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Conservative secondary structure motif of streptavidin-binding aptamers generated by different laboratories

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ABSTRACT

Aptamers that are selected in vitro from random pools of DNA or RNA molecules by SELEX (Systematic evolution of ligands by exponential enrichment) technique have been extensively explored for analytical and biomedical applications. Although many aptamers with high affinity and specificity against specific ligands have been reported, there is still a lack of well characterized DNA aptamers. Here we report the selection of a group of aptamer candidates (85 mer) against streptavidin. Through comparing the predicted secondary structures of all the candidates, a conservative bulge-hairpin structure section (about 29 mer) was found, and then it was determined to be the binding motif to streptavidin. This binding motif was further discovered to also exist in streptavidin-binding aptamers (SBAs) selected by three other laboratories using different methods. The primary sequences of this secondary structure motif are very different, only several nucleotides in the loop and bulge area are critical for binding and other nucleotides are variable. The streptavidin binding of all the SBAs could be competed by biotin implying that they bind to the same site on streptavidin. These results suggest that the evolution of SBA is predominated by specific groups on streptavidin. The highly variable sequence composition of streptavidin-binding aptamer would make the design of aptameric sensor or device based on streptavidin more flexible and easy.

1. Introduction

Aptamers that bind to specific ligands with high affinity and specificity have attracted intense investigation in recent years because of their potential analytical and biomedical applications.[1,2] Many aptamer-based sensors and devices have been reported,[3,4] which shows their great promise in areas ranging from fundamental research to clinical applications. In addition, the first aptamer-based therapeutic agent (Macugen) for treating a form of macular degeneration entered clinical use in 2005.[5] Aptamers are discovered in vitro from random pools of DNA or RNA molecules (about 10^{14}–10^{15} different sequences) using SELEX (Systematic evolution of ligands by exponential enrichment) technique via rounds of affinity capture and amplification.[6,7] It was assumed that aptamers can be raised against any target and any site of a protein target. In the past two decades, various improvements of the original SELEX method have been reported, such as magnetic bead separation, CE-SELEX, cell-SELEX, M-SELEX, MonoLEX, photo-SELEX, tailored-SELEX, and automated selection.[4,8–17] Hundreds of high-affinity aptamers against different targets ranging from small molecules to live cells have been reported, which can be assessed by reference to an online database.[18] The procedures for the discovery of aptamers include upstream selection and downstream characterization of individual aptamers. At the end of SELEX process (upstream selection), the enriched pool containing aptamers is cloned and sequenced, the obtained sequences are usually found to distribute into different families based on their sequence similarities. These aptamer candidates are full-length sequences (70–120 nucleotides) including the fixed sequence at each terminus for PCR amplification. However, not all nucleotides are necessary for direct interaction with the target or for folding into the structure that facilitates target binding. The unnecessary nucleotides would generally lead to low yield and high cost of aptamer synthesis, and increase the aptamer's susceptibility to random degradation as well as the difficulty of further study.[19] Thus, to identify their minimal binding motif are essential for the further application of aptamers. However, compared to the significant progress in SELEX technique, much less reports have been focused on the downstream aptamer characterization. DNA aptamers have been intensively investigated for analytical applications recently because of their relatively high stability against chemically hydrolysis and ubiquitous ribonucleases, and low cost of chemical synthesis compared to RNA. However, a large proportion of the reports on analytical applications of aptamers use the same DNA aptamers, such as aptamers against ATP, thrombin, platelet derived growth factor (PDGF), and

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cocaine.[3,4] One of the possible reasons is that only these DNA aptamers have been well characterized. Thus the short of well characterized DNA aptamers is a main obstacle for broad application of aptamer.

The commonly used methods for determining the binding motif and binding site of aptamers include enzymatic footprinting, partial hydrolysis or in vitro transcription of truncated DNA. The predicted potential minimal sequences then had to be prepared to confirm the binding capacity. However, these approaches are often complicated, expensive, or time-consuming,[20–24] for instance, radioactive labeling was often needed to detect the aptamer fragments, and the cost for chemical synthesis of all the shorter sequences of a full-length RNA aptamer would be very high. In a recent development, a massively parallel sequence-function analysis based on microarray for optimizing aptamer binding motif has shown high efficiency and rich information content.[24,25] Additionally, comparison of the predicted secondary structures of aptamer candidates has also been used by several groups for deducing the aptamer binding motifs,[19,20,26] this method would eliminated the complicated and time-consuming experimental investigation if the deduce is successful.

Streptavidin is widely used in bioanalysis and purification, which is based on the strongest non-covalent interaction known in nature between streptavidin and biotin. However, the difficulty to separate biotinylated molecules from streptavidin sometimes limits their downstream applications, so that several strategies for modifications of both streptavidin and biotin have been reported to overcome these problems.[27,28] Since aptamers possess appropriate binding ability and great flexibility in probe/sensor design, streptavidin-binding aptamer (SBA) can be a good alternative of biotin for developing the streptavidin-based technology.[29] In this study, we generated 30 DNA aptamer sequences by SELEX using streptavidin-coated beads, which could be grouped into more than four families after alignment. By comparing all the predicted secondary structures, we identified a unique secondary structure binding motif in all of these sequences, in which only a few nucleotides in the loop and bulge are conservative. Further investigation found that this secondary structure binding motif also exists in streptavidin-binding aptamers generated by three other different laboratories.

2. Results

2.1. Selection and characterization of SBA

SBAs were generated by the affinity chromatographic SELEX protocol[30,31] using streptavidin-coated sepharose beads. After nine rounds of selection, the enriched pool was cloned and 30 clones were sequenced (Table 1). Two sequences were found to have double copies, respectively, thus totally 28 different sequences were obtained. After alignment, 17 of these sequences could be grouped into 3 families and the rest 11 of them showed low primary sequence homology. In previous reports, the binding motifs of DNA aptamers were determined by comparing the similarities of predicted secondary structures between the sequences in the same family,[19,20] which is based on the assumption that sequences in the same family should bind to the same target with same secondary structure. Aptamers in three different families were found to have different binding motif and bind to three different targets.[19] In the current case, when comparing the predicted secondary structures, we found a consensus secondary structure segment, a bulge-hairpin structure, to exist not only in the sequences of same family but also in all the obtained sequences (as shown in the red rectangular area in Fig. 1). However, the primary sequences of the bulge-hairpin structure between different families were very different (Table 1). This bulge-hairpin structure contains a loop (6–7 nucleotides) and a bulge (3–5 nucleotides) that are linked by a stem with three to five base-pairs length, then this structure is maintained by another stem with different length and the rest of the aptamer sequences folded into different structures. The secondary structure segment in sequence St-2 and St-2-1 was chosen for further investigation.

Table 1
Aptamer candidates selected against streptavidin in our laboratory

Name	Center sequence[a]
St-2	TTGAC**CGC**T-GTGT**G**A**CGC**AACACTCAATTTCTTCCAGCCGGTCCG-
St-20	-TTGAC**CGC**T-GTGT**G**A**CGC**AACACTCAATTTCTTCCAGCCGGTCCA-
St-26	GGGCATA**CGC**–GTGT**G**G**CGC**AACACTATGCCTATTTTAGCCGCTG-
St-10	-AGAAC**CGC**T-GTGT**G**A**CGC**AACACTTCTAATCATGTATCTGTCTCC-
St-22	-**CGC**T-G-GT**G**A**CGC**AACCATAACCTCCGTGCTGCTCAACAGTCAAA-
St-6	-**CGC**-ATCC**G**A**CGC**AGGATTATCCTTCGCAAGTGCTGAGTCCC-
St-12	-**CGC**-ATCC**G**A**CGC**AGGATTATACTCCGCAAGTGCTGAGTCCC-
St-1	-**CGC**-ACAT**G**C**CGC**AATGTTATTCTTCGTAGCTGTTCCGTCTTCGCC-
St-25	-A**CGC**-ACC**G**GT**CGC**AGGTTTATTCTCCGTGTTCGATGTAGGACCGC-
St-7	-**CGC**-ACCT**G**T**CGC**AAGGTTAGCCTTAAGCTGCTTCAATCCTTCATC-
St-19	-**CGC**-ACCT**G**T**CGC**AAGGTTACCCTTAAGGTGCTTCAATCCCTCATC-
St-24	GGA**TGC**-ACCT**G**G**CGC**AAGGTTCCGTGTAGGCTCC-GGCTGTTGTCC
St-11	-AT**CGC**TCACC**G**A-**CGC**AGGTGTTATC-TTCGTAACGCTGCATGCTTG
St-23	-AT**CGC**TCACC**G**A-**CGC**AGGTGTTATC-TTCGTAACGCTGCGTGCTTG-
St-16	-AT**CGC**TCACC**G**G-**CGC**AGGTGTTATC-TTCGTAACGCTGCGTGCTTG-
St-21	-AT**CGC**TCACC**G**G-**CGC**AGGTGTTATC-TTCGTAACGCTGCATGCTTG-
St-18	-AT**CGC**TCACC**G**A-**CGC**AGGTGTTATC-TTCGTAATGCTGCATGCTTG-
St-15	-TA**CGC**ACC**G**GT**CGC**AGGTTATATTAATGCGTACTGCT-CATTCTCC-
St-8	-**CGC**-GGTC**G**G**CGC**GGATTATCTGTACGATGCGTGTGATCCC-
St-9	T**CGC**CACATC**G**A**CGC**AGATGTT-ATCATTCACTTCTTGCTATCCTG- ×2
St-13	-CAGT**TGC**C-CTCC**G**A**CGC**AGGAGATGGATCTCCCATGTCTGGCTCA-
St-30	GGGAGAGC**CGC**T-AATC**G**A**CGC**AGATTCTCTCCCGTTCGCCGGC-
St-3	TGCCGTTGGAGTGTGAA**CGC**TCGTAT**TGC**TCGATTCATCAATTCA- ×2
St-27	GGCCCGCAGATGAAGCA**CGC**ACC**G**AT**CGC**AGGTTGCATCATCGGC-
St-28	-GCCCCGTTGTGAT**TGC**T-CCCC**G**A**CGC**AGGGGTC-ACTCCGGGGGTA-
St-5	TTGGACTAACACAACTTA**CGC**-ATGT**G**A**CGC**AACATTAAGATTGGG-
St-4	-C-ACTTAGTGTATGGCGA**TGC**CCCT**G**T**CGC**AAGGGTCGCAGTTCAC
St-14	-GTAGC-GTGGAGTACCAGCGGATACGCCCTA**CGC**ACC**G**GT**CGC**AGG-

[a] 5′-*ATACCAGCTTATTCAATT*-(center sequence)-*AGATAGTAAGTGCAATCT*-3′, the full-length sequences include two primer hybridization sites and the center random sequence. The underlined and bold section is the conserved nucleotides.

St-2-1 was synthesized and labeled with carboxyfluorescein (FAM) at its 5′ end. The binding assay showed that St-2-1 can strongly bind to streptavidin-coated sepharose beads, this binding could be competed by biotin and streptavidin but not by avidin (Fig. 2A and Fig. S1). The apparent equilibrium dissociation constants (K_d) of St-2-1 was measured to be 40 ± 18 nM by static adsorption method (Fig. 2B).

In order to further confirm the streptavidin binding of the bulge-hairpin structure, St-23-2, a bulge-hairpin structure segment in the clone sequence St-23 (from different family than St-2) was synthesized, meanwhile a slightly different secondary structure segment found in another predicted secondary structure of St-23 (multiple potential structures could be predicted for one sequence), St-23-1, was synthesized as a control sequence (Fig. 3 and Table S1). Additionally, to elucidate the critical nucleotides for the streptavidin binding, nine mutated sequences derived from St-2-1 were also synthesized (Fig. 4 and Table S1). The binding ability of these sequences to streptavidin was evaluated by competing with FAM-labeled St-2-1 for streptavidin binding (Fig. S2, details are in Section 5). The percentage of FAM-labeled St-2-1 being displaced by unlabeled St-2-1 is 84.8 ± 2.7%. As shown in Figure 3, St-23-2 has strong binding

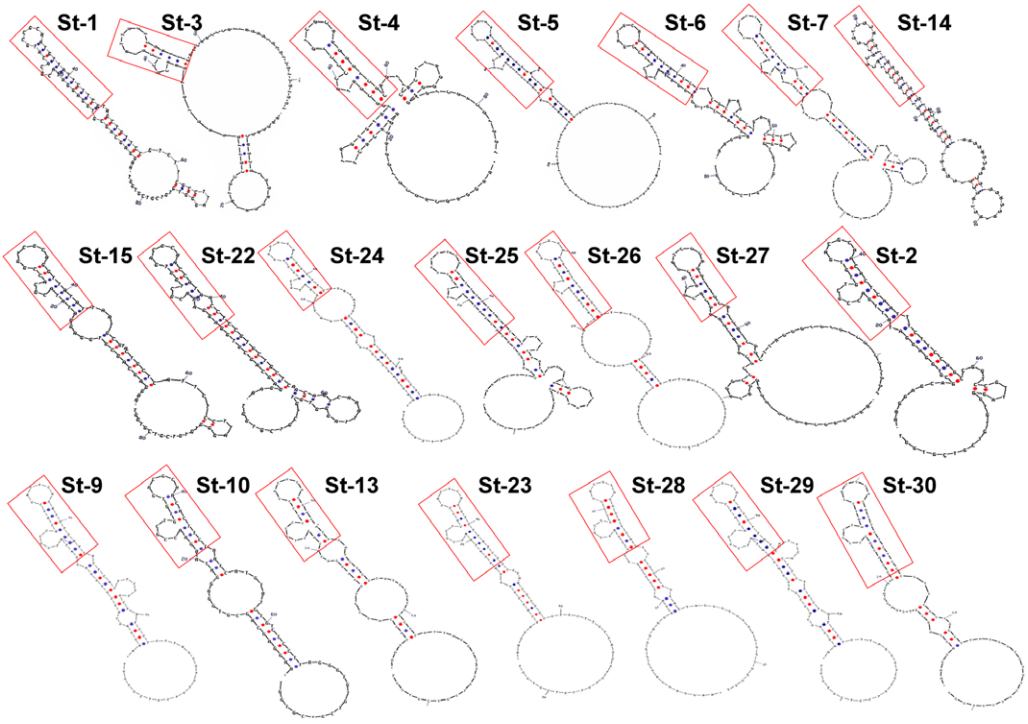

Figure 1. Secondary binding structures of all the aptamer candidates. The similar secondary structure segments are marked in red rectangular area.

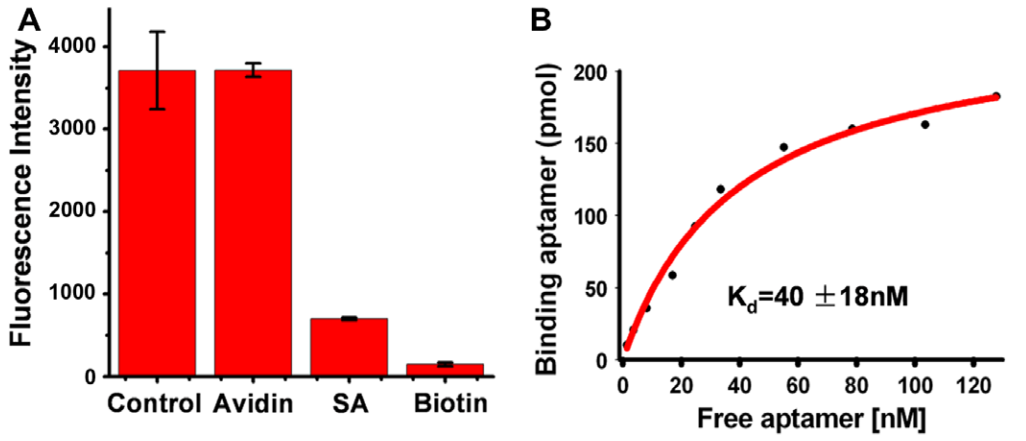

Figure 2. (A) Binding of St-2-1 on streptavidin-coated beads in the absence (Control, buffer) or presence of avidin (5 μM), streptavidin (SA, 5 μM) or biotin (20 μM, streptavidins were saturated). Each data point represents an average ± the standard deviation of three replicates. (B) Binding curves of St-2-1 to streptavidin-coated beads.

ability (percentage of displaced labeled St-2-1 is 70.9 ± 1.0%) to streptavidin, but St-23-1 did not show any competition to St-2-1, which suggests that the bulge-hairpin secondary structure segment is indeed the binding motif of all obtained sequences. Extending the terminal stem of St-2-1 by two base-pairs (St-2-A) did not increase the competition ability much (86.0 ± 3.4%). However, gradually truncating the terminal stem of St-2-1 (St-2-T-1, St-2-T-2, and St-2-T-3) decreased the binding ability gradually, suggesting that the terminal stem section of St-2-1 is essential for maintaining the binding conformation. Removing two nucleotides (St-2-R-1) on the bulge reduced, but not by much, the binding ability (59.6 ± 4.1%) to streptavidin. Nevertheless, removing the whole bulge (St-2-R-2, 13.0 ± 6.8%) or replacing the middle nucleotide G (G8) with T in bulge (St-2-M-1, 21.2 ± 5.4%) greatly decreased the binding ability, suggesting that the bulge and the middle nucleotide G are critical for streptavidin binding. Replacing the nucleotide G18 with T (St-2-M-2, 10.6 ± 7.9%) or G15 with A (St-2-M-3, 12.4 ± 3.0%) in the loop section greatly decreased the binding ability, indicating that this two G's are essential for high affinity binding. By further comparing the primary sequences of the bulge-hairpin structure segment in all the obtained sequences, a secondary structure motif of streptavidin binding was proposed as shown in Figure 5A, in which the two stem sections are highly variable in sequence, the bulge and loop structures are essential, and several nucleotides in the loop and bulge are invariant (underlined and bold section in Table 1). A designed sequence based on the proposed secondary structure with the stem section replaced with all GC or CG base-pairs (St-D-1) was found to have relatively high binding ability (65.6 ± 1.2%), which further suggests that the stems may not bind to streptavidin directly and only maintain the loop and bulge structures for binding.

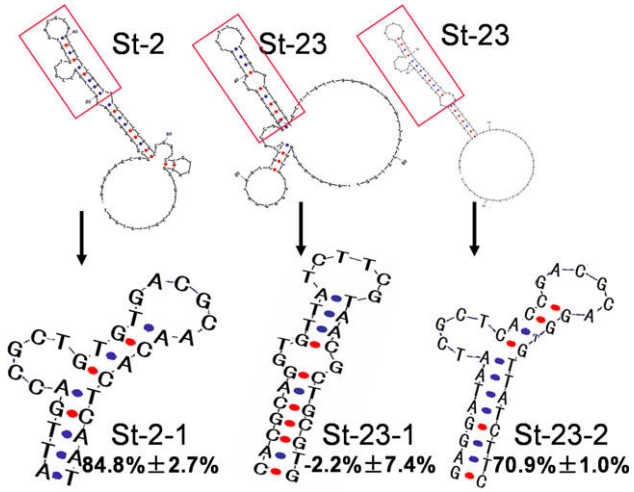

Figure 3. The secondary structures of St-2, St-23 and their truncated sequences. The Percentages denote the percentage of competition of each sequence to FAM-labeled St-2-1. Each data point represents an average ± the standard deviation of three replicates.

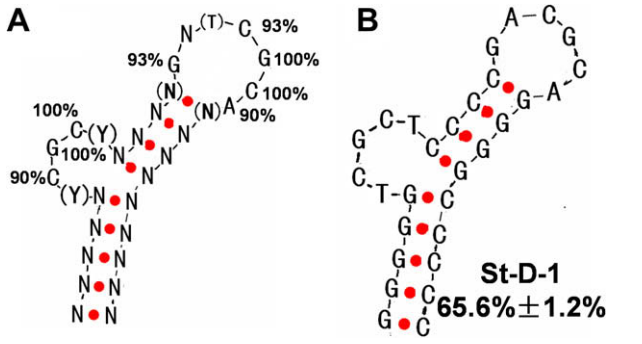

Figure 5. (A) The proposed structure of SBA. The percentage values by the nucleotides represent the frequency of each nucleotide appearing in all sequences evolved in our laboratory. (B) A designed aptamer St-D-1. The Percentages denote the percentage of competition of St-D-1 to FAM-labeled St-2-1. Each data point represents an average ± the standard deviation of three replicates.

2.2. The secondary structure of streptavidin-binding aptamers selected by other laboratories

Three other laboratories have reported DNA aptamer selection against streptavidin with different methods.[32–34] Some high affinity streptavidin-binding aptamers have been generated but less characterized. It is interesting that the bulge-hairpin structure is also found in all of the reported sequences (Table 2).

Strehlitz's laboratory has generated five groups of aptamers with streptavidin-coated magnetic beads by FluMag-SELEX method, with five sequences from each group having K_ds in the range from 59 to 85 nM.[33] As shown in Figure 6A, the bulge-hairpin structure also exists in all these five sequences, and all the synthesized bulge hairpin sequences show certain binding ability to streptavidin. Surprisingly, the critical nucleotides in the loop and bulge of

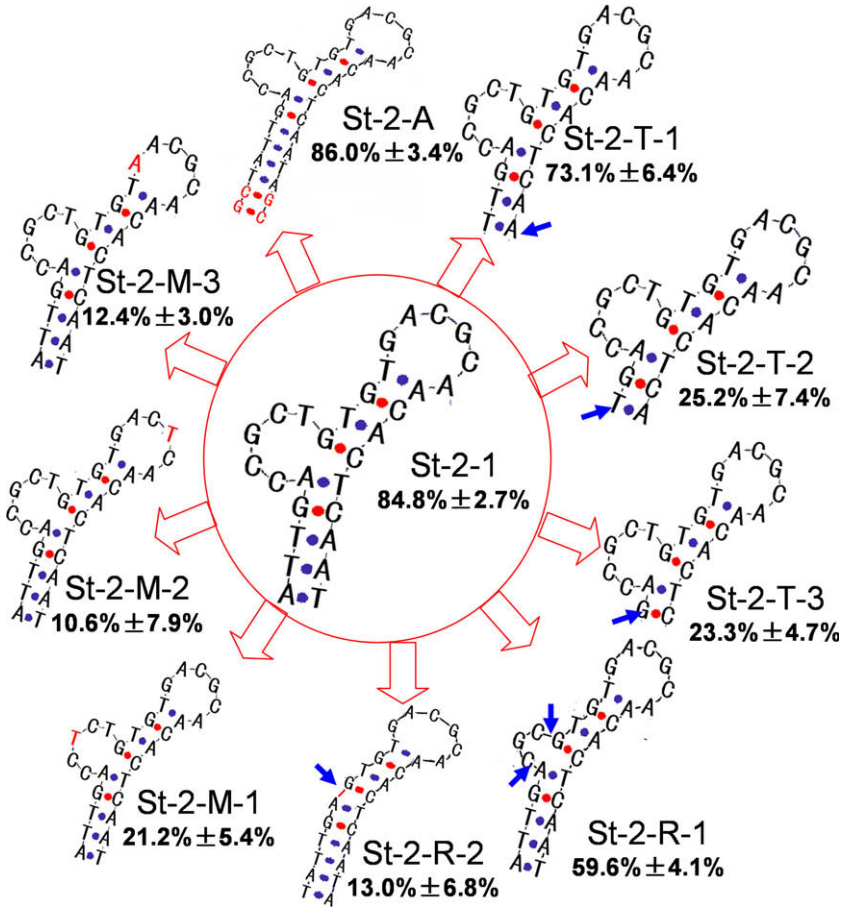

Figure 4. The secondary structures of derivative sequences of St-2-1. The displaced or added nucleotides are shown in red; the place where nucleotides were removed is pointed with arrows. The Percentages denote the percentage of competition of sequences to FAM-labeled St-2-1. Each data point represents an average ± the standard deviation of three replicates.

Table 2
Aptamer sequences selected by other laboratories

Lab	Name	Center sequence[d]
Strehlitz[a]	ABC-2	T**CGC**GTGATT**TGG**TCACAAATTGTATGATCCGGTGTCCTGCAAGTGTTGGTGTTCTCTCA
	ABC-26	GATGCAGCCACCTACATTGCAGGTTTTATAATACAACTA**CGC**GCC**G**TT**CGC**AGGCTAGTT
	ABC-30	CGGGGGGTACG**TGC**CCGT**G**TT**TGC**TCGGCGGC CCCCTCGCTTATTTTTGTGTGCCCTTT
	ABC-31	CTATACTCCACTTTGCTATTTCTCGGTTCCTTCA**CGC**GCC**G**AT**CGC**AGGCTGATGAATTG
	ABC-33	ACAATGT**CGC**TCTCC**G**C**CGC**AGGAGCATTGTCTGTCTTTATGCTTCTCTTTTTTGTTGC
Wang[b]	ABBS-30	-CGA**CGC**ACC**G**AT**CGC**AGGTTCGGGA-
	ABBS-29	-*CTATAGCAAT**GGT**ACGGTACTTCC*AGATA**CGC**TCGTGTTGCTCGATAGC
	ABBS-32,34	-*AGCAAT**GGT**ACGGTACTTCC*TATAA**CGC**CCGTGTTGCTCGGTTAT
Liu[c]	NT-1	-GACTCA**CGC**ATAATT**GGG**TTATTGAGTC-
	NT-2	-GACTCA**TGC**ATAATT**GGG**TTATTGAGTC-
	NT-3	-CTGTGAGACGA**CGC**ACC**G**GT**CGC**A-GGTTTTGTCTCACAG-

[a] 5′-*ATACCAGCTTATTCAATT*-(center sequence)-*AGATAGTAAGTGCAATCT*-3′.
[b] 5′-*TAATACGACTCACTATAGCAATGGTACGGTACTTCC*-(center sequence)-*CAAAAGTGCACGCTACTT TG*-3′
[c] The full-length sequences have about 200–300 nucleotides (see Supplementary data), here only shows the sequence of the secondary structure discussed in the text.
[d] The underlined and bold section is the conserved nucleotides.

our sequence St-2-1 also exist in the high affinity sequences (ABC-31a, 82.8 ± 3.9% and ABC-33a, 62.7 ± 4.7%). Sequence ABC-30a with an additional loop in the terminal stem has median affinity (48.9 ± 3.6%). Further displacing the loop with two AT or GC base-pairs (ABC-30b, 53.4 ± 3.2% and ABC-30c, 78.1 ± 6.6%) improved the binding ability.

Wang's laboratory has reported three aptamers with K_ds in the range from 59 to 104 nM selected with streptavidin-coated magnetic beads.[32] As shown in Figure 6B, the bulge-hairpin structures are also found in these three sequences, however only one synthesized sequence (ABBS-1) that has the same critical nucleotides with St-2-1 shows high affinity (84.0 ± 3.0%), and the other two sequences (ABBS-2, 1.3 ± 3.5% and ABBS-3, 13.8 ± 3.8%) with a much larger loop have much lower binding ability than St-2-1, suggesting that the size of the loop is important for binding. The loop size of high-affinity aptamers is found in the range of 5–7 nucleotides based on above sequences.

Liu's laboratory has generated SBAs with K_ds ranging from 3 to 166 nM using streptavidin–agarose by error-prone PCR and nonhomologous random recombination techniques. The reported sequences have 200–300 nucleotide lengths and share two binding motifs.[34] As shown in Figure 6C, by comparing the secondary structures of these six reported sequences, the bulge-hairpin secondary structure is found in three of these long sequences and a two-loop hairpin structure is found in the other two long sequences. The synthesized bulge hairpin binding motifs (NT-1 and NT-2) showed binding ability to streptavidin, in which NT-1 (46.8 ± 2.3%) with a CGC bulge had much higher binding ability than NT-2 (17.5 ± 0.8%) with a TGC bulge. Considering the nucleotide G (G15) at the 5′ end of the loop of St-2-1 has been demonstrated to be important for binding (St-2-M-3 in Fig. 4), the lower binding ability of NT-1 than St-2-1 may due to the absence of G base at the 5′ end of the loop. The synthesized two-loop hairpin structure (NT-3a) showed relatively weak binding ability (22.8 ± 3.2%). Further changing the second loop to bulge (NT-3b) by removing one nucleotide considerably enhanced the binding ability (46.8 ± 1.5%), suggesting that the bulge structure is important for high affinity binding.

All the above results show that the SBAs generated by three other groups also possess a similar secondary binding motif to that of the SBAs generated in our laboratory, even though the primary sequences are low homologous except for several conservative nucleotides in the loop and bulge section. The same conservative secondary structure motif of all the SBAs and the fact that their binding can be competed by biotin suggest that all the aptamers bind to the same site on streptavidin.

3. Discussion

By comparing all the predicted secondary structures of candidate aptamer sequences, the binding motif of SBAs has been easily determined without complicated and time-consuming experimental investigation. Although the actual SBA sequences from different laboratories, or even generated in the same laboratory are mostly different, they seem to share the same secondary structure binding motif. In many previous reports on aptamer selection, consensus sequences were usually found through sequence alignment and analysis, which could be the actual aptamer binding motif,[35,36] a part of the aptamer binding motif,[24,37] or even sequences unrelated to binding.[33] In comparison, in the current study, no consensus primary sequence but consensus secondary structure motif has been identified in all the candidate sequences, implying that determination of the aptamer binding motif based on their primary sequence may not always be efficient, and determination of binding motif based on the secondary structure could be an alternative method. RNA secondary structure prediction has been reported to evaluate RNA structure–function relationships, such as in understanding and manipulating the ability of small RNAs to regulate gene expression, improving microRNA identification and siRNA design strategies, identification of siRNA target sites, facilitating the interpretation of mutations, and determining evolutionary relationships between catalytic RNA molecules that are so distantly related they are scarcely alignable.[38,39] There are an increasing number of noteworthy computational methods for predicting RNA and DNA secondary structures from sequences, many of which are available on internet.[38,40] Because structure determination of nucleic acids is often experimentally difficult despite tremendous advances in crystallography, nuclear magnetic resonance spectroscopy, and chemical modification, computational methods can be an important and simple tool for generating hypotheses about aptamer structure and binding ability.

Further comparing the binding ability of all synthesized sequences in this paper, it can be found that besides the bulge-hairpin structure, only several nucleotides in the loop and bulge region are critical for high affinity binding, while other nucleotides in this structure are variable (Fig. 5A). Many aptamer-based sensors or devices have been developed by introducing an additional DNA operator that hybridizes with partial or whole aptamer sequence but allows dehybridization when aptamer binds to target molecule.[41,42] However, the introduction of the DNA operator always requires careful optimization to avoid newly introduced hybridization sequence that would hinder the recognition and affinity of the aptamer toward target. In this sense, the highly var-

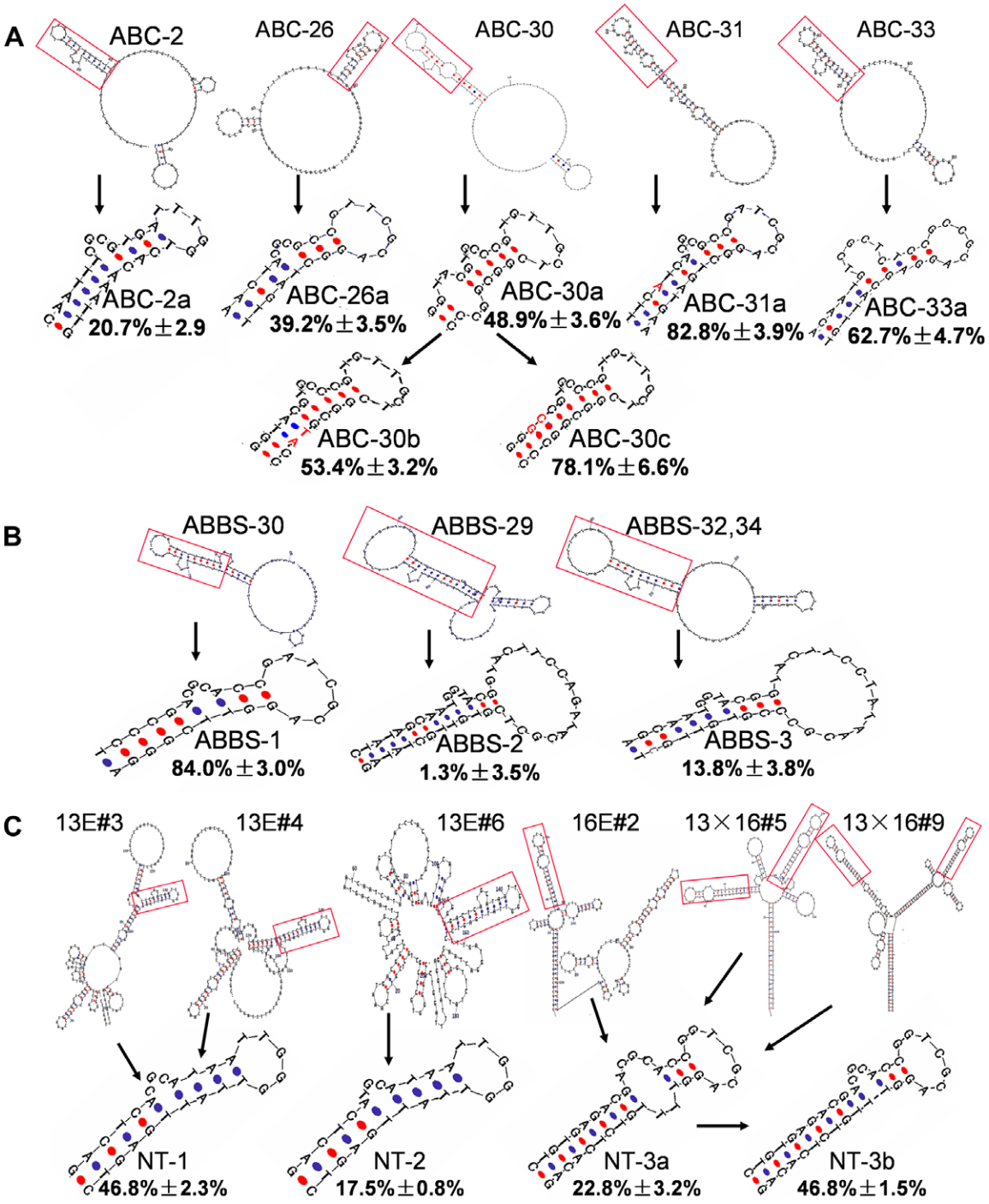

Figure 6. The predicted secondary structure of aptamers generated by (A) Strehlitz's laboratory, (B) Wang's laboratory, (C) Liu's laboratory and their truncated and derived sequences. The Percentages denote the percentage of competition of sequences to FAM-labeled St-2-1. Each data point represents an average ± the standard deviation of three replicates.

iable sequence composition of streptavidin-binding aptamer will make the design of aptameric sensor or device more flexible and easy.

The hypostasis of SELEX is an evolutionary procedure, including enrichment and amplification of the sequences existing in a random DNA or RNA pool (about 10^{14}–10^{15} sequences), as well as mutation occurred as a result of the intrinsic error rate of the polymerase in amplification. It was assumed that aptamers can be raised against any target and any site of a protein target. Two different DNA aptamers that bind to different sites on thrombin have been reported by different laboratories.[43,44] However, based on the above results, all the selected sequences, even generated from different laboratories with different DNA libraries, different binding buffers and different supports for immobilizing streptavidin, have the same secondary structure motif and bind to the same site on streptavidin. This implies that this binding site dominate the evolution of aptamers, which is probably because this site is more accessible than other sites, or have more amino acid residues that can form non-covalent interaction with groups on DNA molecules than other sites. In general, targets that are positively charged under physiological conditions (pH 7.0–7.4) are regarded as excellent targets. Systemin is a peptide composed of 18 amino acids with the

sequence AVQSKPPSKRDPPKMQTD, its isoelectric point (pI, ~9.7) is much higher than that of streptavidin (pI, 5~near-neutral) (http://en.wikipedia.org/wiki/Streptavidin), however when we selected aptamers against biotin labeled systemin using streptavidin-coated beads, we finally got the enriched DNA pool that only bound to streptavidin and could be competed by biotin (data not shown). This further suggests that the binding site on streptavidin is very favorable for aptamer evolution. Despite a large number of papers about aptamer selection published in the past two decades, there are no reliable rules that can be used to predict the success of selection. It is hard to judge whether a target molecule or a selection strategy is suitable for the selection process, some unknown factors may determine the direction of aptamer evolution.

4. Conclusion

DNA streptavidin-binding aptamers are evolved using streptavidin-coated beads. By comparing their predicted secondary structures, they are found to possess a common secondary structure motif for binding, in which only several nucleotides in the bulge and loop region are conservative. Furthermore, this conservative secondary structure motif has been also found in aptamers selected by three other laboratories using different libraries and different supports for streptavidin-coating. The similarity comparison of secondary structure between aptamer candidates is further demonstrated to be an effective and easy way to determine the binding motif of aptamers. All aptamers selected for streptavidin bind to the same site on streptavidin because they have the same binding motif and their binding can be interrupted by biotin. This high similarity of all aptamers selected by different laboratories suggests that some factors can affect the direction of aptamer evolution, which may result in the failure of aptamer selection for specific target or site of interest.

5. Materials and methods

5.1. Materials

All DNA sequences were synthesized by Sunbiotech Co. Ltd (Beijing, China), and unless otherwise indicated, were purified using PAGE. The fluorescein-labeled sequences were purified by HPLC in our laboratory. Streptavidin Sepharose High Performance was purchased from GE Healthcare (GE Healthcare, Sweden), and all the other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). The buffer used for all experiments, unless otherwise indicated, was Tris–HCl buffer of 25 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, and 0.02% Tween20, at pH 7.4. All solutions were prepared with deionized water purified by a Milli-Q system (Millipore, USA). All the fluorescence measurements were recorded by SpectraMax M5 (Molecular Devices Corporation, USA).

5.2. Selection of streptavidin-binding aptamers

A DNA library composed of 45 random nucleotides flanked by 20-nt primer hybridization sites (5′-AAG GAG CAG CGT GGA GGA TA-$(N)_{45}$-TTA GGG TGT GTC GTC GTG GT-3′) was purified by HPLC and used for SELEX. The following primers purified by HPLC were used for amplification of the selected oligonucleotides during the selection process: 5′ primer, 5′-FAM-AAG GAG CAG CGT GGA GGA TA-3′ and 3′ primer, 5′-ACC ACG ACG ACA CAC CCT AA-3′.

Each ssDNA pool dissolved in binding buffer (140 mM NaCl, 2.5 mM KCl, 1.6 mM KH_2PO_4, 15 mM Na_2HPO_4, 0.02% Tween20, pH 7.4–7.6) was heated to 95 °C for 5 min, and cooled on ice for 15 min, then kept at 25 °C for 5 min prior to use. For the initial selection round, 20 μl of washed streptavidin-coated beads were re-suspended in 1000 μl of DNA library (10 nmol). After an incubation of this mixture at 25 °C for 30 min with mild shaking, the complex was transferred to an empty column and washed with 2 × 150 μl of binding buffer. The beads were aspirated and used for PCR directly. The entire selected ssDNA pool was amplified in 10 parallel PCR reactions. Each contained 1 × PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 300 μM dNTPs, 1.2 μM each primer, and 2.5 units *Taq* DNA in 50 μl of reaction. The cycling protocol was 15 cycles of: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min. The PCR products were mixed and used for asymmetric PCR. 10 parallel asymmetric PCR reactions (50 μl) contained 1 × PCR buffer, 300 μM dNTPs, 1.2 μM 5′ primer, 2.5 units *Taq* DNA, and 1.2 μl of above PCR product. After desalted using NAP-5 column (GE Healthcare, UK) and dried, the asymmetric PCR products were used for next round SELEX process. In order to acquire aptamers with high specificity, a counter-selection step was performed from the second round of selection, namely, 30 μl of sepharose high performance beads (GE Healthcare, Sweden) without streptavidin were incubated with the asymmetric PCR products dissolved in 200 μl of binding buffer. After centrifuge, the supernatant was incubated with streptavidin-coated beads. In order to obtain aptamer with high affinity, the volume of the streptavidin-coated beads were scaled down gradually from 20 to 2 μl; the wash strength was enhanced gradually by extending wash time (from 1 to 10 min), increasing the number of washes (from 3 to 5). After nine rounds of selection, the selected ssDNA pool was PCR-amplified by using unlabeled primers and cloned into *Escherichia coli* by using the TA cloning kit and 30 clones were sequenced by Beijing Genomics Institute (BGI) (Beijing, China).

5.3. Characterization of streptavidin-binding aptamer

Predicted secondary structures of all the obtained sequences,[45] were compared carefully to identify structure similarities in all sequences. Then a common secondary structure motif found in this way, named St-2-1 (5′-ATTGACCGCTGTGTGACGCAACACTCAAT-3′), was synthesized and labeled with FAM at its 5′end. 0.5 μM of FAM-labeled St-2-1 was incubated in 100 μl of Tris–HCl buffer in the absence or presence of avidin (5 μM), streptavidin (5 μM) or biotin (20 μM) at 25 °C for 30 min. Then 10 μl of streptavidin-coated beads were added. After 60 min incubation, the samples were transferred into an empty column and washed with 100 μl of Tris–HCl buffer, then the columns were observed under UV-light (Fig. S1). The bound aptamers were eluted with 100 μl of 0.5 M NaOH, and their fluorescence intensities were measured on a 96-well plate (Costar) by SpectraMax M5 with excitation at 488 nm and emission at 530 nm.

The affinity of St-2-1 to streptavidin was measured by incubating increasing amounts of FAM-labeled St-2-1 with 20 μl of streptavidin-coated beads in 6 ml of binding buffer at 25 °C for 15 min, after centrifuge, the concentrations of free St-2-1 were determined by measuring the fluorescence of the supernatants. The amount of bound aptamers ($Aptamer_{bound}$) was calculated as:

$$Aptamer_{binding} = aptamer_{total} - aptamer_{free}.$$

The apparent equilibrium dissociation constants (K_d) of the aptamer to streptavidin were obtained by fitting the amount of bound aptamer on the concentration of the free aptamers to the equation $Y = B\ \max X/(K_d + X)$, using SigmaPlot (Jandel, San Rafael, CA).

5.4. Competition assay of mutated sequences

All mutated sequences were diluted with Tris–HCl buffer, heated to 95 °C for 5 min, cooled on ice for 15 min, then at 25 °C

for 5 min prior to use. For competition assay: 0.4 μM fluorescein-labeled St-2-1 was incubated with 20 μM competitive sequence in 100 μl at 25 °C for 30 min. Then 10 μl of streptavidin-coated beads were added. After 60 min incubation, the sample was transferred into an empty column and washed with 100 μl of Tris–HCl buffer, then eluted with 100 μl of 10 mM biotin. The fluorescence intensities of the eluates were measured on a 96-well plate (Costar) by SpectraMax M5 with excitation at 488 nm and emission at 530 nm. The percentage of competition was calculated on the basis of the binding of the St-2-1 without competitor (Figs. S2 and S3):

$$\text{Percentage of competition} = (FI_0 - FI_c) \times 100\%/FI_0,$$

where FI_0 is the fluorescence intensity of the eluate without competitor, FI_c is the fluorescence intensity of the eluate with competitive sequence.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.054.

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