carboxyfluorescein (FAM) were further incubated with target and negative control cells and observed under a fluorescence microscope. The FAM-labeled aptamers SN8, HN2, and HN16 labeled their respective target cells [Fig. 8(a)].

FIG. 6. Determination of dissociation constant based on flow cytometry analysis. (a) HN2 aptamer with HuCCT-1 cell line; (b) HN2 aptamer with MMNK-1 cell line; (c) HN16 aptamer with HuCCT-1 cell line; (d) HN2 aptamer with MMNK-1 cell line; (e) SN8 aptamer with SNU-478 cell line; (f) SN8 aptamer with MMNK-1 cell line.

TABLE I. Sequences and dissociation constants (K_d) of the selected aptamers.

Aptamer	K_d	Sequence (5'-3')
SN8	$(29 \pm 6.8 \text{ nM})$	GGCAGGAAGACAAACAGGCCGACTCGCCCCACACACCGACCGCTCGT ATCATGCGGTGGTCTGTGGTGCTGT
HN2	$(41 \pm 5.1 \text{ nM})$	GGCAGGAAGACAAACACCGGCGGAGTGCCACATCCCACGCGAGCCTGCTGT TCCGTGGTCTGTGGTGCTGT
HN16	$(37 \pm 5.8 \text{ nM})$	GGCAGGAAGACAAACACGGGGCGGCCGAAACAATGCGTTTCGACCCAGCA ACTCGTGGTCTGTGGTGCTGT

FIG. 7. 3D structures and free energies of the aptamers (a) SN8: -10.83 and $-10.78 \text{ kcal mol}^{-1}$ for the left- and right-most structures, respectively. (b) HN2: -10.09 and $-9.13 \text{ kcal mol}^{-1}$ for the left- and right-most structures, respectively. (c) HN16: $12.03 \text{ kcal mol}^{-1}$.

FIG. 8. Cells stained with 5'FAM-labeled aptamers (green) and Hoechst (blue). (a) Fluorescent images of three specific aptamers to their respective target cell lines; SNU-478 (SN8) and HuCCT-1 cells (HN2 and HN16); (b) Fluorescent images of the three aptamers to the negative control cell line (MMNK-1).

The merged images display the nucleus in blue (Hoechst) and the aptamers bound to the cell surface receptors (green). As shown in Fig. 8(b), little green staining was observed on the surfaces of the negative control cells, demonstrating that the aptamers had minimum affinity towards the negative control cell line. For the HuCCT-1 cell lines, the fluorescence intensity observed for HN2 and HN16 aptamers was consistent with their relatively low K_d values (41 and 36.6 nM, respectively; Table I).

F. Binding selectivity of CCA-specific aptamers

Binding selectivity tests were conducted to determine each aptamer's specificity towards the target cell lines, as well as other cancer cells; the latter group included BxPC-3 (pancreatic adenocarcinoma cell line), HepG2 (human liver cancer cell line), Huh-28 (cholangiocarcinoma cell line), Kku-100 (cholangiocarcinoma cell line), Huh-7 (hepatocyte-derived cellular carcinoma cell line), and Hep3B (human liver cancer cell line) (Table II). The SNU-478-specific aptamer SN8 and the HuCCT-1-specific aptamers HN2 and HN16 captured $\geq 50.0\%$ of their target cells and $\leq 50\%$ of the non-target cells (Table II). With the aim of determining the

TABLE II. Binding specificity of seven CCA-specific aptamers to various cell lines. + = cell capture rate of 0%–24.9%; ++ = 25.0%–49.9%; +++ = 50.0%–74.9%; ++++ = 75.0%–100%.

Cell lines	Aptamers			Antibody Ber-Ep4
	SN8	HN2	HN16	
SNU-478	+++	+	+	++++
HuCCT-1	+	++	++	+++
MMNK-1	+	+	+	+++
BxPC-3	+	+	+	++
Hepg2	+	+	+	++
Huh-28	+	+	+	+++
Kku-100	+	+	+	+++
Huh-7	+	+	+	+++
Hep3B	+	+	+	++

biomarker capacity of these aptamers, a comparative study was conducted with magnetic beads coated with anti-mouse IgG1 monoclonal antibody (clone Ber-EP4) specific for two (34 and 39 kDa) glycopolyptide membrane antigens. These glycoproteins, called epithelial cell adhesion molecules (EpCAM), are membrane glycoproteins that are highly expressed by most carcinomas and are well-known diagnostic and prognostic markers for a variety of cancers.^{35,36} This oncogene has been widely studied since its discovery ~40 years ago, and it is involved in the cell cycle and proliferation of cancer cells.^{37,38} The selected aptamers performed well in recognizing their targets with high selectivity when compared to the Ber-Ep4 coated magnetic beads, which captured all cancerous cells used in the study at similar rates (Table II). The aptamers uncovered herein thereby hold promise for targeted therapy without affecting healthy or non-specific cell lines.

IV. CONCLUSIONS

Three highly specific aptamers were obtained for both human inter-hepatic and intrahepatic cell lines. The PCR amplicon bands produced were similar in intensity to those generally observed after performing 15+ rounds of selection on a conventional platform; therefore, the use of our novel microfluidic platform saves both time and effort. Dissociation constants of the aptamers were determined using flow cytometry and microscopy, and the aptamers only bound to the target cell lines. Further efforts are needed to identify the cellular ligands of SN8, HN2, and HN16. When the binding site of the aptamers is validated, comparison of the sensitivity and specificity of the aptamer with that of an antibody may be explored in more detail. In conclusion, we demonstrated the use of a microfluidic platform for the selection of three high-affinity aptamers specific to CCA cells by performing an automated, on-chip Cell-SELEX process. In the future, this system could be modified for use in personalized medicine or drug discovery.

ACKNOWLEDGMENTS

The authors would like to acknowledge financial support from Taiwan's National Health Research Institute (NHRI-EX104-10428EI) and Ministry of Science and Technology in Taiwan (MOST 105-2221-E-007-007; MOST 105-2119-M-007-009). Partial financial support from Taiwan's Ministry of Education's "Towards a World-Class University Project" (104N2751E1) is also greatly appreciated.

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