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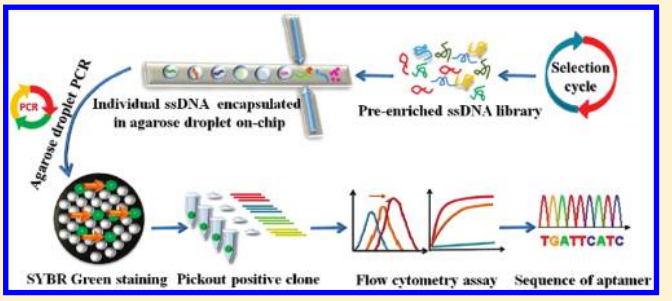
Highly Parallel Single-Molecule Amplification Approach Based on Agarose Droplet Polymerase Chain Reaction for Efficient and Cost-Effective Aptamer Selection

Wei Yun Zhang, Wenhua Zhang, Zhiyuan Liu, Cong Li, Zhi Zhu, and Chaoyong James Yang*

Key Laboratory of Analytical Science, Key Laboratory for Chemical Biology of Fujian Province, State Key Laboratory of Physical Chemistry of Solid Surfaces and Department of Chemical Biology, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China

Supporting Information

ABSTRACT: We have developed a novel method for efficiently screening affinity ligands (aptamers) from a complex single-stranded DNA (ssDNA) library by employing single-molecule emulsion polymerase chain reaction (PCR) based on the agarose droplet microfluidic technology. In a typical systematic evolution of ligands by exponential enrichment (SELEX) process, the enriched library is sequenced first, and tens to hundreds of aptamer candidates are analyzed via a bioinformatic approach. Possible candidates are then chemically synthesized, and their binding affinities are measured individually. Such a process is time-consuming, labor-intensive, inefficient, and expensive. To address these problems, we have developed a highly efficient single-molecule approach for aptamer screening using our agarose droplet microfluidic technology. Statistically diluted ssDNA of the pre-enriched library evolved through conventional SELEX against cancer biomarker Shp2 protein was encapsulated into individual uniform agarose droplets for droplet PCR to generate clonal agarose beads. The binding capacity of amplified ssDNA from each clonal bead was then screened via high-throughput fluorescence cytometry. DNA clones with high binding capacity and low K_d were chosen as the aptamer and can be directly used for downstream biomedical applications. We have identified an ssDNA aptamer that selectively recognizes Shp2 with a K_d of 24.9 nM. Compared to a conventional sequencing−chemical synthesis−screening work flow, our approach avoids large-scale DNA sequencing and expensive, time-consuming DNA synthesis of large populations of DNA candidates. The agarose droplet microfluidic approach is thus highly efficient and cost-effective for molecular evolution approaches and will find wide application in molecular evolution technologies, including mRNA display, phage display, and so on.



Aptamers are single-stranded DNA or RNA sequences that can bind their target molecules with high affinity and specificity.[1] Owing to their significant advantages over antibodies, including quick and reproducible synthesis, easy and controllable modification, long-term stability, ability to sustain reversible denaturation, nontoxicity, lack of immunogenicity, and fast tissue penetration,[2] aptamers have been widely applied for target validation,[3] drug delivery,[4] biomolecule detection,[2c] therapeutics,[5] diagnostics,[6] and biosensing.[7] Aptamers are selected from a large library consisting of 10^{14}−10^{15} random sequences, which involves two major steps: (1) enrichment of high binding affinity sequences and (2) screening of high binding affinity sequences from the enriched library. The enrichment process is also called SELEX (systematic evolution of ligands by exponential enrichment), which involves progressive selection of aptamers by 8−30 rounds of partitioning and amplification from the library.[1a−c]

Over the past two decades since the invention of aptamer technology,[1a−c] great efforts have been made to develop innovative methods for fast and efficient enrichment of aptamers. For example, taking advantage of the excellent separation efficiency of capillary electrophoresis (CE), CE−SELEX has been developed by which aptamers can be enriched after 1−4 rounds of selection.[8] Surface plasmon resonance−SELEX (SPR−SELEX) technology also provides an improved methodology to efficiently and rapidly enrich aptamers with high affinity after only two selection cycles.[9] More recently, microfluidic SELEX technology has been developed, and as few as 1 round of enrichment was reported to enrich high-affinity aptamer sequences.[10] These innovative enrichment methods will further advance the aptamer technology in a wide range of applications.

In contrast to the development of enriching methods, the screening process has remained a rarely explored area over the past two decades. In a normal screening process, the enriched library is cloned into plasmids, which are then transfected into bacteria. Bacteria are then grown, and colonies are picked and sequenced in large quantities to obtain aptamer candidates. Alternatively,

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the enriched library can be sequenced via next-generation sequencing technologies without the need for traditional cloning methods. After analysis of hundreds to thousands of sequences, the discovered consensus sequences with high repeats are chemically synthesized and their binding affinities are screened individually. Such a process is time-consuming, labor-intensive, inefficient, and expensive for several reasons. First, not all of the sequences with high repeats are aptamer sequences.[11] Second, sequences with low repeat numbers are often ignored,[11a,12] leading to loss of some good but low-frequency binding sequences. Furthermore, it is not rare to find sequences with good binding efficiency but lack of selectivity.[11c] Finally, chemical synthesis of long DNA sequences is an expensive and time-consuming process, especially when fluorophore labeling is needed for affinity assessment.

If the binding property of each individual sequence in the enriched library could be evaluated prior to DNA sequencing and synthesis, the wasteful, time-consuming DNA sequencing and chemical synthesis of a large amount of aptamer candidates could be avoided, and consequently, aptamers could be rapidly identified in a high-throughput manner. Toward this end, we have developed a novel method for efficient screening and characterization of aptamers from an enriched single-stranded DNA (ssDNA) library by employing high-throughput single-molecule emulsion polymerase chain reaction (PCR) based on agarose droplet microfluidic technology.[13] With the new method, we were able to identify aptamer sequences with good binding affinity and selectivity against cancer biomarker protein Shp2[14] from an enriched DNA library without the need for DNA cloning, sequencing, and chemical synthesis. The agarose droplet microfluidic approach is highly efficient and cost-effective for aptamer screening and may find wide application in other molecular evolution technologies, including mRNA display, phage display, and so on.

EXPERIMENTAL SECTION

Materials and Reagents. Glass (B270 glass, 150 μm thick) for microfluidic chip fabrication was purchased from Shaoguang Co., Ltd. (Changsha, China). The 749 fluid and 5225C formulation aid were purchased from Dow Corning Co. Silicone oil AR 20 (10836-500 ML), octadecyltrichlorosilane (OTS; 104817-25G), and agarose (A2576-5G, ultralow gelling temperature) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium periodate and sodium cyanoborohydride were purchased from Alfa Aesar (Ward Hill, MA). The initial library (unselected library, 5′-AGC GTC GAA TAC CAC TAC AG-N_{40}-CTA ATG GAG CTC GTG GTC AG-3′, 80-mer), sense primer (5′-AGC GTC GAA TAC CAC TAC AG-3′), and antisense primer (5′-CTG ACC ACG AGC TCC ATT AG-3′) were purchased and ssDNA of the 10th library was sequenced from Sangon Biotechnology Co., Ltd. (Shanghai, China). EasyTaq DNA polymerase and EasyTaq buffer were supplied by Beijing TransGen Biotech Co., Ltd. (Beijing, China). SYBR Green was obtained from Biovision Biotechnology Co., Ltd. (Xiamen, China). Streptavidin-coated Sepharose beads were purchased from GE Healthcare (Piscataway, NJ).

Instruments. DNA amplification was performed in a Peltier thermal cycler (BIO-RAD, Richmond, CA). Images of agarose beads were taken with an inverted fluorescence microscope (Nikon Eclipse Ti-U, Tokyo, Japan). The DNA concentration was determined with a NanoDrop spectrometer (Thermo Scientific, Waltham, MA). The binding affinity of aptamer candidates was determined using flow cytometry (FACSAria, BD Biosciences, Franklin Lakes, NJ).

Aptamer Enrichment. The plasmid pGEX4T1-Shp2 (a gift from Dr. Gensheng Feng at the University of California, San Diego) was transformed into the engineering strain BL-21, and the glutathione S-transferase (GST)-tagged Shp2 protein was expressed. After purification with glutathione Sepharose beads by affinity chromatography, the Shp2−GST fused protein was linked to glutathione Sepharose beads via the selective GST−glutathione interaction for positive selection. To remove DNA sequences that bind to GST protein or bare bead surfaces, GST beads were used as a negative control in the selection. The DNA library (200 pmol) dissolved in 200 μL of binding buffer (phosphate-buffered saline (PBS) spiked with 0.5 mM $MgCl_2$ and 0.1 mg/mL yeast tRNA) was first denatured before incubation with GST beads for counterselection to remove sequences binding to GST beads. Unbound sequences were then incubated with positive Shp2−GST beads. DNA sequences bound to the target-coated beads were eluted, collected, and then amplified by PCR with carboxyfluorescein (FAM)-labeled sense primer and biotin-labeled antisense primer. After denaturation in 0.1 M NaOH, the selected sense ssDNA was separated from the biotinylated antisense ssDNA by streptavidin-coated Sepharose beads and used for the next round of selection. Ten rounds of enrichment were performed.

Design and Fabrication of the Microfluidic Chip. The cross-channel patterns were drawn using AutoCAD software and then printed to a dark mask film. The microchannels on the mask were photolithographically transferred onto a glass surface and etched to produce a 310 μm (width) × 146 μm (depth) cross-channel in a well-stirred bath containing diluted HF/HNO_3 solution. Four 1.8 mm diameter holes were drilled on the etched glass at channel terminals using a mechanical drill (TBM115, Proxxon, Greenville, WI). The etched glass was thermally bonded at 580 °C for 2 h to an identical thick featureless glass in a muffle furnace (Ney Centurion Q50, Dentsply Neytech, Burlington, NJ). After bonding, the microchannels were rinsed with 2-propanol, acetone, piranha solution (H_2SO_4/H_2O_2 = 3/1, v/v), and deionized water and dried with nitrogen gas. Glass channels were then hydrophobically treated with a 0.1% solution of OTS (purity >90%) in dry toluene for 5 min. The treated channels were rinsed with dry toluene, 2-propanol, and deionized water.

Agarose Droplet Generation and Single-Molecule Emulsion PCR. The flow-focusing microfluidic devices consisted of a cross-junction with three inputs and one outlet channel through which agarose droplet flowed out of the device. The PCR cocktail was prepared at 65 °C by mixing 4% ultralow gelling temperature agarose, 5 U/μL EasyTaq DNA polymerase, 10× EasyTaq buffer, a 2.5 mM concentration of each dNTP, 10 μM primers, and the diluted ssDNA of the 10th library against Shp2 protein. The dispersed-phase liquid admixed with PCR cocktail was pumped into the junction through an inlet channel where it met the continuous-phase silicone oil, which flowed from two orthogonally oriented channels. This geometry caused the silicone oil streams to focus the dispersed phase into a thin stream that broke into droplets as it flowed through a constriction into the outlet channel. The geometry of the device and the physics of the fluids at the micrometer scale ensured the stable and reproducible breakup of the dispersed phase into uniform droplets. The droplets were collected into the PCR tube, cooled at 4 °C for 1 h, and then amplified by PCR. The thermal cycling conditions were as follows: 94 °C for 3 min (initial denaturation), 25 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, followed by a

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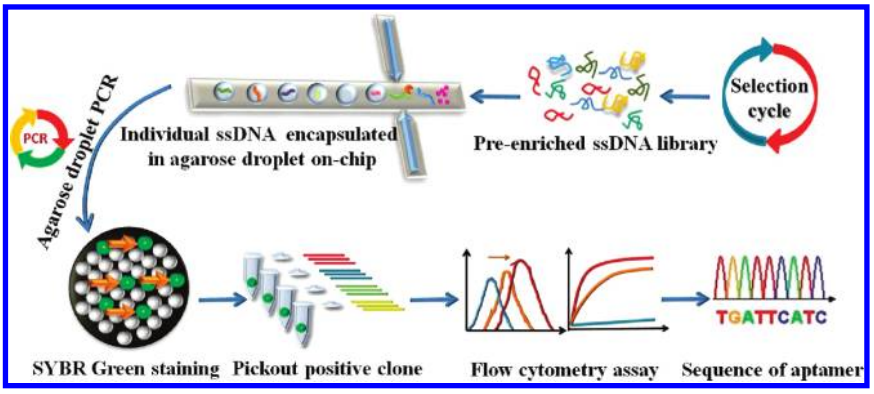

Figure 1. Schematic of aptamer screening by agarose droplet microfluidic technology. Single DNA sequences of an enriched library obtained by traditional SELEX are encapsulated individually into agarose droplets for high-throughput single-copy DNA amplification. The resulting agarose droplets are cooled to become agarose beads and stained with SYBR Green to pick out highly fluorescent beads containing DNA colonies. The binding affinity of DNA in each fluorescent bead against the target molecule is screened. DNA sequences with a low K_d value and good selectivity can be directly used as aptamers or can be sequenced and synthesized for further study.

single final extension at 72 °C for 5 min in a Peltier thermal cycler. Agarose droplets were solidified by cooling to 4 °C for 1 h. After removal of the oil phase in the supernatant, agarose beads were stained in 60 μL of oil phase with 3 μL of 20-fold SYBR Green.

Binding Affinity Evaluation for Positive Clones. An image of individual clones was taken with an inverted fluorescence microscope exposed for 3 s at 100-fold magnification. In detail, agarose beads were diluted with oil, and bright green fluorescent beads packing amplified ssDNA stained by SYBR Green (named positive clones) were picked out manually with a capillary tube. Thirty positive clones were obtained and amplified separately with FAM-labeled sense primer and biotin-labeled antisense primer, followed by incubation with streptavidin-coated Sepharose beads. After denaturation in NaOH (0.1 M), FAM-labeled ssDNA was separated from the biotinylated antisense ssDNA strand, purified with a NAP-5 desalting column, quantified with the NanoDrop spectrometer, and concentrated by vacuum freeze-drying. Consequently, the FAM-labeled ssDNA (100 nM) dissolved in 200 μL of binding buffer (100 mg of tRNA and 5 mL of 1 M $MgCl_2$ were added to 1 L of phosphate-buffered saline) was denatured by heating at 95 °C for 5 min, cooled on ice for 10 min, and subsequently incubated with Shp2 protein beads at 37 °C for 45 min in the dark. After incubation, the beads were washed three times with binding buffer by means of filtration, resuspended in 400 μL of binding buffer, and analyzed by flow cytometry.

K_d Determination of Potential Aptamers. To obtain the K_d values, Shp2 protein beads were incubated with various concentrations of FAM-labeled potential aptamers and then subjected to flow cytometry analysis three times. The K_d values were then calculated by fitting the dependence of the fluorescence intensity of specific binding on the concentration of aptamers to the one-site saturation equation $Y = B_{max}X/(K_d + X)$ using SigmaPlot software. Potential aptamers with a low K_d value were amplified, cloned, and sequenced. Subsequently, to make sure obtained sequences were aptamers, they were chemically synthesized and then their K_d values were redetermined.

Selectivity Test. To verify whether aptamers screened from the 10th library could specifically recognize Shp2 protein, a 100 nM concentration of the FAM-labeled 10th library and aptamers were incubated with target protein Shp2 or control protein GST in 200 μL of binding buffer at 37 °C for 45 min in the dark and the fluorescence intensity was determined by flow cytometry. The FAM-labeled unselected library and RS (random sequence, 5′-TAC TAG ACC GCC ACC CCG TGC AGC TTG AGT TCA CAA TGC GGT GAA CTC TTA ACC TCA GTC GGA CGA TGT TGT GTC AAA AC-3′) were used as the control for nonspecific binding.

RESULTS AND DISCUSSION

The working principle of our agarose droplet microfluidic approach for aptamer screening is schematically depicted in Figure 1. Our agarose droplet microfluidic technology capitalizes on the unique thermoresponsive sol−gel switching property of ultralow gelling temperature agarose for highly efficient individual ssDNA amplification and amplicon trapping.[13] The ultralow gelling temperature agarose has a melting point of about 56 °C and a gelling point around 16 °C. Once melted, agarose remains in the liquid phase until the temperature drops below 16 °C, which ensures easy generation of agarose droplets on chip at room temperature. In addition, at all PCR temperatures, agarose remains in the liquid phase, where PCR takes place with high efficiency. On the other hand, after PCR amplification, the solution form of the agarose droplets can be switched to the solid gel phase by simply cooling the solution below the gelling point. Once solidified, agarose beads will remain solid unless the temperature rises above 56 °C. As a result, DNA products amplified in the droplet will retain their monoclonality even after the oil phase is removed.[13] In the aptamer selection experiment, as shown in Figure 1, single DNA sequences of the enriched library obtained by traditional SELEX technology are encapsulated individually into agarose droplets for high-throughput single-copy DNA amplification. The resulting agarose droplets are cooled to become agarose beads, which are then stained with SYBR Green. Agarose beads containing DNA clones are fluorescent and can be picked out easily. The binding ability of DNA sequences in each individual agarose bead is rapidly screened via flow cytometry. Positive clones with good binding affinity are then further characterized for their dissociation constant (K_d) and selectivity. DNA clones with a low K_d value and good selectivity can be directly used as aptamers without the need for sequencing and chemical synthesis or can be sequenced and synthesized for further study. The ability to segregate and amplify individual sequences in the enriched library with the agarose

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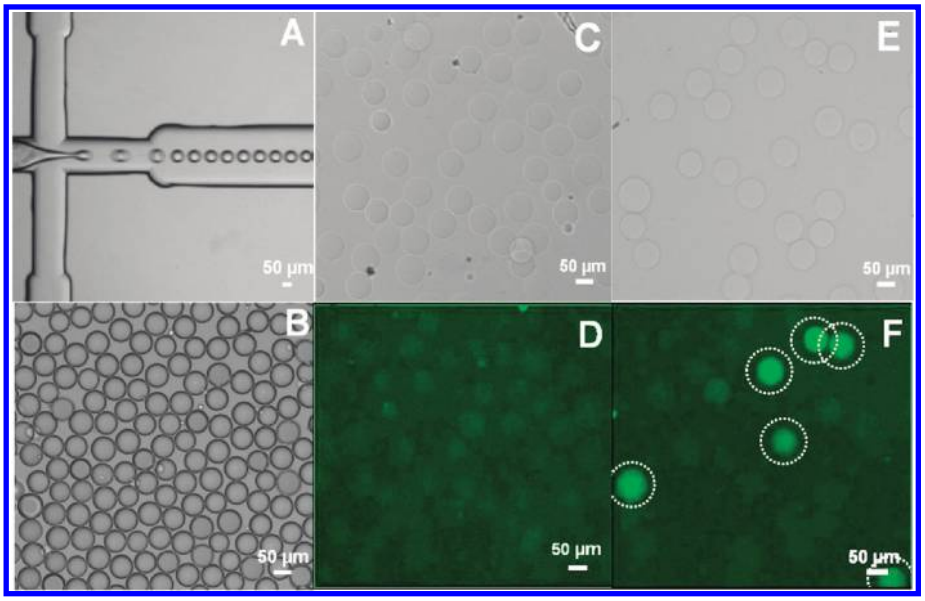

Figure 2. Generation of agarose droplets on chip (A). Uniform agarose droplets in oil (B). Bright-field (C, E) and fluorescence-field (D, F) microscope images of agarose beads after PCR amplification and SYBR Green staining at DNA template concentrations of 0 copies/droplet (C, D) and 0.3 copy/droplet (E, F).

droplet microfluidic approach is the key to the proposed method, which allows rapid and efficient evaluation of the binding affinity and selectivity of individual sequences without the need for DNA sequencing and chemical synthesis.

To demonstrate the feasibility of our agarose droplet microfluidic approach for aptamer screening, a cancer biomarker Shp2 protein[14] with a GST tag was chosen as an aptamer selection target using GST as the control. Conventional SELEX was performed to enrich the DNA library. The single-stranded DNA library was first allowed to interact with excess GST immobilized on Sepharose beads, and the DNA sequences that did not bind to GST beads were collected and incubated with Shp2 beads. After washing, the bead−ssDNA complexes were collected, and the DNA was amplified by PCR with sense primers and biotin-labeled antisense primers. After denaturation in NaOH (0.1 M), the selected sense ssDNA was separated from the biotinylated antisense ssDNA strand on streptavidin-coated Sepharose beads and used for the next round of selection. After 10 rounds of selection (10th library), DNA sequences that bound to GST beads were efficiently removed, while Shp2 protein-specific aptamer candidates were enriched (Figure S1, Supporting Information).

Agarose droplet PCR was then performed to amplify individual DNA sequences in the enriched library to generate monoclonal agarose beads. The enriched 10th library was statistically diluted and encapsulated into uniform agarose droplets on chip so that on average there would be no more than one DNA template in one agarose droplet. Uniform individual agarose droplets were consistently generated on chip at room temperature at a frequency higher than 500 Hz (Figure 2A). Highly uniform monodisperse droplets in oil with a mean diameter of 63 μm were large enough to be picked out and monitored under a bright-field microscope (Figure 2B). After droplet generation, single-molecule emulsion PCR in picoliter volume droplets was performed to generate clonal agarose beads. Images of agarose beads after PCR amplification and stained by SYBR Green were displayed in a bright-field (Figure 2C,E) and fluorescence-field (Figure 2D,F) microscope, respectively. Beads with no DNA template added were used as the control group (Figure 2C,D). Beads with 0.3 copy of DNA template/droplet were used as the experimental group (Figure 2E,F). Droplets with DNA template, after amplification, droplet solidification, and dye staining, produced highly fluorescent beads (positive clones) as highlighted with a white dotted circle in Figure 2F, while empty droplets yielded weakly fluorescent beads (Figure 2D). For demonstration, 30 positive clonal beads were picked out with a capillary tube and kept separately. Then each of these positive clones was further amplified with FAM-labeled primer by conventional PCR and single-stranded DNA to produce enough single-stranded FAM-labeled aptamer candidate sequences for affinity evaluation.

To evaluate the binding ability of the DNA sequence from each clonal bead, flow cytometry was applied. The transition of the signal strength from the unselected library to the 10th library showed a detectable onset of enrichment; thus, the fluorescence intensity of the 10th library was set as the criterion for binding affinity evaluation. Out of 30 positive clones analyzed, a majority of positive clones showed no or weak binding to target protein Shp2 as indicated by their small shift of fluorescence intensity in flow cytometry (Figure S2, Supporting Information). By contrast, the 15th, 18th, and 21st positive clones displayed a fluorescence shift larger than that of the 10th library against target protein Shp2. No observable binding was displayed for the 15th, 18th, and 21st positive clones with control protein GST (Figure 3B). Consequently, their K_d values were further characterized by flow cytometry. K_d values of the 15th and 21st positive clone were 354.0 ± 359.9 and 68.7 ± 43.9 nM (Figure S3A,C, Supporting Information), respectively, while the K_d value of the 18th positive clone was beyond the nanomolar range (Figure S3B).

During the single-molecule amplification step, the enriched DNA library (10th library) was diluted to 0.3 template/droplet to ensure single-molecule amplification. Under this condition, the Poisson distribution equation suggested that there would be 74.1% beads containing no sequence, 22.2% beads containing a single sequence, 3.3% beads containing two sequences, and 0.3% beads containing three sequences. As a result, positive beads with

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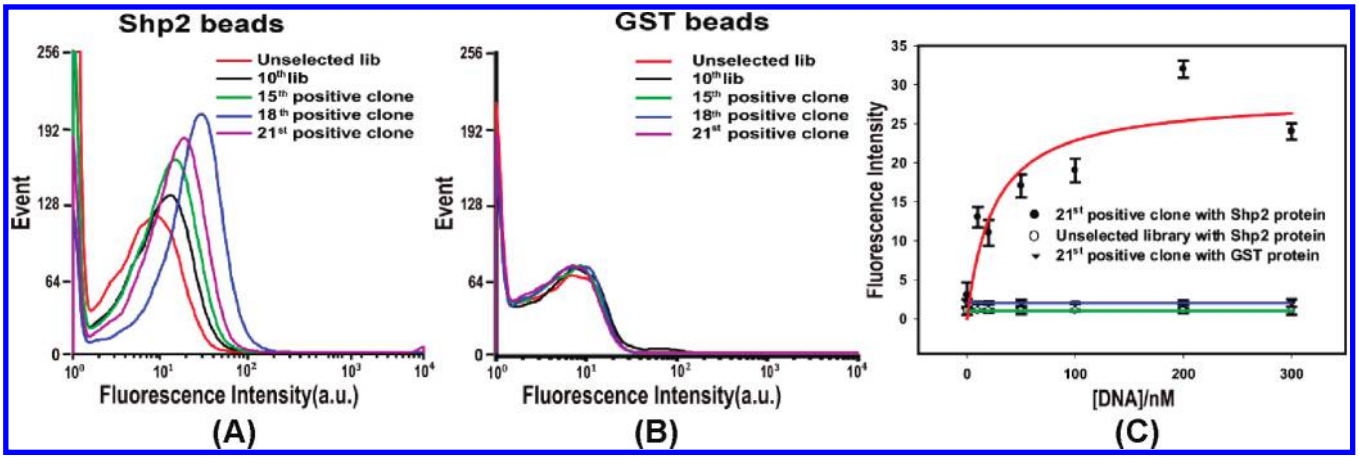

Figure 3. Binding assay of DNA in clonal beads with Shp2 protein (A) and GST protein (B) monitored by flow cytometry. Compared with the unselected library, the 10th library exhibited a clear fluorescence intensity shift. Among 30 positive clones, 3 possible aptamers (15th, 18th, and 21st positive clones) displayed an obvious fluorescence shift with target protein Shp2 (A) and did not show an observable fluorescence shift with control protein GST (B). K_d determination of the 21st positive clone (C). The mean fluorescence intensity for each concentration of the 21st positive clone−Shp2 protein interaction was examined with three replicates. The K_d of the 21st positive clone was 24.9 ± 14.6 nM.

only one sequence accounted for 85.7% of all positive beads containing sequences. Because of its low K_d value and good selectivity, the PCR product of the 21st positive clone was cloned and sequenced. All 10 clonies of the 21st positive clone showed an identical sequence (5′-AGC GTC GAA TAC CAC TAC AGC CCG CAC TCA ACC ACC GTT CCT TTG TTT AAT TTT GCA CAT CTA ATG GAG CTC GTG GTC AG-3′), suggesting that the 21st positive clone was indeed a monoclonal bead. To confirm the binding ability of the 21st positive clone, the identified sequence was synthesized and its K_d value was redetermined using flow cytometry. The synthesized sequence bound to the target Shp2 protein with a K_d value of 24.9 ± 14.6 nM and showed no affinity toward the control GST protein (Figure 3C). Accordingly, the sequence of the 21st positive clone was chosen as a good aptamer because of its high binding affinity and good selectivity. The ability to identify a high binding affinity sequence from an enriched library without the need for lengthy and expensive DNA sequencing and synthesis clearly demonstrated the feasibility of our agarose droplet PCR approach for rapid, inexpensive, and efficient aptamer screening.

After further structure analysis and optimization (Table S1 and Figure S4A−C, Supporting Information), the core binding sequence of aptamer 21 was identified as aptamer 21-11 with the sequence 5′-AGC GTC GAA TAC CAC TAC AGC CCG CAC TCA ACC ACC CTG GTG GTC AG-3′, and the K_d value of aptamer 21-11 was 43.4 ± 9.4 nM (Figure S4D), which was similar to that of the full-length aptamer. To further test the selectivity of aptamer 21-11, several proteins, including thrombin, trypsin, lysozyme, BSA, GST, and Shp2, were incubated with FAM-labeled aptamer 21-11 and subjected to flow cytometry analysis, respectively. No obvious binding of aptamer 21-11 was observed for the proteins tested except for Shp2 (Figure S5A−E, Supporting Information), while significant binding toward Shp2 protein was observed (Figure S5F), indicating that aptamer 21-11 can bind selectively to Shp2 protein.

As a control experiment, the 10th enriched library was screened for aptamers with the conventional cloning−sequencing−synthesis−screening method. The enriched DNA library was cloned and transfected into bacteria. About 30 clones were picked out and sequenced. Sequence alignment indicated the existence of 2 homologue sequences each repeated twice in the 30 tested sequences (Table S2, Supporting Information). Chemical synthesis of these two sequences was performed. Unfortunately, there was no clear binding of both sequences toward target protein Shp2. To identify the best aptamer sequence from the library, one may have to synthesize all of the rest of the sequences. However, this would be an expensive and time-consuming attempt. The failure of finding good binding sequences from the enriched library with the conventional screening method clearly suggested the advantages of our single-molecule PCR approach based on agarose droplet microfluidics.

CONCLUSIONS

We have developed a novel method for efficient screening and characterization of aptamers from an enriched ssDNA library by employing high-throughput single-molecule emulsion PCR based on agarose droplet microfluidic technology. Taking advantage of the unique thermoresponsive sol−gel switching property of ultralow gelling temperature agarose, highly efficient individual ssDNA amplification of the enriched library and amplicon trapping were achieved. After single-molecule emulsion PCR in agarose droplets which serve as microreactors, the binding ability and K_d value of individual ssDNA sequences were determined by flow cytometry. Only the amplified ssDNAs with high binding affinity and a low K_d value were chosen as aptamers and sequenced. Compared to the conventional cloning−sequencing−synthesis−screening work flow, our agarose droplet microfluidic approach avoids large-scale DNA sequencing and expensive and time-consuming DNA synthesis of large populations of aptamer candidates. Therefore, the agarose droplet microfluidic approach allows rapid screening of the binding capability and selectivity of each DNA sequence in an enriched library prior to knowing their exact sequence information, avoiding sequencing and synthesizing nonbinding or weakly binding sequences; hence, it is rapid, efficient, and cost-effective. The agarose droplet microfluidic approach could also be further applied to other molecular evolution methods, including mRNA display, phage display, and so on.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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AUTHOR INFORMATION

Corresponding Author

*Phone: (+)00865922187601. Fax: (+)00865922189959. E-mail: cyyang@xmu.edu.cn.

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