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Self-folded Primers as an Alternative to Single-stranded Primers in an Oligonucleotide Library Designed for Aptamer Screening

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In recent years, oligonucleotide libraries have been used to screen for high-affinity and high-selectivity aptamers that target proteins, peptides, small molecules, or cells. The members of a traditional oligonucleotide library contain a randomized central region with a single-stranded primer region at each end for amplifying target-bound central sequences. However, the single-stranded primers may interact with complementary sequences in the central region of the oligonucleotide and interfere with target-binding. The authors proposed a new library design that uses self-folded motifs to replace the single-stranded primers. For proof of concept, highly structured intercalated motifs (i-motifs) were used to construct the primer regions, and DNA oligonucleotides containing a known streptavidin-binding aptamer in the central region were used to evaluate the performance of the i-motif primers. The applicability of i-motif primers for polymerase chain reaction (PCR) was evaluated as well. The experimental results indicate that the self-folded i-motif primers do not interrupt streptavidin binding to the central region of the DNA oligonucleotide, and that they are PCR-friendly. Finally, when these self-folding primers were used to construct the members of an otherwise traditional DNA library, the target molecules were successfully collected, demonstrating that such primers are a viable alternative to the traditional single-stranded primers in the members of an oligonucleotide library used for aptamer screening.

Keywords: Self-folded primers, Intercalated motif (i-motif), Single-stranded primers, Oligonucleotide library, Aptamer screening

INTRODUCTION

The term "aptamer" refers to an oligonucleotide sequence that is able to adopt a distinct conformation, which allows it to bind to a molecule of interest with high affinity and selectivity. The traditional aptamer screening method, systematic evolution of ligands by exponential enrichment (SELEX), involves repetitive rounds of partitioning target-bound aptamers from unbound oligonucleotides, and amplifying the target-bound aptamers by polymerase chain reaction (PCR) [1,2]. In recent years, a simpler and more efficient approach called CE-SELEX has been developed, which employs high resolution capillary electrophoresis (CE) to conduct the partitioning process [3-9]. Aptamers are usually screened from a randomized nucleic acid pool that contains a large number of fragments (*e.g.*, 10^{12}-10^{13} different sequences), each composed of a randomized central region (30-60 nucleotides) with a fixed, single-stranded primer region at each end (~20 nucleotides) for amplifying target-bound central sequences (Fig. 1A). Because the single-stranded primers may interact with complementary sequences in the central region of the oligonucleotide and interfere with target-binding, many strategies have been developed to modify the traditional oligonucleotide libraries [10-16].

To the authors' knowledge, no study has investigated the use of highly structured, self-folded forward and reverse primers to replace the standard single-stranded primers in an oligonucleotide library to be used for aptamer screening. The corresponding author previously published a

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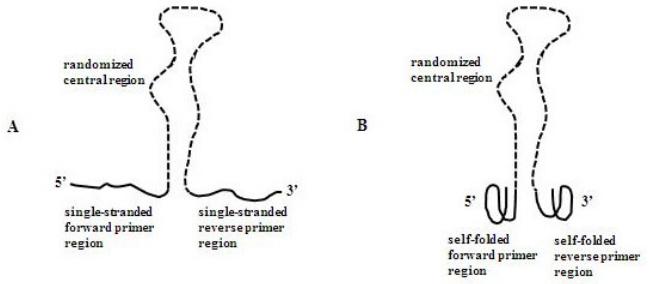

Fig. 1. Schematic illustration of (A) an oligonucleotide that includes traditional single-stranded primers, and (B) an oligonucleotide that includes newly designed, self-folded primers for an oligonucleotide library to be used for aptamer screening. The illustration includes conceptual self-folding primer regions, rather than the actual conformation of the i-motifs used.

Perspective article that proposed a new configuration for SELEX library oligonucleotides that uses conformation-changeable DNA or RNA sequences as primers [17], and suggested intercalated motifs (i-motifs) as a potential choice for such primers. Because i-motifs self-fold (at slightly acidic to neutral pH conditions [18-20]), the authors used them to construct the primer regions (Fig. 1B) for this study, and experimentally evaluated the performance of such primers. A typical i-motif sequence consists of four cytosine tracts separated by three variable regions. A protonated cytosine (C^+) in one tract can pair with an unprotonated one (C) in another tract *via* hydrogen bonds, holding the two cytosine tracts together, and resulting in the subsequent formation of a four-stranded quadruplex structure. Previous studies have demonstrated that certain cytosine-rich sequences, such as 5'-CCCCCTTTCCCCCTTTCCCCCTTTCCCCC-3', abbreviated as $C_5(T_3C_5)_3$, and 5'-CCCCTTTCCCCTTTCCCCTTTCCCC-3', abbreviated as $C_4(T_3C_4)_3$, form stable quadruplex structures under slightly acidic to neutral pH environment [21]. In the current study, the authors used these sequences as the forward and reverse primers, respectively, to build a new type of oligonucleotide library.

For proof of concept, the authors first used a DNA oligonucleotide instead of a DNA library to evaluate the performance of the i-motif primers. Rather than using 40 random nucleotides in the central region, the DNA oligonucleotide contained a 40-nucleotide sequence that is known to target the protein streptavidin, flanked by two i-motif primer regions. The authors compared the performance of the i-motif primer-containing oligonucleotide with two other oligonucleotides that contain traditional single-stranded primers.

Finally, a DNA library was constructed whose members each contain a central region composed of 40 random nucleotides, with an i-motif primer region at each end. The sequences of the forward ($C_5(T_3C_5)_3$) and reverse ($C_4(T_3C_4)_3$) primers are distinct enough to allow the differentiation of the 5' and 3' ends of the oligonucleotides for PCR purposes. The applicability of i-motif primers for PCR amplification was also investigated.

EXPERIMENTAL

Chemicals and Materials

Taq DNA polymerase (recombinant), Taq DNA polymerase PCR buffer (10×, without $MgCl_2$), magnesium chloride (50 mM and 1 M), a dNTP set (100 mM each dATP, dCTP, dGTP, and dTTP), streptavidin protein, and nuclease-free water were purchased from Invitrogen (Carlsbad, CA). Potassium phosphate monobasic, HEPES, and sodium hydroxide were purchased from Thermo Fisher (Waltham, MA). All DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). The Agilent DNA 1000 kit was purchased from Agilent Technologies (Santa Clara, CA). The alkaline wash solution (0.1 M NaOH), and bare fused silica capillaries with 50 μm internal diameter and 375 μm outer diameter were purchased from SCIEX (Redwood City, CA).

Design of DNA Oligonucleotides and DNA Libraries

For this study, a 98-nucleotide DNA oligonucleotide named "i-motif primers with streptavidin aptamer" was constructed. The central region of the DNA (underlined in Table 1) contained a 40-nucleotide sequence that has been proven to bind to the protein streptavidin [15,22]. The forward primer region of the oligonucleotide consisted of four cytosine tracts (with five cytosines in each tract)

Table 1. The Names and Sequences of the Streptavidin Aptamer-containing DNA Oligonucleotides Used in this Study. The 40-Nucleotide Streptavidin-binding Sequence is Underlined

Name	Sequence
i-Motif primers with streptavidin aptamer	5'-TTCCCCCTTTCCCCCTTTCCCCCTTTCCCCC ACTACTATTGACCGCTGTGTGACGCAACACTCAATTTCAC CCCCTTTCCCCTTTCCCCTTTCCCCAA-3'
Traditional primers 1 with streptavidin aptamer	5'-AGCAGCACAGAGGTCAGATG ACTACTATTGACCGCTGTGTGACGCAACACTCAATTTCAC CCTATGCGTGCTACCGTGAA-3'
Traditional primers 2 with streptavidin aptamer	5'-CTTCTGCCCGCCTCCTTCC ACTACTATTGACCGCTGTGTGACGCAACACTCAATTTCAC GGAGACGAGATAGGCGGACACT-3'

Table 2. The Names and Sequences of Members of the DNA Libraries Used in this Study. "N" Represents a Random Nucleotide

Name	Sequence
i-Motif primers library	5'-TTCCCCCTTTCCCCCTTTCCCCCTTTCCCCC NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN CCCCTTTCCCCTTTCCCCTTTCCCCAA-3'
Traditional primers 1 library	5'-AGCAGCACAGAGGTCAGATG NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN CCTATGCGTGCTACCGTGAA-3'
Traditional primers 2 library	5'-CTTCTGCCCGCCTCCTTCC NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN GGAGACGAGATAGGCGGACACT-3'

separated by three loops containing three thymines per loop, abbreviated as $C_5(T_3C_5)_3$. The reverse primer region consisted of four cytosine tracts (with four cytosines in each tract) separated by three loops containing three thymines per loop, abbreviated as $C_4(T_3C_4)_3$. In addition, the forward primer region included TT at its 5' end, and the reverse primer region contained AA at its 3' end. This design reduces the chance of forming forward-reverse primer dimers during the PCR process. To compare the effects of different types of primers on streptavidin binding to the central region of the DNA oligonucleotides, two additional oligonucleotides were constructed, named "traditional primers 1 with streptavidin aptamer" and "traditional primers 2 with streptavidin aptamer," respectively (Table 1). These two DNA oligonucleotides retained the 40-nucleotide central sequence for streptavidin binding (underlined in Table 1); however, the forward and reverse primer regions were different from those in the DNA "i-motif primers with streptavidin aptamer." The authors chose two sets of single-stranded primers that have been widely used to construct DNA libraries for SELEX aptamer screening [3,4,6,23].

Three DNA libraries were constructed as well. The central region of the DNA library members consisted of 40 random nucleotides, flanked by a primer at each end (Table 2). The sequences of the forward and reverse primers in the three libraries were the same as those in the respective DNA oligonucleotide listed in Table 1. The three libraries are thus named "i-motif primers library," "traditional

primers 1 library," and "traditional primers 2 library," respectively.

CE Experimental Setup

First, the $C_5(T_3C_5)_3$ and $C_4(T_3C_4)_3$ i-motif-forming DNA molecules to be used as primers were separately dissolved in nuclease-free water to a final concentration of 100 μM. Next, 5.0 μl of each 100 μM DNA was mixed with 10.0 μl of the 1× binding/running buffer at pH 6.7 (25 mM HEPES, 5 mM KH_2PO_4, and 1 mM $MgCl_2$, adjusted to pH 6.7 using 1 M NaOH), heated at 95 °C for 2 min, allowed to cool to room temperature, and then split into two 7.5 μl aliquots. The first aliquot of each DNA was mixed with 2.5 μl of the 1× binding/running buffer at pH 6.7 to make a 25 μM DNA solution. The second aliquot of each DNA was mixed with 2.5 μl of 100 μM streptavidin protein (diluted with 1× binding/running buffer at pH 6.7) to make a "25 μM DNA-25 μM streptavidin" solution, and then incubated at room temperature for 20 min.

Second, each of the three DNA oligonucleotides listed in Table 1 was dissolved in nuclease-free water to a final concentration of 100 μM. Next, 5.0 μl of each 100 μM DNA was mixed with 10.0 μl of the 1× binding/running buffer at pH 6.7, heated at 95 °C for 2 min, allowed to cool to room temperature, and then split into two 7.5 μl aliquots. The first aliquot of each DNA was mixed with 2.5 μl of the 1× binding/running buffer at pH 6.7 to make a 25 μM DNA solution. The second aliquot of each DNA was mixed with 2.5 μl of 100 μM streptavidin protein, prepared as above, to make a "25 μM DNA-25 μM streptavidin" solution, and then incubated at room temperature for 20 min.

An uncoated fused-silica capillary was installed on a SCIEX P/ACE™ MDQ Capillary Electrophoresis system with UV detection set at 254 nm (Redwood City, CA). The capillary was 60.2 cm total length from inlet to outlet (L_{total}); had a 50.0 cm effective length from inlet to detector ($L_{effective}$); and had a 50 μm internal diameter. The capillary was rinsed with the alkaline wash solution, nuclease-free water, and 1× running buffer at pH 6.7 (25 mM HEPES, 5 mM KH_2PO_4, and 1 mM $MgCl_2$, pH 6.7), respectively, before use. The DNA solutions and the DNA-streptavidin solutions were individually run on the CE system using the 1× running buffer at pH 6.7. Each sample was injected onto the capillary at 1.0 psi for 5.0 s, and then separated at 30.0 kV for 20 min under normal polarity (*i.e.*, an anode is placed at the capillary inlet, and a cathode is placed at the capillary outlet). The entire experiment was repeated three times.

Third, each of the three DNA libraries listed in Table 2 was dissolved in nuclease-free water to a final concentration of 500 μM. Next, 5.0 μl of each 500 μM DNA library was mixed with 2.5 μl of the 1× binding/running buffer at pH 6.7, heated at 95 °C for 2 min, allowed to cool to room temperature, and then mixed with 2.5 μl of 100 μM streptavidin and incubated at room temperature for 20 min. The "250 μM DNA-25 μM streptavidin" solutions were individually run on the CE system using the 1× running buffer at pH 6.7. Each sample was injected onto the capillary at 1.0 psi for 5.0 s, and then separated at 30.0 kV for 20 min under normal polarity. Because the streptavidin-DNA complexes migrate out of the capillary tube earlier than the unbound DNA fragments, the time window for collecting only the complexes at the capillary outlet (t_{out}) was calculated according to the migration time of the complexes (t_{det}, the time needed for the complexes to reach the detection point), using the following equation:

$$t_{out} = t_{det} \times L_{total}/L_{effective} = t_{det} \times 60.2 \text{ cm}/50.0 \text{ cm}$$

The streptavidin-DNA complexes were collected into an outlet vial containing 10 μl of the 1× binding/running buffer at pH 6.7. For each sample, the collection process was repeated three times, all into the same vial.

PCR Amplification and DNA Identification

The collected streptavidin-DNA complexes from each DNA library were PCR-amplified using an Eppendorf Mastercycler® thermal cycler (Enfield, CT). The PCR reaction master consisted of 200 μM dNTPs, 0.5 μM forward primer (Table 3), 0.5 μM reverse primer (Table 3), 0.025 units/μl Taq DNA polymerase, and 7.5 mM $MgCl_2$ in 1× Taq DNA polymerase PCR buffer (20 mM Tris pH 8.4 and 50 mM KCl). Twenty cycles of denaturation (94 °C, 45 s), annealing (58 °C, 45 s), and extension (72 °C, 45 s) were performed. A negative control that used nuclease-free water as the PCR template was run in parallel for each sample. The length and amount of the PCR products were identified using an Agilent DNA 1000 kit on an Agilent

Table 3. PCR Primers Used to Amplify the Collected Streptavidin-DNA Complexes

Name of the DNA Library	PCR Primers
i-Motif primers library	PCR forward primer: 5'-TTCCCCCTTTCCCCCTTT-3'
	PCR reverse primer: 5'-TTGGGGAAAGGGGAAAGG-3'
Traditional primers 1 library	PCR forward primer: 5'-AGCAGCACAGAGGTCAGATG-3'
	PCR reverse primer: 5'-TTCACGGTAGCACGCATAGG-3'
Traditional primers 2 library	PCR forward primer: 5'-CTTCTGCCCGCCTCCTTCC-3'
	PCR reverse primer: 5'-AGTGTCCGCCTATCTCGTCTCC-3'

2100 Bioanalyzer (Santa Clara, CA).

Determination of the Dissociation Constant (K_d)

The streptavidin-DNA binding affinity was measured using a PerkinElmer LS55 Fluorescence Spectrometer (Waltham, MA). One µl of 10 nM streptavidin was added to 3.33 ml of the 1× binding/running buffer at pH 6.7 in a quartz cuvette; this procedure was repeated to produce a total of three aliquots. For each DNA oligonucleotide listed in Table 1, 10 µM was titrated from 0.1 µl to 100 µl into one of the streptavidin aliquots. The streptavidin fluorescence intensities were monitored at 332 nm (*i.e.*, 280 nm excitation, 332 nm emission); all experiments were repeated three times. The data collected from the fluorescence titrations was fit to the following equation using KaleidaGraph 4.0 (Synergy Software, Reading, PA):

$$\text{Fraction bound} = [\text{streptavidin-DNA complex}]/[\text{streptavidin}]_{\text{total}} = [\text{DNA}]/([\text{DNA}] + K_d)$$

RESULTS

Figures 2A and 2B illustrate the electropherograms of the 25 µM DNA $C_5(T_3C_5)_3$ solution at pH 6.7, and the 25 µM streptavidin-25 µM DNA $C_5(T_3C_5)_3$ mixture at pH 6.7, respectively. Electropherograms of the 25 µM DNA $C_4(T_3C_4)_3$ solution at pH 6.7, and the 25 µM streptavidin-25 µM DNA $C_4(T_3C_4)_3$ mixture at pH 6.7 are shown in Figs. 2C and 2D, respectively. Compared to the DNA itself, the streptavidin-DNA complex is larger and thus has lower electrophoretic mobility. Under normal polarity and in a bare fused-silica capillary, all species in the sample move toward the cathode in the presence of electroosmotic flow;

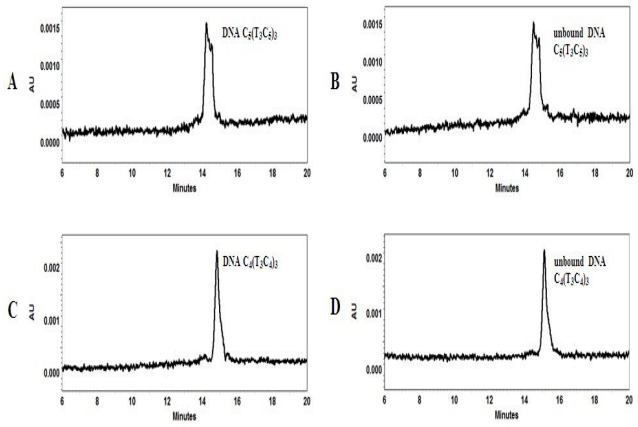

Fig. 2. CE electropherograms of (A) the 25 µM DNA $C_5(T_3C_5)_3$ solution at pH 6.7, (B) the 25 µM streptavidin-25 µM DNA $C_5(T_3C_5)_3$ mixture at pH 6.7, (C) the 25 µM DNA $C_4(T_3C_4)_3$ solution at pH 6.7, and (D) the 25 µM streptavidin-25 µM DNA $C_4(T_3C_4)_3$ mixture at pH 6.7. Note: The electropherogram displays UV absorbance (Y axis, in artificial units, AU) *versus* migration time (X axis, in minutes) at 254 nm. Figure 3 has the same axes as Fig. 2.

the streptavidin-DNA complex migrates off the capillary before the unbound DNA. In Figs. 2B and 2D, no streptavidin-DNA complex peaks were observed before the unbound DNA i-motif peak. In addition, the unbound DNA peaks showed a similar area and height to the DNA control in Figs. 2A and 2C.

Electropherograms of the 25 µM DNA "i-motif primers with streptavidin aptamer" solution at pH 6.7 and the 25 µM streptavidin-25 µM DNA "i-motif primers with streptavidin

aptamer" mixture at pH 6.7 are shown in Figs. 3A and 3B, respectively. Electropherograms of the 25 μM DNA "traditional primers 1 with streptavidin aptamer" solution at pH 6.7 and the 25 μM streptavidin-25 μM DNA "traditional primers 1 with streptavidin aptamer" mixture at pH 6.7 are shown in Figs. 3C and 3D, respectively. Figures 3E and 3F illustrate the electropherograms of the 25 μM DNA "traditional primers 2 with streptavidin aptamer" solution at pH 6.7 and the 25 μM streptavidin-25 μM DNA "traditional primers 2 with streptavidin aptamer" mixture at pH 6.7, respectively. As shown in Figs. 3B, 3D, and 3F, streptavidin-DNA complex peaks were observed in all three samples (enclosed in a dashed rectangle). The ratio of streptavidin-DNA complexes to unbound DNA fragments was determined by calculating the ratio of the area of the streptavidin-DNA complex peak ($A_{complex}$) to the area of the unbound DNA peak ($A_{unbound}$). The proportion of the complexes formed between streptavidin and DNA "traditional primers 1 with streptavidin aptamer" ($A_{complex}$:$A_{unbound}$ ≈ 1:20) is much lower than those between streptavidin and DNA "i-motif primers with streptavidin aptamer" ($A_{complex}$:$A_{unbound}$ ≈ 10:1), and between streptavidin and DNA "traditional primers 2 with streptavidin aptamer" ($A_{complex}$:$A_{unbound}$ ≈ 10:1).

Figures 4A-4C illustrate the predicted secondary structures of the DNA "i-motif primers with streptavidin aptamer," DNA "traditional primers 1 with streptavidin aptamer," and DNA "traditional primers 2 with streptavidin aptamer" generated by RNAstructure software, version 6.3 (https://rna.urmc.rochester.edu/RNAstructure.html). The predications indicate that the streptavidin-binding motif located in the central region of the DNA oligonucleotides could correctly fold in the DNA "i-motif primers with streptavidin aptamer" and DNA "traditional primers 2 with streptavidin aptamer" (see Figs. 4A and 4C), whereas this

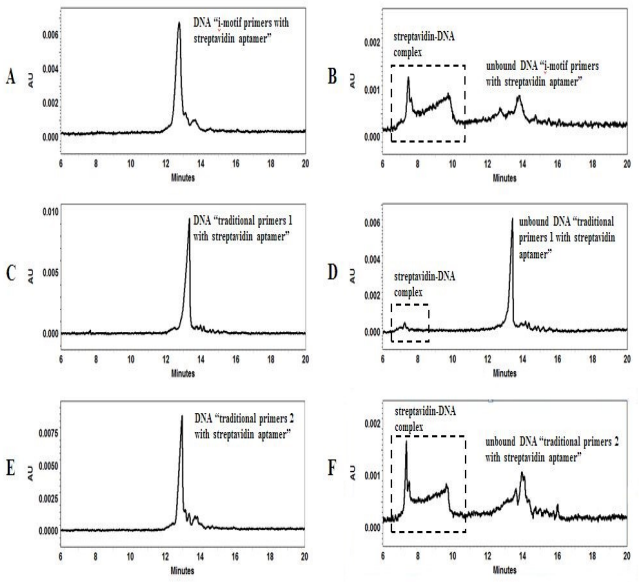

Fig. 3. CE electropherograms of (A) the 25 μM DNA "i-motif primers with streptavidin aptamer" solution at pH 6.7, (B) the 25 μM streptavidin-25 μM DNA "i-motif primers with streptavidin aptamer" mixture at pH 6.7, (C) the 25 μM DNA "traditional primers 1 with streptavidin aptamer" solution at pH 6.7, (D) the 25 μM streptavidin-25 μM DNA "traditional primers 1 with streptavidin aptamer" mixture at pH 6.7, (E) the 25 μM DNA "traditional primers 2 with streptavidin aptamer" solution at pH 6.7, and (F) the 25 μM streptavidin-25 μM DNA "traditional primers 2 with streptavidin aptamer" mixture at pH 6.7. Note: The streptavidin-DNA complex peaks are enclosed in a dashed rectangle.

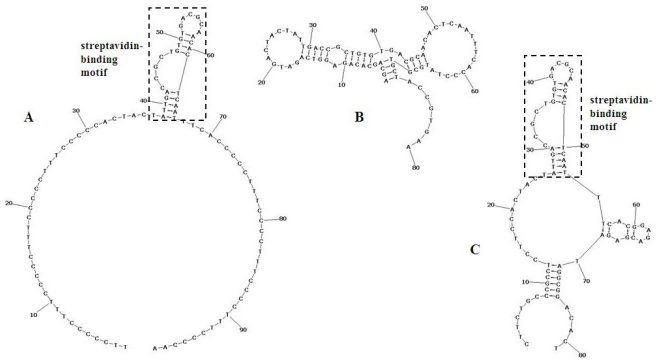

Fig. 4. Predicted secondary structures of (A) the DNA "i-motif primers with streptavidin aptamer," (B) the DNA "traditional primers 1 with streptavidin aptamer," and (C) the DNA "traditional primers 2 with streptavidin aptamer." Because the RNA structure software cannot predict i-motif structures, the i-motif primers in Fig. 4A were output as single-stranded. Note: The streptavidin-binding motif is enclosed in a dashed rectangle.

stem-loop structure was disrupted in the DNA "traditional primers 1 with streptavidin aptamer" due to its primer regions interacting with complementary sequences in the central region of the DNA (see Fig. 4B).

To confirm the applicability of i-motif primers for PCR amplification, the authors incubated streptavidin with the DNA library "i-motif primers DNA library," in which $C_5(T_3C_5)_3$ serves as the forward primer, and $C_4(T_3C_4)_3$ as the reverse primer (see Table 2). The streptavidin-DNA complexes were separated from the unbound DNA fragments at pH 6.7 by CE, and collected into a vial containing 10 µl of the binding/running buffer (pH 6.7). The collected complexes in solution were used as the PCR templates for DNA amplification. Figure 5 shows the gel electrophoresis results of DNA identification. The peaks at the 47 s and 100 s positions in the electropherogram are the lower and upper markers included in the Agilent 1000 DNA kit. There was no peak at the 60 s position in the negative control, which used nuclease-free water as the PCR template (Fig. 5A). The appearance of the PCR-amplified DNA products at the 60 s position in Fig. 5B (enclosed in a dashed rectangle) indicates that the i-motif forward and reverse primers can successfully be used to amplify target-bound central sequences. The complexes of streptavidin-DNA "traditional primers 1 library" and streptavidin-DNA "traditional primers 2 library" were also separated by CE, collected, and then used as PCR templates for DNA amplification. These two samples were successfully amplified as well (data not shown), which is expected because these traditional single-stranded primers have long been used for PCR in SELEX.

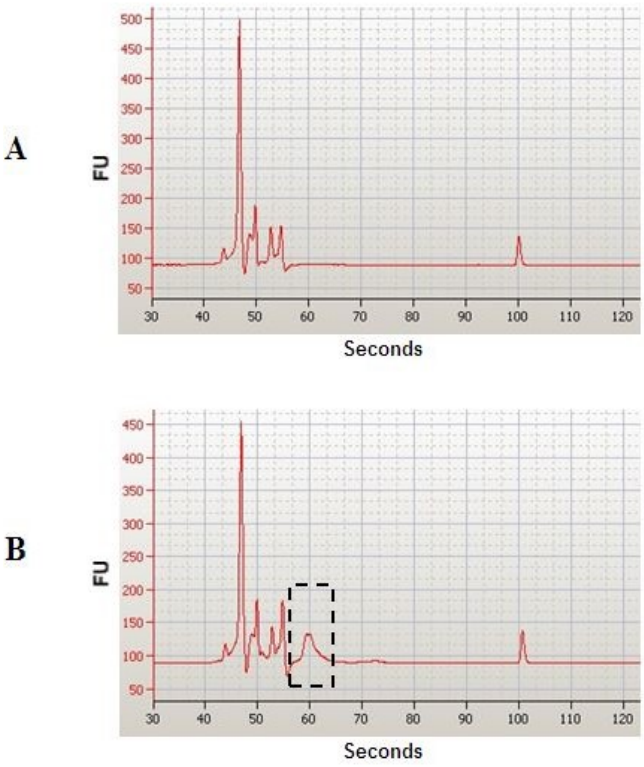

Fig. 5. Identification of the amplified PCR products by gel electrophoresis. Electropherograms of (A) the negative control, with nuclease-free water used as the PCR template, and (B) the amplified streptavidin-DNA complex collected using the "i-motif primers library." The peak at ~60 s is enclosed in a dashed rectangle. Note: The electropherogram displays fluorescence intensity (Y axis, in fluorescence units, FU) *versus* migration time (X axis, in seconds).

DISCUSSION

First, the authors investigated whether the folded DNA $C_5(T_3C_5)_3$ and $C_4(T_3C_4)_3$, which serve as the primers in the DNA "i-motif primers with streptavidin aptamer," bind to streptavidin themselves. Since no protein-DNA complex peaks were observed between 6 min and 11 min in Figs. 2B and 2D, and the unbound DNA i-motif peak demonstrated a similar area and height to the DNA control (compare Fig. 2B with Fig. 2A, and Fig. 2D with Fig. 2C), the authors assert that there are no apparent interactions between these DNA i-motifs and streptavidin.

Next, the authors investigated whether the folded i-motif primer regions interfere with streptavidin binding to the central region of the DNA. For comparison, two DNA oligonucleotides containing traditional single-stranded primers were studied in parallel. As shown in Fig. 3, streptavidin bound to all three DNA samples. However, only the i-motif primers-containing DNA and the traditional primers 2-containing DNA demonstrated significant binding ($A_{complex}$:$A_{unbound}$ ≈ 10:1) (see Figs. 3B and 3F), which suggests that their primer sequences did not anneal to the central region of the DNA. Furthermore, the dissociation constants between streptavidin and both the i-motif primers-

containing DNA (K_d ≈ 24 nM), and the traditional primers 2-containing DNA (K_d ≈ 23 nM) were similar to that between streptavidin and the primer-free DNA aptamer (K_d ≈ 21 nM) [15], indicating that these primers had no significant effect on aptamer-target binding. The traditional primers 1-containing DNA demonstrated a much lower binding affinity to streptavidin ($A_{complex}$:$A_{unbound}$ ≈ 1:20) (see Fig. 3D), indicating the existence of interactions between its primer regions and the central region of the DNA. The K_d value between streptavidin and the traditional primers 1-containing DNA could not be determined due to the low binding affinity.

The predicted secondary DNA structures provide an explanation for the different binding affinities. As shown in Figs. 4A and 4C, the primer regions of the DNA "i-motif primers with streptavidin aptamer" and the DNA "traditional primers 2 with streptavidin aptamer" did not interrupt the formation of the structure that is responsible for streptavidin binding, a 29-nucleotide hairpin with a CCGCT bulge (enclosed in a dashed rectangle). Note that because the RNAstructure software cannot predict i-motif structures, the i-motif primers in the DNA "i-motif primers with streptavidin aptamer" were output as single-stranded in Fig. 4A. Figure 4B illustrates that part of the single-stranded traditional primers 1 annealed to the central region of the DNA oligonucleotides, thus disrupting the streptavidin-binding motif.

Traditional primers 1- and traditional primers 2-containing DNA libraries have been widely used for aptamer screening [3,4,6,23]. Researchers have also designed and used numerous other single-stranded primers for library construction. It is extremely difficult, if not impossible, to identify primer sequences that will seldom or never interact with any central aptamer sequences in an oligonucleotide library. The inconsistent performance of the two traditional primer examples as uncovered in this study highlights the importance of developing alternatives to single-stranded primers for an oligonucleotide library. Even though the DNA "i-motif primers with streptavidin aptamer" demonstrated a similar, rather than better, streptavidin-binding activity to DNA "traditional primers 2 with streptavidin aptamer" (both resulted in $A_{complex}$:$A_{unbound}$ ≈ 10:1) in this study, the advantage of using self-folded primers over single-stranded traditional primers 2 is the reduced likelihood that the primers will anneal to the central regions of aptamers included in future oligonucleotide library members.

After comparing the performance of the folded i-motif primers with the single-stranded primers, the authors investigated whether the i-motif primer regions are PCR-friendly, which is critical for SELEX aptamer selection. Figure 5 shows that the collected streptavidin-bound DNA fragments from an i-motif primer-containing library were successfully amplified. Therefore, the i-motif primers can be incorporated into an oligonucleotide library for aptamer screening.

Previous studies have demonstrated that more than 50% of the DNA $C_5(T_3C_5)_3$ and $C_4(T_3C_4)_3$ molecules fold into a quadruplex structure at neutral pH [21]. The transitional pHs of DNA $C_5(T_3C_5)_3$ and $C_4(T_3C_4)_3$, where 50% of the molecules are folded, are 7.2 and 7.1, respectively [21]. To stimulate the majority of the DNA $C_5(T_3C_5)_3$ and $C_4(T_3C_4)_3$ to stably fold, a slightly acidic solution environment (pH = 6.7) was applied in this study. The authors have also tested the performance of these i-motif primers at physiological pH (7.2-7.4). As shown in Supplementary Fig. 1, the DNA "i-motif primers with streptavidin aptamer" also demonstrated significant binding to streptavidin at pH 7.3; however the binding activity ($A_{complex}$:$A_{unbound}$ ≈ 3:1) is lower than that at pH 6.7. Since less than 50% of the $C_5(T_3C_5)_3$ and $C_4(T_3C_4)_3$ are properly folded at pH 7.3, and our goal was to evaluate self-folded i-motifs as primers, we did not choose physiological pH as the experimental condition for this work.

The authors selected DNA $C_5(T_3C_5)_3$ and DNA $C_4(T_3C_4)_3$ as primers in this study because they are currently the two best i-motif candidates, according to data from the Waller group [21]. The authors have also tested other i-motif primers. For example, DNA "alternate i-motif primers 1 with streptavidin aptamer" contained a $C_5(T_4C_5)_3$ forward primer and $C_3(T_3C_3)_3$ reverse primer; DNA "alternate i-motif primers 2 with streptavidin aptamer" contained a $C_3(T_3C_3)_3$ forward primer and $(C_3TAA)_4$ reverse primer. The transitional pHs of DNA $C_5(T_4C_5)_3$, $C_3(T_3C_3)_3$, and $(C_3TAA)_4$ are 6.7, 6.7, and 6.5, respectively [21]. As shown in Supplementary Fig. 2, these i-motif primers-containing DNAs also demonstrated significant binding to streptavidin at pH 6.7. But because only about half of the DNA

$C_5(T_4C_5)_3$, $C_3(T_3C_3)_3$, and $(C_3TAA)_4$ primers are folded under the experimental condition of pH 6.7, no further investigations on these alternate i-motif primers' libraries were conducted.

This proof-of-concept study indicates that DNA "i-motif primers library" could be used to screen aptamers for cancer diagnosis and treatment, as the extracellular pH of tumor microenvironments is typically acidic (pH 6.5 to 6.9). Future development of i-motif-containing libraries may involve modifying the length and/or base composition of the cytosine tracts, as well as alteration of the length and/or base composition in the loop region of the i-motifs, to allow the i-motif primer regions to stably fold at physiological pH.

Concluding Remarks

This feasibility study demonstrates that self-folded i-motif primers can be used as an alternative to traditional single-stranded primers in an oligonucleotide library for aptamer screening. With a deliberate sequence design, i-motif primer-containing libraries could be functional at both a slightly acidic environment and at physiological conditions. Other types of self-folded primers might also be developed with the prerequisite that they do not bind to the target of interest themselves. The authors assert that these new oligonucleotide libraries would substantially reduce the loss of high-affinity aptamers during the SELEX process.

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Conflict of Interest Statement

The authors declare that they have no competing financial interests.

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