ABSTRACT

We present a microchip for isolation of aptamers that bind to target ligands at prespecified temperatures. The device uses integrated resistive heaters and sensors to control the temperatures of (poly)dimethylsiloxane (PDMS) microchambers for temperature-specific selection and bead-based amplification of aptamers. Aptamers are isolated from a randomized DNA library at specified temperatures, and amplified onto microbeads using bead-based polymerase chain reaction (PCR). As a proof of concept, the device was used to isolate aptamers for human immunoglobulin E (IgE), with the enriched pool of candidate aptamers exhibiting a much higher and temperature-dependent affinity for the target protein. The procedure was performed with significantly less reagent use in a much shorter time period (4 hours) than with conventional devices (up to 2 days).

INTRODUCTION

Aptamers are oligonucleotides that display highly specific affinity for target molecules such as proteins, small molecules, nucleic acids, and whole cells, and have applications to clinical diagnostics and therapeutics. In recent years the recognition abilities of aptamers have been coupled with various transduction methods to generate novel diagnostic tools [1]. In addition, these molecules have enabled rapid advances in therapeutics for diseases such as macular degeneration and various types of cancer [2]. Aptamers have several advantages over the most common form of affinity molecule, antibodies, such as their in vitro production (ensuring consistent binding performance), easily modifiable structure, and highly specific affinity to target molecules. Additionally, in recent years “smart” aptamers have been generated which bind with specific equilibrium constants, kinetic parameters, and at specific temperatures [3].

Aptamer sequences are developed by an evolutionary process known as Systematic Evolution of Ligands by Exponential Enrichment, or SELEX. In practice, this process is labor-intensive, requiring months of work by a highly trained technician to discover an aptamer sequence. Conventional SELEX technology, such as column-based separation of binding strands, is inefficient. Some work in recent years has attempted to make the process more efficient, for example by introducing robotic control of the experimental process. However this produces aptamers with lower affinity, requires large capital input, and still uses substantial quantities of reagents and process time.

Recently, microfluidic technology has been used to improve various aspects of the SELEX process. Applications of microfluidics to selection have shown orders-of-magnitude improvement in efficiency, due to the much higher surface area to volume ratios inherent in microfluidic devices [4]. Microfluidic PCR devices have demonstrated the ability to amplify nucleic acids more rapidly and efficiently than conventional systems [5]. Additionally, fully integrated microfluidic devices have demonstrated considerable improvements in process speed and efficiency in other fields [6]. While chip-level integration of SELEX has been lacking [7], recent work shows great promise [8].

Here we present an integrated microchip capable of temperature-specific isolation of aptamers that bind to target ligands. Our device isolates nucleic acids binding to human immunoglobulin E (IgE) at 37°C, amplifies them on-chip, and collects the resulting single-stranded DNA (ssDNA). Results from off-chip analysis and on-chip PCR indicate proper isolation and amplification of binding strands. An enriched pool of aptamer candidates is shown to have greatly enhanced affinity to the target molecule, binding and releasing target molecules at predetermined temperatures. The on-chip isolation process requires fewer reagents and less time that conventional procedures.

PRINCIPLE AND DESIGN

Isolation of Aptamers

Aptamers are typically isolated using multiple rounds of the SELEX process (Figure 1). In SELEX, binding sequences are isolated from a random library (Figure 1a–1c), chemically amplified via the polymerase chain reaction (PCR) (Figure 1d), and single strands are then collected (Figure 1e) so that the process can be repeated. Our microchip consists of two chambers, one of which performs selection and separation of candidate aptamers, and one which amplifies and collects ssDNA (as shown by the dotted lines in Figure 1).

Temperature-Specific Isolation

To control the temperature dependence of aptamer binding, the temperature of the isolation chamber is...
Figure 2: Temperature-specific isolation. At temperature $T_1$, target molecules are exposed to the oligomer library (a), and non-binding oligomers are removed by washing (b). The chamber temperature is then set to $T_2$ and aptamer candidates are eluted by washing (c).

precisely controlled during experimentation. The randomized nucleic acid library is exposed to the target molecules in the chamber at the desired binding temperature, $T_1$ (Figure 2a). Non-binding strands are then removed by washing, while holding the chamber temperature constant at $T_1$ (Figure 2b). The chamber is then set to the desired release temperature, $T_2$, and as washing continues aptamer candidates are released from the beads (Figure 2c). After elution, aptamer candidates are passed to the amplification chamber, where they are captured by microbeads coated with reverse primers prior to PCR.

Chip Design

The microchip primarily consists of two chambers for selection and amplification (Figure 3). The selection chamber is a 400 μm tall cuboid, with two inlet/outlets restricted to a height of 10 μm and one 400 μm tall inlet for insertion of microbeads. The amplification chamber is a 4 μL cylinder 400 μm tall with two inlet/outlets. One such inlet allows for insertion of PCR reagents, including microbeads, while the other is only 10 μm tall so that microbeads may be retained while the supernatant solution is removed. The use of bead retention structures (weirs) throughout the microchip allows for precise control of buffer conditions during each step of the isolation process, by retaining desired nucleic acids on the microbeads while the solution is changed. Between the two chambers is a serpentine channel which serves to mix ssDNA from the selection chamber with PCR reagents via diffusion. Resistive heaters and sensors are placed directly beneath each microchamber to adequately control chamber temperature. The chip is coated with Parylene C to minimize adsorption of reactants and vapor losses.

EXPERIMENTAL

Materials and Equipment

All DNA used in these experiments was purchased from Integrated DNA Technologies (IDT), with sequences as follows. Library: 5'-CTA CCT ACG ATC TGA CTA GCN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN GCT TAC TCT CAT GTA GTT CC-3'. Forward primer: 5'-FAM-Spacer-CTAC TCT GAC TAG C-3'. Reverse primer: 5'-Dual Biotin-Spacer-G GAA CTA CAT GAG AGT AAG C-3'. Library and primer sequences are based on a conventional SELEX protocol targeting IgE [9]. Reagents used for PCR include 5× GoTaq Flexi PCR mix (Promega), 25 mM MgCl$_2$, 10 mM dNTPs (Promega), 50 μg/mL BSA (Sigma), GoTaq enzyme (Promega), and streptavidin-coated polymer microbeads (Streptavidin Plus Ultralink, Pierce). Beads used in the selection procedure were Bio-Rad Affi 10 Gel activated media with 80 μm average diameters. Target protein used was human IgE, (Athens Research).

Device Fabrication

Devices were fabricated using contact lithography. Briefly, glass slides were coated with chrome and gold (15 nm and 150 nm thick, respectively), patterned using optical lithography, and etched to produce resistive heaters and temperature sensors. A 1 μm film of silicon dioxide was then applied via plasma-enhanced chemical vapor deposition (PECVD), with a silicon hard mask defining openings for electrical connections. Molds for soft lithography were defined using optical lithography of layered SU-8 on silicon wafers. PDMS was cast onto these to produce microfluidic channels, chambers, weirs, and the mixer. The PDMS fluidic network was then bonded to the glass slide following oxygen plasma treatment, and the entire chip was coated with 1 μm of Parylene C via CVD prior to packaging (Figure 4a).

Experimental Procedure

Following fabrication, the resistive temperature sensors were calibrated in an environmental chamber and the mixer was tested using green dye to measure mixing efficiency (Figure 4b). Prior to testing, target-coated beads were loaded into the selection chamber until fully packed, and the bead inlet was sealed with wax. Beads
were then washed once with 1× phosphate buffered saline (PBS) prior to testing, and the entire chip was exposed to a 50 μg/mL BSA solution to prevent non-specific adsorption of DNA. The chamber temperature was then set to the desired selection temperature (T₁), 37°C, and three 30 μL aliquots of 10 μM library DNA solution in 1× PBS modified with 1 mM MgCl₂ (PBSM) was introduced to the selection chamber at 5 μL/min. Following exposure to the library, the chamber was then rinsed with ten 30 μL aliquots of 1× PBSM, also at 37°C, to remove unbound or weakly bound ssDNA. The selection chamber was then set to the elution temperature (T₂), 57°C, and after 5 minutes of incubation, four 30 μL aliquots of modified PBS were inserted at 5 μL/min to elute candidate aptamers.

Buffer containing candidate aptamers was mixed with PCR reagents and microbeads, and introduced to the amplification chamber. Remaining unamplified solution was separated from the chip and stored. With the amplification chamber filled, the inlets were sealed with wax and the solution thermally cycled. Fluorescence intensity of the beads confirms final surface concentration of DNA. The chamber was then held at 95°C for 5 minutes to dehybridize bead-bound DNA, and buffer was then flowed at 1 μL/min to remove amplified ssDNA.

Binding analyses were performed in the selection chamber using a fresh chip containing fresh IgE-coated beads. A sample of enriched library DNA was further amplified off-chip, again using FAM-labeled forward primers. This was then purified with streptavidin-coated microbeads; ssDNA was eluted at 95°C, and resuspended in 1× PBSM. Sample concentration was then measured with UV/VIS and normalized to 1 μM. For affinity was measured. As with the affinity measurements, enriched pool had much higher affinity for the target protein, IgE.

In addition to measuring the increased affinity of the enriched pool for IgE, the temperature-dependence of the affinity was measured. As with the affinity measurements, a 1 μM solution of fluorescently-labeled aptamer candidates in 1× PBSM was exposed to a chamber packed

**RESULTS AND DISCUSSION**

**PCR Profiles of Isolation of Binding Oligomers**

A portion of each buffer sample used for washing the beads in the selection chamber was removed and stored for testing following isolation and amplification. These samples were analyzed off-chip using conventional PCR, in parallel with on-chip amplification and collection. To measure the effect of on-chip aptamer isolation on the randomized pool, each of the ten buffer aliquots which washed the target-coated beads at 37°C was amplified off-chip and tested using gel electrophoresis. This generated a clearly defined gradient of concentration, with bands corresponding to initial washes fluorescing brighter than those corresponding to the following washes (Figure 5). This indicates that as selection proceeded, fewer weakly bound strands of library DNA were being removed from the bead-bound targets in the selection chamber, increasing the selection stringency.

Amplification of the washes at 57°C provided a similar measure of the effects of selection. By amplifying each sample of eluted aptamer candidates, we illustrate both the presence of candidates and the efficiency of the elution protocol. Following the isolation washes at 37°C, ssDNA was eluted at 57°C, mixed with PCR reagents on-chip, and subjected to 17 cycles of PCR amplification. Measurements of the fluorescent intensity of microbeads following on-chip bead-based PCR of four aliquots of aptamer candidates showed a discrete change from a strong signal following amplification of the first sample, to a minimal signal in ensuing tests (Figure 6). This sudden change indicated that the desired aptamer candidates were almost entirely eluted in the first wash at 57°C. Future experiments can thus be optimized to minimize the number of PCR amplifications, thus reducing both process time and reagent consumption.

**Affinity Measurements of Enriched Pool**

Finally, the affinity of the enriched pool of aptamer candidates for IgE was tested. To generate a quantity of DNA large enough for testing, the enriched pool was further amplified off-chip using conventional PCR. Following isolation and resuspension of the target ssDNA in 1× PBSM, the concentration of the sample was measured with UV/VIS and normalized to 1 μM. For comparison, randomized library was purchased containing a FAM modification at the 5' terminus, and diluted to 1 μM in 1× PBSM. Samples of each (5 μL) were individually inserted into a microchamber containing microbeads freshly coated with IgE and maintained at 37°C, incubated for 5 minutes, washed with buffer, and micrographed during fluorescent excitation. The increased affinity of the enriched pool versus the randomized library was easily viewable using an optical microscope (Figure 7). Analysis of fluorescent intensity of micrographs after incubation of each 5 μL aliquot confirms that the enriched aptamer pool had much higher affinity for the target protein, IgE.

In addition to measuring the increased affinity of the enriched pool for IgE, the temperature-dependence of the affinity was measured. As with the affinity measurements, a 1 μM solution of fluorescently-labeled aptamer candidates in 1× PBSM was exposed to a chamber packed
with microbeads coated with IgE until the fluorescent signal saturated. Pure buffer was then flowed at 1 μL/min, while the temperature of the chamber was changed in increments of 3°C. While buffer was continuously flowed, the chamber was maintained at each temperature for 5 minutes. The pool exhibited highly temperature-dependent binding to IgE, with maximum binding at 37°C as desired (Figure 8). These temperature-sensitive aptamer candidates were isolated much faster and more efficiently than with conventional technology (Table 1).

Table 1: Comparison of integrated microfluidic aptamer isolation to conventional methods

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<tr>
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<th>Microfluidic Isolation</th>
<th>Conventional Isolation</th>
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<tbody>
<tr>
<td>Sample Volume</td>
<td>30 μL</td>
<td>~250 μL</td>
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<td>Assay Time</td>
<td>4 hours</td>
<td>~2 days</td>
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CONCLUSION

We have developed a microfluidic chip which isolates and amplifies target-binding nucleic acids, or aptamers. By implementing a solid-phase isolation scheme in which target molecules are immobilized onto microbeads in a microchamber, a highly efficient selection procedure was developed. Coupled to an on-chip bead-based PCR procedure, the chip allows rapid, efficient, and temperature-specific aptamer selection. The use of microbeads in both the isolation and amplification chambers allows for very efficient fluid handling and precise control over buffer conditions for every step of the process. As a proof of concept, thermally-responsive aptamers were isolated targeting human IgE. The chip-based procedure required fewer reagents and far less time than conventional procedures for aptamer isolation.

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