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A Simple Method for Eliminating Fixed-Region Interference of Aptamer Binding During SELEX

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ABSTRACT: Standard libraries for systematic evolution of ligands by exponential enrichment (SELEX) typically utilize flanking regions that facilitate amplification of aptamers recovered from each selection round. Here, we show that these flanking sequences can bias the selection process, due in part to their ability to interfere with the fold or function of aptamers localized within the random region of the library sequence. We then address this problem by investigating the use of complementary oligonucleotides as a means to block aptamer interference by each flanking region. Isothermal titration calorimetry (ITC) studies are combined with fold predictions to both define the various interference mechanisms and assess the ability of added complementary oligonucleotides to ameliorate them. The proposed blocking strategy is thereby refined and then applied to standard library forms of benchmark aptamers against human α-thrombin, streptavidin, and vascular endothelial growth factor (VEGF). In each case, ITC data show that the new method effectively removes fixed-region mediated interference effects so that the natural binding affinity of the benchmark aptamer is completely restored. We further show that the binding affinities of properly functioning aptamers within a selection library are not affected by the blocking protocol, and that the method can be applied to various common library formats comprised of different flanking region sequences. Finally, we present a rapid and inexpensive qPCR-based method for determining the mean binding affinity of retained aptamer pools and use it to show that introduction of the pre-blocking method into the standard SELEX protocol results in retention of high-affinity aptamers that would otherwise be lost during the first round of selection. Significant enrichment of the available pool of high-affinity aptamers is thereby achieved in the first few rounds of selection. By eliminating single-strand (aptamer-like) structures within or involving the fixed regions, the technique is therefore shown to isolate aptamer sequence and function within the desired random region of the library members, and thereby provide a new selection method that is complementary to other available SELEX protocols.

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Introduction

Nucleic acid aptamers are single stranded DNA or RNA molecules that fold into specific secondary structures and bind molecular targets with high affinity, much like antibodies (Jayasena, 1999). They typically are selected by an iterative combinatorial chemistry technique termed systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak, 1990; Tuerk and Gold, 1990), where large pools (10^{12}–10^{16}) of random nucleic-acid sequences are interrogated against a particular molecular target. During the selection, bound sequences are panned from unbound fractions, amplified, and further screened against the target until a relatively small set of tight-binding aptamers has been successfully enriched (Djordjevic, 2007). The selection of these high-affinity ligands is currently a major area of investigation that is driven by the need for specific tight-binding reagents in diagnostic (Charlton et al., 1997; Fang et al., 2001; Gupta et al., 2011; Li et al., 2002), therapeutic (Bless et al., 1997; Hicke et al., 1996; Nimjee et al., 2005;

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Rusconi et al., 2002; Siddiqui and Keating, 2005) and biosensing (Baker et al., 2006; Liu et al., 2006; Minunni et al., 2004; Zhou et al., 2010) applications. To date, most aptamers have been selected against proteins, but aptamers against other targets including peptides, carbohydrates, small organic molecules or even whole cells have been reported (Daniels et al., 2003; Hesselberth et al., 2003; Jenison et al., 1994; Jeong et al., 2001; Leva et al., 2002; Mannironi et al., 1997; Sassanfar and Szostak, 1993; Wang et al., 2000).

Typically, SELEX libraries are generated using combinatorial chemical synthesis, with all library members having an equivalent length ranging from 80 to 100 nucleotides (nt). Each member contains a random core region of fixed length (40–60 nt) flanked by common fixed regions (~20 nt each) that facilitate enriched aptamer pool amplification at each round of selection. Recently, the impact these fixed regions have on the selection process has been a subject of discussion (Legiewicz et al., 2005; Pan and Clawson, 2009; Pan et al., 2008). Beyond their prescribed function as primer annealing sites, the fixed regions can contribute to aptamer fold, though the impact of this on aptamer discovery is limited due to the invariant sequences of the two fixed regions (Legiewicz et al., 2005). The deleterious effects of the fixed regions on aptamer discovery are therefore thought to be of greater concern. Portions of the fixed regions can either self-hybridize or anneal to complementary sequences in the core region to create unwanted secondary structures that compromise selection of potentially useful aptamers. As shown in this study and by others, fixed regions can further complicate the discovery of aptamers by SELEX through other mechanisms (Chang et al., 2009; Legiewicz and Yarus, 2005; Nonaka et al., 2010; Zhang et al., 2010). Several strategies have therefore been proposed to minimize or even remove unwanted effects of the fixed regions on the aptamer selection process. The most developed and frequently applied include (1) minimizing the length of each fixed region (Pan et al., 2008; Vater et al., 2003), (2) completely removing and regenerating the fixed regions before and after each selection step using restriction digest and ligation reactions, respectively (Jarosch et al., 2006; Lai and DeStefano, 2011), and (3) replacing the fixed regions with different sequences following each or a certain selection step (Shtatland et al., 2000). Though sometimes effective, this collection of strategies has known limitations, including, but not limited to, the potential for a reduction in PCR efficiency when short priming sites are used, and a loss of library material during the cleavage and re-ligation of the new fixed regions between selection rounds. Additionally, replacement of single-strand fixed regions during the selection process does not abrogate the potential for losing a tight-binding aptamer, particularly during early selection rounds, through binding interference effects involving those regions.

In this article, we carefully evaluate a fourth strategy that involves annealing the complementary sequence to each fixed region of an aptamer library during the selection process in order to reduce artifacts that might compromise or eliminate the discovery of a tight-binding ligand within the combinatorial random region of the library. This idea was first proposed by Shtatland et al. (2000) and has also been discussed by Wen and Gray (2004) for use with genomic SELEX libraries. However, neither group conducted an analysis of the concept's utility, due in part to concerns that the double-stranded tails formed might also interfere with aptamer structure, binding or selection. As a result, the value and possible limitations of this flanking-region blocking strategy on aptamer selection are not known. Here, we report results from a set of model studies specifically designed to evaluate the merits of the idea on combinatorially synthesized aptamer libraries, which are known to possess much larger diversities than their genomic counterparts, and to establish an effective protocol for incorporating the concept into the screening process. The goal of this work is therefore not the discovery of a new aptamer, but rather the evaluation of a protocol that might improve aptamer discovery. When applied to a set of benchmark aptamers, the new protocol proves very effective in minimizing unwanted interference effects caused by the fixed regions, allowing for efficient selection of tight-binding ligands that might otherwise be lost from the library during the early critical rounds of selection. The general workflow for the refined method is outlined in Figure 1, along with that for a standard selection step of SELEX for comparison. Because it eliminates any single-strand structures within the fixed regions, the technique isolates aptamer fold and function within the random region of the library members. The protocol therefore complements current standard and modified SELEX approaches by offering a non-enzymatic route to minimizing fixed-regions interference during aptamer selection.

Materials and Methods

Materials

Human α-thrombin, purified from human plasma and resuspended in phosphate buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4), was purchased from Haematologic Technologies (Essex Junction, VT). Streptavidin was purchased from Sigma–Aldrich (Oakville, ON, Canada) and recombinant human VEGF-165 from BioLegend (San Diego, CA). All proteins were either exchanged or reconstituted in 1× aptamer folding (AF) buffer (1× AF buffer is 20 mM Tris–HCl pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$). Because some of the aptamers utilized in this work were originally selected in different buffers, preliminary studies were conducted to ensure that all proteins and aptamers studied here are fully functional in AF buffer. MyOne carboxylic acid and streptavidin C1 Dynabeads from Invitrogen Life Technologies (Burlington, ON, Canada) were supplied as a 10 mg mL^{-1} stock solution. Details for preparing protein-loaded Dynabeads can be found in the Supporting Information.

Library Designs and Synthesis

Each member of the ssDNA aptamer library was comprised of a core 40-mer random region (N_{40}) flanked by a 5′

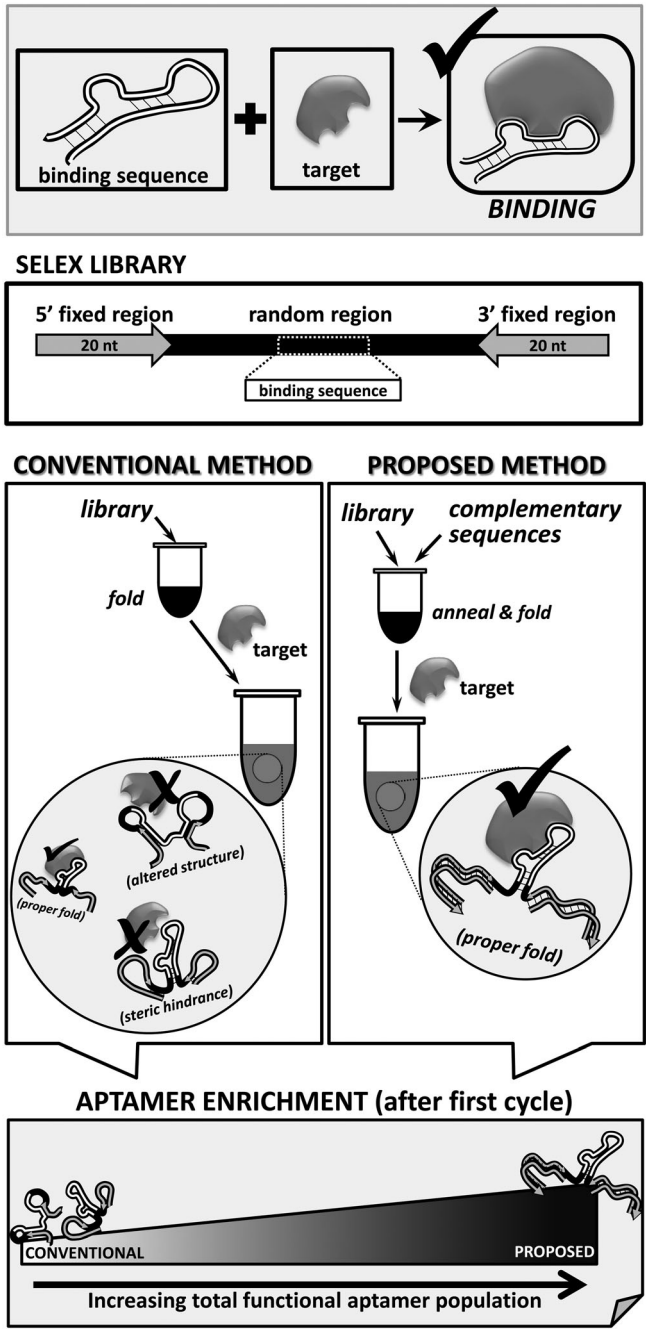

Figure 1. Comparison of standard and proposed protocols for SELEX-based selection of aptamers. **Top box**: Aptamers bind their target with high affinity through the specific fold of their consensus sequence. **Second box**: A typical aptamer library construct used in SELEX contains a random core region flanked by ~20 nt fixed regions to facilitate PCR amplification. Mechanisms by which the flanking regions can interfere with conventional SELEX, but which are ameliorated by the proposed method are then highlighted. In the proposed method, the fixed regions are blocked using complementary sequences. The aptamer fold is therefore confined within the random region and is able to bind its target with high affinity, allowing for efficient SELEX-type enrichment of high-affinity aptamers.

universal 20-mer flanking sequence and a 3′ universal 20-mer primer binding sequence. Two libraries were synthesized, differing in the sequences of the two flanking regions used. In each, the random region was created combinatorially by mixing A:C:G:T at a molar ratio of 3:3:2:2.4 in order to achieve equal probability incorporation of each nucleotide within the core region (Pollard et al., 2001). The two independent sets of 20-mer flanking sequences used in this study are among the most frequently applied to SELEX-based aptamer selection (Hamula et al., 2008; Janssen et al., 2011; Mehta et al., 2012; Mosing et al., 2005; Qian et al., 2009; Tran et al., 2010). Indeed, the selection of each of the current benchmark aptamers against thrombin, streptavidin, and VEGF used engineered 80-mer libraries comprised of one of these two flanking-sequence pairs and associated library templates, hereafter referred to as L1 and L2. Both flanking sequences of each library had their complement synthesized, where the complement to the 3′ flanking sequences (3′-Comp-L1 or 3′-Comp-L2) also served as the reverse primer (REV-L1 or REV-L2) for PCR amplification. All oligonucleotides were synthesized and HPLC purified by Integrated DNA Technologies, Inc. (IDT; Coralville, IA), and reconstituted in 1× AF buffer. Tables I and II summarize the two library formats used in this study, as well as the primers and blockers applied to them. Engineered aptamer sequences used in this study, which include the 29 nt streptavidin aptamer (SBA29), the 15 and 29 nt thrombin aptamers (TBA15 and TBA29, respectively), and the 25 nt vascular endothelial growth factor aptamer (VBA25), are summarized in Table SI of the Supporting Information. For certain selection studies identified in the Results and Discussion section, the appropriate library form of either SBA29 (10^{12} sequences added), TBA15 (10^{12} sequences added), or TBA15 and TBA29 (10^{6} sequences of each added), was introduced into the ssDNA library (1 nmole; 10^{14} sequences) to permit detection of the aptamer in the bound and unbound pools during the first round of selection. Otherwise, the basic unsupplemented library in either blocked or unblocked form was used.

Isothermal Titration Calorimetry (ITC)

All aptamers with the exception of the anti-VEGF aptamers were diluted to a final concentration of 30 μM in 1× AF buffer. VEGF aptamers were diluted to a final concentration of 20 μM. Prior to ITC measurements, working concentrations of aptamers were heated to 95°C and slowly cooled down to 25°C at a rate of 0.5°C min^{-1} in a Mastercycler® ep thermocycler (Eppendorf, Mississauga, ON, Canada) to ensure proper folding. In cases where complementary oligonucleotides were added, this folding protocol also ensured their proper annealing to the flanking regions. ITC measurements were performed at 25°C using a MicroCal iTC_{200} system (GE Healthcare, Piscataway, NJ). The sample cell contained 200 μL of target protein (3 μM; 2 μM for VEGF) also prepared in 1× AF buffer. The injection syringe contained 40 μL of diluted aptamer. A first injection of 0.4 μL

Table I. DNA primers, blockers, and Library-1 sequences used in this study.

Name	Sequence (5′–3′)	Comments
FOR-L1	AGCAGCACAGAGGTCAGATG	5′ amplification primer
5′-Comp-L1	CATCTGACCTCTGTGCTGCT	5′ complementary blocker
REV-L1/3′-Comp-L1	TTCACGGTAGCACGCATAGG	3′ amplification primer/complementary blocker
Library-1 (L1) (80 nt)	AGCAGCACAGAGGTCAGATG–$N_{(40)}$–CCTATGCGTGCTACCGTGAA	$N_{(40)} = 40$ randomized nucleotides

preceded the 15 injections of 2.5 μL. Run parameters were set for an injection rate of 0.5 μL s^{-1} with a 150 s time interval between injections. The syringe rotation speed was set at 1,000 rpm. The results were analyzed using Origin 7.0 software (OriginLab Corp., Northampton, MA) by fitting to a single-site binding model.

SELEX-Based Aptamer Screening

Selection of streptavidin-binding aptamers is described in full here as an example of the general protocol employed. A 2 μL portion of washed MyOne streptavidin C1 Dynabeads ($\sim 10^6$ beads) was incubated with 1 nmole ($\sim 10^{14}$ sequences) of the selected folded aptamer library supplemented with equimolar amounts of its respective complementary oligonucleotides 5′- and 3′-Comp in 500 μL of 1× binding buffer (20 mM Tris–HCl pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.02% Tween-20) for 1 h at room temperature with gentle rotation. Unbound and weakly bound aptamers, including the library form of the 29 nt streptavidin aptamer (SBA29-L1), were removed by conducting a series of 500 μL washes in 1X binding buffer. All remaining bound aptamers and oligonucleotides were eluted from the beads in 1× denaturing buffer (40 mM Tris–HCl pH 8.0, 3.5 M urea, 10 mM EDTA, 0.02% Tween-20) at 85°C. Conventional SELEX cycles (Djordjevic, 2007) were also performed exactly as described above, but without addition of the complementary blocking sequences to the library prior to folding.

Similarly, pre-blocked and standard SELEX-based screens of the 80 nt Library-1 containing the 15 nt thrombin aptamer (TBA15-L1) and/or the 29 nt thrombin aptamer (TBA29-L1) were also performed as described above, but using carboxylic acid functionalized beads displaying immobilized human α-thrombin prepared according to the protocol described in the Supplementary Material.

Recovered members were amplified using Platinum Taq (Invitrogen; Burlington, ON) and 250 nM of forward (FOR-L1 or FOR-L2) and phosphorylated reverse (REV-L1 or REV-L2) primers. Amplification was stopped at the maximum number of cycles before artifact formation was observed on a one-dimensional agarose gel (i.e., the maximum number of cycles where no higher molecular weight amplification products could be observed). Single-stranded products were regenerated using lambda exonuclease (NEB; Whitby, ON) before the next round of selection (Civit et al., 2012). Further details can be found in the Supporting Information.

Real-Time Amplification of Elution Pools

Desalted eluted library fractions were amplified in a CFX Real-Time PCR detection system (Bio-Rad, Mississauga, ON) in the presence of either SYBR Green or a TaqMan™ probe against the target aptamer (e.g., the hydrolysis probe 5′-FAM-CCG CTG TGT GAC GCA ACA CTC AA-Iowa Black FQ -3′ was used for the SBA29-L1 aptamer). For the latter real-time amplification, a 5 μL aliquot of each eluted library fraction was mixed with iQ Supermix (Bio-Rad), 300 nM each of the forward and reverse primers, as well as 200 nM of the respective TaqMan™ hydrolysis probe to form a 25 μL reaction volume. Quantification of total library members eluted in each fraction was achieved by mixing a 5 μL aliquot of an eluted fraction with iQ SYBR Green Supermix (Bio-Rad) and 300 nM of each required primer (Tables I and II). The thermal cycling conditions were as follows: initial denaturation for 3 min at 95°C, then 50 PCR cycles each comprised of denaturation at 95°C for 30 s, and annealing/extension at 59.5°C for 30 s. For the TBA29-L1 primer pair, the annealing/extension temperature was 55°C for 30 s. All results were analyzed using CFX Manager Software (Bio-Rad), which automatically computes and reports a threshold cycle value for each real-time amplification curve.

qPCR-Based Bulk Affinity Determination

A new qPCR-based method was developed to permit rapid and inexpensive determination of the mean binding affinity,

Table II. DNA primers, blockers, and Library-2 sequences used in this study.

Name	Sequence (5′–3′)	Comments
FOR-L2	TCGCACATTCCGCTTCTACC	5′ amplification primer
5′-Comp-L2	GGTAGAAGCGGAATGTGCGA	5′ complementary blocker
REV-L2/3′-Comp-L2	TTCGCACACACGGACTTACG	3′ amplification primer/complementary blocker
Library-2 (L2) (80 nt)	TCGCACATTCCGCTTCTACC–$N_{(40)}$–CGTAAGTCCGTGTGTGCGAA	$N_{(40)} = 40$ randomized nucleotides

recorded in terms of the equilibrium dissociation constant K_D, of the eluted library to the target. Briefly, the protein target (e.g., human α-thrombin) was immobilized on NUNC Immobilizer Amino plates (Thermo Fisher; Edmonton, AB) according to the manufacturer's instructions (Harlow and Pfundheller, 2010) at a concentration of 90 nM in 100 mM sodium phosphate buffer pH 7.4. The eluted aptamer pool was serially diluted into eight different concentrations (twofold dilutions starting at 100 nM) and the set incubated in functionalized wells for 1 h at 25°C with gentle mixing at 300 rpm. Aptamer pools in the equilibrated wells were successively washed three times with 300 μL 1× binding buffer and the retained members then eluted in 50 mM NaOH at 70°C and neutralized with 20 mM Tris buffer pH 7.4 containing 50 mM HCl. The neutralized material was mixed with iQ SYBR Green supermix (Bio-Rad) containing 250 nM of each required primer (Tables I and II). Thermal cycling conditions were as follows: initial denaturation for 3 min at 95°C, then 40 cycles each comprised of denaturation at 95°C for 30 s and annealing/extension at 64°C for 30 s. Bound aptamer fractions were determined through comparison to a standard curve constructed from C_q data for serially diluted Library-2 members. The resulting binding isotherm data were nonlinearly fitted to the Langmuir equation to determine the dissociation constant for the pool of eluted members (Moreau and Schaeffer, 2012).

Results and Discussion

Building on the arguments first proposed by Shtatland et al., we posit that each fixed-region sequence within a given selection library can interfere with aptamer fold and function through its potential to adopt stable secondary structures created through either (1) self-association, or (2) association with complementary nucleotides within the random core region, the opposing fixed region, or both. Each of these types of unwanted structures can occur either within an individual aptamer or between complementary regions of different members of the library. Below, we provide examples of these possible interference effects and then show how pre-blocking of the fixed regions can serve to eliminate their occurrence.

Secondary Structures Formed Within Fixed Regions Can Interfere With Aptamer Binding Through Steric Hindrance Effects

Arguably the most extensively studied of all known aptamers, the 15 nt thrombin binding aptamer (TBA15) tightly and specifically binds the fibrinogen exosite of the serine protease human α-thrombin (Bock et al., 1992). Figure 2A reports ITC data (Table SII; Supporting Information) that show that TBA15 binds α-thrombin with nanomolar affinity at physiological conditions through an enthalpically driven reaction that is compensated by a loss in entropy. Insertion of the TBA15 consensus sequence into the 40 nt core region of the 80 nt SELEX Library-1 template results in a sequence (TBA15-L1) that is predicted by Mfold (Zuker, 2003) to form no base pairs that *directly* interfere with TBA15's known G-quadruplex structure required for thrombin binding (Fig. 2B). However, Mfold also predicts a large stem loop formed within the 5′ flanking region adjacent to the aptamer binding sequence, as well as a second moderate-sized stem loop within the 3′ flanking region. ITC data for titrating this 80-mer library version of the thrombin aptamer (TBA15-L1) into a solution of human α-thrombin show evidence of, at most, very weak binding (Fig. 2B, bottom), indicating that the presence of the flanking regions strongly inhibits binding, presumably through steric hindrance imposed by the two stem loop structures. Thus, although Mfold predictions are two-dimensional and therefore rather crude, we find they correlate with ITC data by identifying changes in library-aptamer fold or other structural aspects that may influence aptamer function.

Fixed Regions Can Eliminate Aptamer Fold and Function Through Hybridization With Complementary Sequences Within the Core Region

Known aptamers having nM affinity to streptavidin share a 29 nt consensus sequence (SBA29) that folds into a bulge-hairpin motif (Fig. 3A) (Bing et al., 2010). ITC data for titrating SBA29 into a streptavidin solution (Fig. 3A, Table SII; Supporting Information) show that the strong affinity is derived from a very favorable binding enthalpy that is only modestly compensated by entropy. Presentation of SBA29 within the random region of the standard 80 nt SELEX Library-1 template results in a ssDNA sequence (SBA29-L1) that is predicted by Mfold to no longer display the bulge-hairpin motif of SBA29 due to the formation of more stable secondary structures comprised of regions of the 29 nt aptamer and complementary sequences within both the 5′ and 3′ fixed regions of the library (Fig. 3B). As a result, ITC data for titrating the 80-mer Library-1 version of the streptavidin aptamer (SBA29-L1) into a solution of streptavidin show no evidence of a binding interaction (Fig. 3B). Thus, we find that the SELEX protocol, in its standard configuration, can preclude the enrichment and identification of desired tight-binding aptamers through a number of mechanisms involving the single-stranded flanking regions.

Pre-Blocking of Fixed Regions With Complementary Sequences Restores Binding by Eliminating Interferences Due to Steric Hindrance or Loss of Required Aptamer Structure

To test this hypothesis, we first investigated the effect of annealing 5′-Comp-L1, 3′-Comp-L1 or both oligonucleotides to the library form of the 15-mer thrombin aptamer (TBA15-L1), with each Comp-L1 sequence added to a concentration equimolar to that of TBA15-L1. ITC data (Fig. 2C–E) show that the hybridization of both blocking oligonucleotides to TBA15-L1 (Fig. 2E) restores binding affinity to that of the original consensus aptamer (TBA15) ($K_D = 20.00 \pm 2.74$ nM; it is in fact seen to be slightly

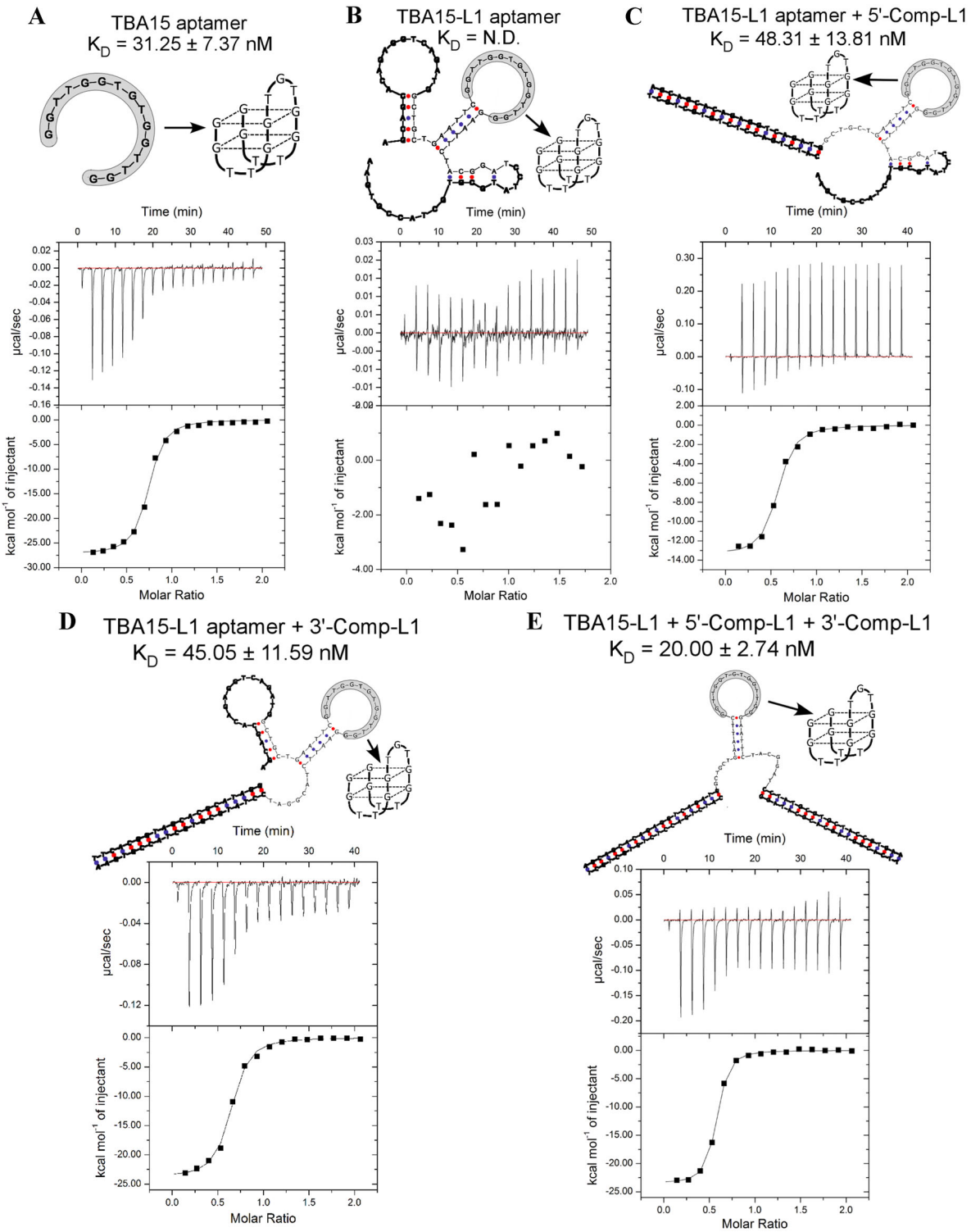

Figure 2. Unwanted hybridization of sequences within the fixed regions can impede aptamer binding through steric hindrance effects. **A**: The 15 nt thrombin-binding consensus sequence (TBA15) folds into a G-quadruplex structure (top panel) that binds thrombin with nanomolar affinity as measured by isothermal titration calorimetry (bottom). Both the raw titration data and the differential heats fitted to a single-site binding model are shown in the bottom panel. **B**: The 80 nt thrombin aptamer library construct (TBA15-L1) containing the TBA15 consensus sequence (highlighted) is predicted by Mfold to adopt a structure that includes two stem-loops adjacent to the known G-quadruplex binding site. Nucleotides in the fixed region are denoted in bold face type. ITC data show that TBA15-L1 binds human α-thrombin very weakly at most (bottom). Addition and hybridization of either 5′-Comp-L1 (**C**) or 3′-Comp-L1 (**D**) results in partial restoration of binding affinity. **E**: Hybridization of both complementary sequences results in complete restoration of binding affinity. ITC experiments were performed in duplicate, from which average K_D values and standard errors were computed and reported. All base-pairing and secondary structure predictions were determined using the program Mfold (Zuker, 2003).

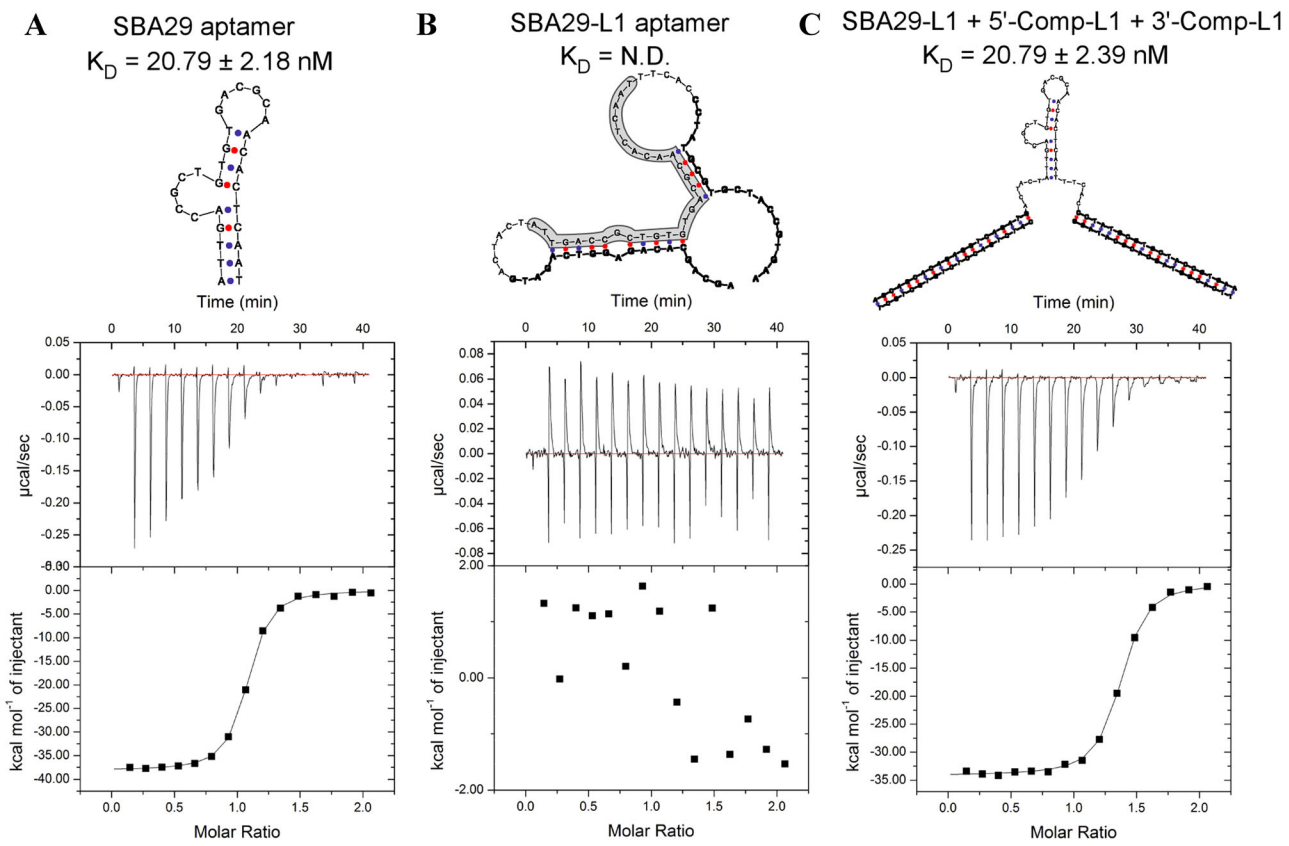

Figure 3. Unwanted hybridization between complementary regions of the flanking sequences and the core sequence can eliminate aptamer fold and function. **A**: The 29-mer streptavidin aptamer consensus sequence (SBA29) adopts a characteristic bulge-hairpin structure (top panel) that ITC data (bottom panel) show binds streptavidin with nanomolar affinity at physiological conditions. **B**: The 80 nt aptamer library construct (SBA29-L1) containing the 29-mer consensus sequence (highlighted) is predicted by Mfold to lack the bulge-hairpin motif required for streptavidin binding due to hybridization of nucleotides within the fixed regions (bold face) to nucleotides within the SBA29 consensus sequence itself (top). ITC data (bottom) show that SBA29-L1 does not bind to streptavidin. **C**: Hybridization of both complementary sequences to the fixed regions of SBA29-L1 is predicted by Mfold to restore SBA29s characteristic bulge-hairpin secondary structure (top), and ITC data (bottom) show that binding affinity is restored and identical to that for SBA29. ITC experiments were performed in duplicate, from which average K_D values and standard errors were computed and reported.

improved, albeit to a nearly insignificant degree when experimental errors are taken into account), while adding only one of the oligonucleotides (Fig. 2C and D) partially restores affinity ($K_D = 48.31 \pm 13.81$ nM for 5′-Comp-L1 or $K_D = 45.05 \pm 11.59$ nM for 3′-Comp-L1). These results therefore suggest that interferences from the flanking regions can be eliminated through pre-blocking with the respective complementary oligonucleotides.

Similarly, when 5′-Comp-L1 and 3′-Comp-L1 are added in equimolar amounts to the library 1 version of the streptavidin aptamer (SBA29-L1), Mfold predicts full recovery of SBA29's bulge-hairpin motif required for binding, which is corroborated with ITC data showing a full restoration of binding affinity ($K_D = 20.79 \pm 2.39$ nM) to streptavidin (Fig. 3C). Thus, we find that pre-blocking can serve to eliminate the most obvious and worrisome mechanisms by which the single-stranded fixed regions of a library interfere with or eliminate binding, and therefore selection, of desired tight-binding aptamers.

Pre-Blocking of Fixed Regions Does Not Interfere With Otherwise Properly Functioning Library Members

To investigate the general utility of pre-blocking in rendering a combinatorial aptamer library suitable for SELEX-based screening, we extended our studies to include two additional aptamers: VBA25 and TBA29. VBA25, a 25 nt DNA aptamer against the vascular endothelial growth factor (VEGF) (Gold and Janjic, 1998), was the first aptamer approved for therapeutic use by the FDA. It therefore serves as a benchmark for therapeutic aptamers (Kanakaraj et al., 2013). The 29 nt aptamer TBA29 binds to the heparin-binding site of human α-thrombin, as opposed to the fibrinogen exosite targeted by TBA15 (Tasset et al., 1997). ITC data (Table SII; Supporting Information) show that each of these consensus aptamers binds its target with nM affinity at physiological conditions ($K_D = 3.94 \pm 0.78$ nM for TBA29; $K_D = 36.76 \pm 4.91$ nM for VBA25). Insertion of either TBA29 or VBA25 into the 40 nt random

region of the 80 nt SELEX Library-1 template results in a sequence (TBA29-L1 or VBA25-L1, respectively) where the flanking regions are predicted by Mfold to not alter directly the known structures of the two aptamers. ITC data for titration of TBA29-L1 or VBA25-L1 into a solution of human α-thrombin or $VEGF_{165}$ (Table SII; Supporting Information), respectively, reveal a binding affinity that is equivalent to the isolated consensus sequence ($K_D = 5.49 \pm 1.46$ nM for TBA29-L1; $K_D = 34.48 \pm 3.17$ nM for VBA25-L1), indicating that, in these cases, the stem-loop structures formed within the flanking regions do not inhibit aptamer function. Here, the addition of both complementary oligonucleotides yields no significant change in binding affinity ($K_D = 5.71 \pm 2.31$ nM for TBA29-L1; $K_D = 35.46 \pm 6.96$ nM for VBA25-L1), demonstrating that the proposed protocol eliminates pathways for significant loss of promising aptamers without adversely affecting the properties and selection of otherwise properly functioning library members.

Pre-Blocking of Fixed Regions Is Equally Effective on Other Library Formats

The various SELEX library designs successfully used to date differ both in library member length and, more importantly, in the sequences of the fixed regions employed. In addition, fixed-region sequences have been replaced in certain studies after each or a selected panning round to reduce persistent interference to aptamer fold (Shtatland et al., 2000). To be of general utility, our pre-blocking of fixed regions strategy must therefore be effective on different libraries and library formats. For the 29 nt thrombin aptamer TBA-29, Figure 4A and B (see also Table SII; Supporting Information) show that when the flanking regions of TBA29-L1 are replaced with

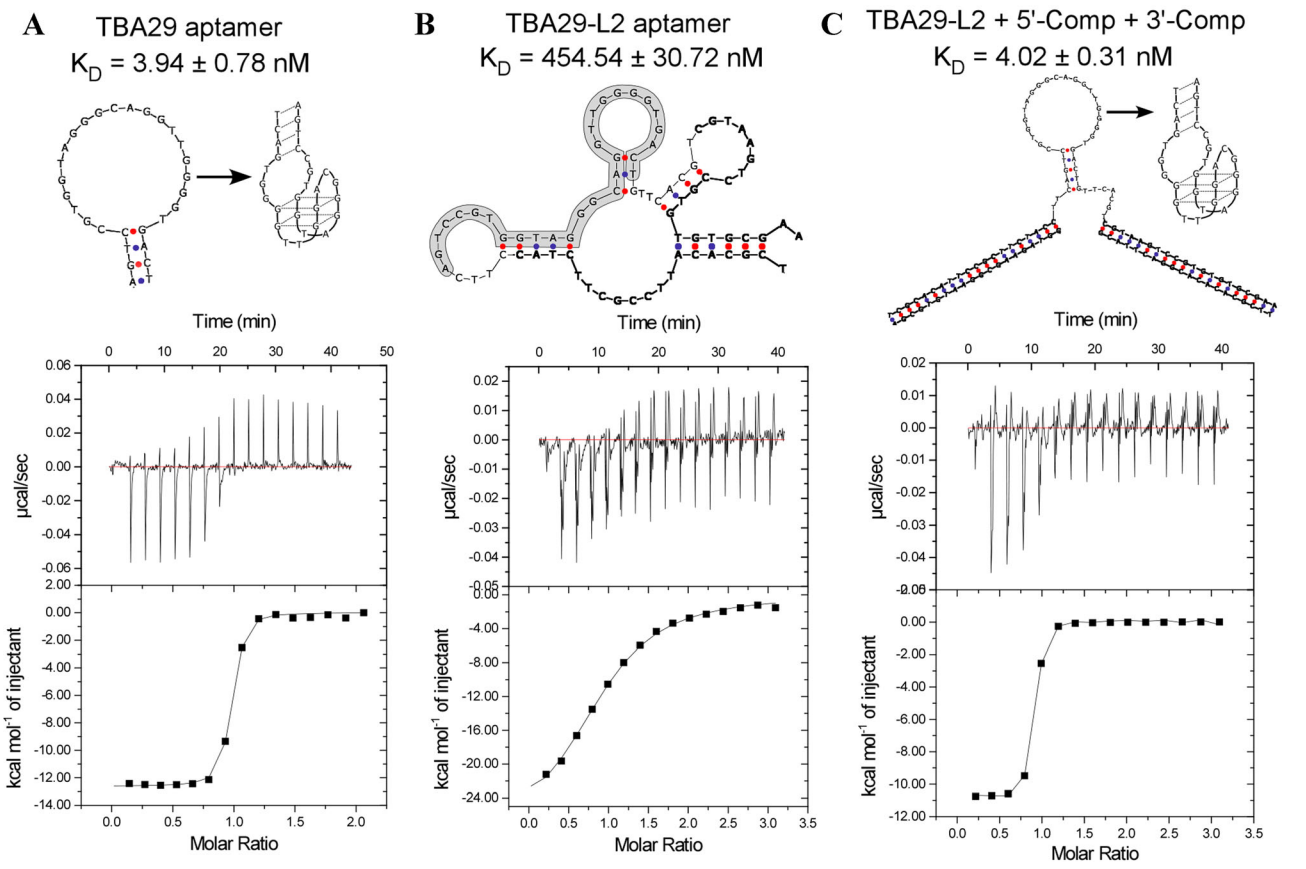

Figure 4. Application of methodology described in Figure 3 to the 29-mer thrombin aptamer consensus sequence (TBA29). **A**: TBA29 folds into a G-quadruplex structure (top panel). ITC data (bottom panel) showing TBA29 binds human α-thrombin with nanomolar affinity at physiological conditions. **B**: When the flanking regions are replaced with those from Library-2, the resulting 80 nt aptamer library construct (TBA29-L2) containing the 29-mer consensus sequence (highlighted) is predicted by Mfold to contain base-pairing that interferes with the known G-quadruplex structure required for binding. This result is significantly different from the Library-1 version of the same aptamer (TBA29-L1), which retains the G-quadruplex structure. ITC data (bottom) show that TBA29-L2 binds thrombin with greatly weakened affinity compared to its Library-1 counterpart. **C**: Hybridization of both complementary sequences to the fixed regions of TBA29-L2 is predicted by Mfold to eliminate all base pairs that serve to interfere with the G-quadruplex secondary structure (top), and ITC data (bottom) show full restoration of binding affinity to TBA29 or TBA29-L1 (Table SII; Supporting Information). ITC experiments were performed in duplicate, from which average K_D values and standard errors were computed and reported.

those of Library-2, the resulting sequence (TBA29-L2) is predicted by Mfold to form base pairs between random and fixed sequences that serve to inhibit formation of the G-quadruplex structure required for binding to thrombin. ITC data (Fig. 4B) for titrating this 80-mer Library-2 version of the thrombin aptamer into a solution of human α-thrombin shows evidence of greatly weakened binding affinity ($K_D = 454.54 \pm 30.72$ nM) at physiological condition, compared to that of TBA29 alone ($K_D = 3.94 \pm 0.78$ nM).

As with Library 1, when 5′-Comp-L2 and 3′-Comp-L2 are added in equimolar amounts to the Library-2 version of this thrombin aptamer (TBA29-L2) and allowed to anneal to their complementary fixed regions, Mfold predicts recovery of TBA29's G-quadruplex structure required for binding. More importantly, ITC data show full restoration of binding affinity ($K_D = 4.02 \pm 0.31$ nM) to thrombin (Fig. 4C). The results in Figures 2–4 therefore show that unblocked flanking regions will impact the structures and affinities of a fraction of library members presenting a functional aptamer sequence within the random region, and that pre-blocking of the flanking regions can provide a general strategy to eliminate those deleterious effects on aptamer fold and function, regardless of the chosen target, library format or fixed region sequences employed.

Pre-Blocking of Fixed Regions With Complementary Sequences Provides a Novel Avenue for Successful Enrichment of High-Affinity Aptamers During the Crucial First Round of SELEX

In SELEX, the potential for inadvertent loss of desired tight-binding aptamers is highest in the first round of selection since there are, at most, only a few (<10) representations of each sequence in the starting pool. Establishing methods that provide more efficient partitioning, retention and subsequent amplification of strong-binding aptamers in the first round of SELEX are therefore likely to enhance the quality of the selection process.

Currently there are several methods by which panning can be performed (see, e.g., Cox et al., 2002; Latulippe et al., 2013; Xiao et al., 2012), each having specific advantages and disadvantages. Among the most often used are those based on magnetic particles (Kikuchi et al., 2003; Qian et al., 2009; Stoltenburg et al., 2005; Wang et al., 2009), which offer a facile and reproducible means of controlling stringency during a selection step. They also allow for simple handling and a modest level of parallelization (Stoltenburg et al., 2007). To evaluate the impact of pre-blocking on aptamer retention and amplification, we therefore performed an initial round of SELEX on aptamer library 1 (L1), supplemented with either SBA29-L1 or TBA15-L1, using as the target either streptavidin or α-thrombin attached to super-paramagnetic beads. Parallel selections were performed either in a conventional manner (where the complementary sequences were omitted) (Fig. 1; left panel) or using the proposed method with both complementary sequences (5′-Comp-L1 and 3′-Comp-L1) added to and equilibrated with the supplemented L1 library ($\sim 10^{14}$ members) prior to the selection (Fig. 1; right panel). For both selections, the molar ratio of target to library members was set at 1:100. Real-time amplification results for the detection of SBA29-L1 within the wash and elution pools for the first round of library L1 panning against immobilized streptavidin are presented in Figure 5A. Relative quantities were calculated using ΔC_q values after correcting for fluorescence drift, where ΔC_q is defined as the difference in threshold cycle value between the test sample and the negative control sample (where no streptavidin-coated beads were added). The results are expressed as the fold difference

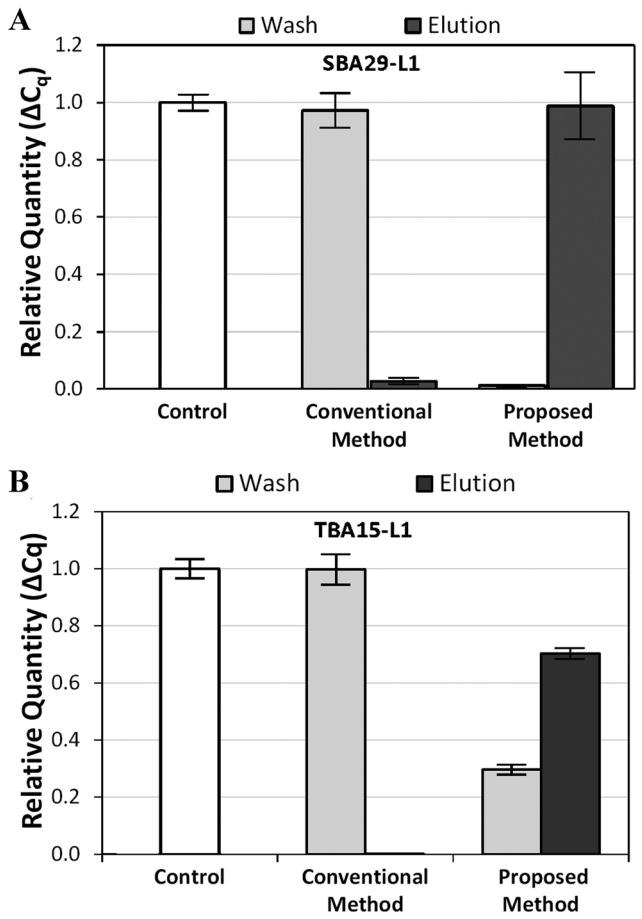

Figure 5. Pre-blocking the fixed regions of a library permits successful enrichment of high affinity aptamers during the early rounds of SELEX. **A**: Conventional SELEX screening was performed using an aptamer library containing SBA29-L1 to permit detection and tracking of this representative high-affinity aptamer during the first round of SELEX. Magnetic beads coated with the target protein (streptavidin) were equilibrated with the library and then washed with binding buffer. Elutions were carried out in a denaturing buffer at 85°C containing 3.5 M urea. A parallel SELEX screening using the blocking method described here was also performed. Real-time amplification with a TaqMan probe generated against the target aptamer was used to characterize aptamer loss and the level of enrichment. Real-time amplification using SYBR green as readout was used to determine the total quantity of library members retained and eluted using each method. The relative quantity of SBA29-L1 in each fraction (wash at 140 mM NaCl and pooled elutions) was determined using the difference in threshold cycle value relative to the control (where no streptavidin was added). **B**: Results for corresponding screenings of the L1 aptamer library containing TBA15-L1 against immobilized α-thrombin.

relative to the control sample, assuming 100% amplification efficiency. For the conventional screening (L1 aptamer library without addition of complementary sequences), the wash fraction—identical to the binding/wash conditions most often used in SELEX—removes essentially all (97.2 [±6.0]% at 95% confidence) of the SBA29-L1 from the pool of retained library members, consistent with the loss in binding affinity when the flanking regions are left unblocked (Fig. 3B). Consequently, a vanishingly small amount of SBA29-L1 is detected in the final elution fraction (2.8 [±1.2]% at 95% confidence), indicating that this benchmark high-affinity aptamer could be falsely screened out of the binding pool of L1 library members in the initial round of SELEX.

When both 5′-Comp-L1 and 3′-Comp-L1 are added to the L1 aptamer library prior to first-round SELEX-based screening (Fig. 1; right panel), real-time amplification results indicate that little (1.1 [±0.15]% at 95% confidence) of the now properly folded high-affinity SBA29-L1 is removed in the wash fraction of the L1 library (Fig. 5A). Virtually all of the SBA29-L1 binds to the target and is therefore recovered in the elution fraction (98.9 [±11.7]% at 95% confidence), resulting in significant enrichment of SBA29-L1 within the retained and eluted L1 library members. The results therefore support our ITC data that show recovery of aptamer binding when 5′-Comp-L1 and 3′-Comp-L1 are used to eliminate fixed-region interference effects (Fig. 3C).

First-round SELEX screening of aptamer library L1 against thrombin-coated magnetic beads yielded similar results, with the corresponding qPCR data sets only showing significant enrichment of TBA15-L1 in the bound fraction of the L1 aptamer library when the fixed-region blocking strategy is applied (Fig. 5B).

As recently outlined (Wilson et al., 2013), nearly all high-affinity aptamers discovered to date are selective toward a single epitope on the corresponding target, with the most documented exception being the pair of DNA aptamers selective towards the fibrinogen (TBA15) and heparin (TBA29) exosites of human α-thrombin, respectively. This exception provided us with a particularly useful system to investigate if our proposed protocol generally results in retention and enrichment of tight-binding aptamers irrespective of aptamer sequence or binding exosite. We therefore performed an initial round of SELEX on aptamer library L1 supplemented with the Library-1 forms of the two thrombin aptamers TBA15-L1 and TBA29-L1. Parallel selections were performed (Fig. 1, left and right panels) as previously described. Real-time PCR results for the partitioning of the two thrombin aptamers (TBA15-L1 and TBA29-L1) within the bound and unbound pools of the L1 aptamer library are presented in Figure 6A for conventional SELEX and Figure 6B for the pre-blocked form of the library.

When conventional SELEX screening was applied (Fig. 6A), 3× washing of the equilibrated beads removes all (99.9 [±1.6]% at 95% confidence) of TBA15-L1 from the retained pool of library members, a result consistent with the loss in binding affinity when the flanking regions are left

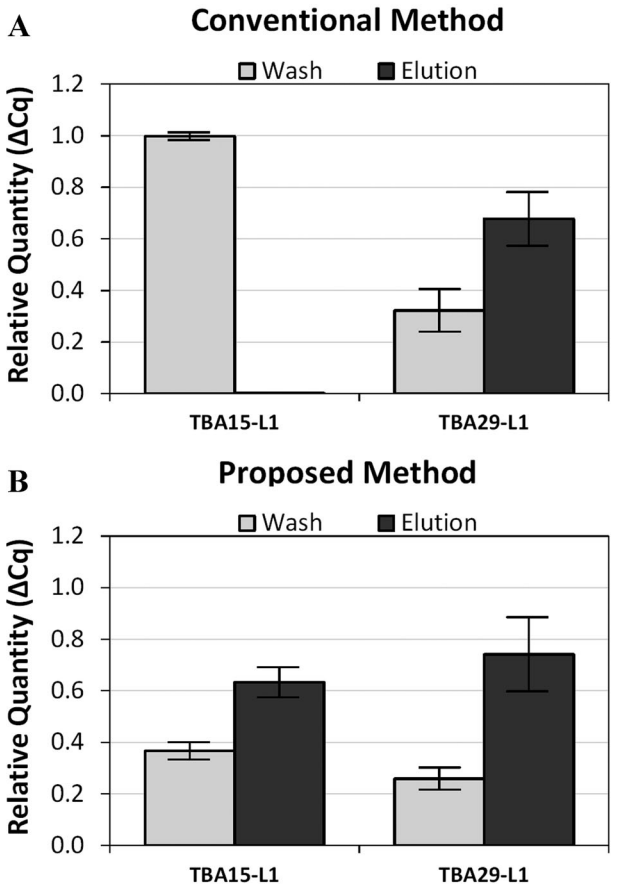

Figure 6. Pre-blocking the fixed regions permits successful enrichment of both benchmark thrombin aptamers during the first round of SELEX against immobilized α-thrombin. **A**: Partitioning of TBA15-L1 and TBA29-L1 in a conventional SELEX screening of Library 1 against magnetic beads coated with human α-thrombin. **B**: Partitioning of TBA15-L1 and TBA29-L1 in a parallel SELEX screening of the same system using the pre-blocking method described. The preblocking strategy successfully enriches both thrombin aptamers at the completion of the first selection round.

unblocked (Fig. 3B). No statistically significant amount of TBA15-L1 was detected in the elution fraction (0.1 [±0.1]% at 95% confidence), confirming that this benchmark high-affinity aptamer candidate is falsely screened out of the binding pool in the initial round of conventional SELEX. A majority (67.7 [±10.4]% at 95% confidence) of TBA29-L1 in the starting pool is retained in the first selection round, consistent with the lack of influence of the flanking sequences on the fold and function of this aptamer in its Library-1 form.

As shown in Figure 6B, addition of 5′-Comp-L1 and 3′-Comp-L1 to the library results in the retention and enrichment of both TBA15-L1 and TBA29-L1 in the first round of the L1 library selection. Moreover, based on the difference in cycle threshold values from real-time amplification with SYBR green used as a general amplification reporter, pre-blocking of the library also results in the retention and recovery of 3.1 ± 0.8 (P-value < 0.001) times more aptamer sequences than was achieved using the

conventional SELEX protocol, a result that suggests that up to two-thirds of potential aptamer sequences embedded within the random core region of the library may be compromised with respect to either fold or function through interference from the flanking regions. We find that interference effects related to unblocked flanking sequences may therefore severely impact aptamer discovery.

Pre-Blocking of Fixed Regions With Complementary Sequences Accelerates Enrichment of High-Affinity Aptamers as Measured by a Simple New qPCR-Based Method for Mean-Affinity Determination

In conventional SELEX, panning is typically terminated if an order-nM K_D is recorded for a pool of library members recovered and amplified after a given cycle. We therefore asked if the number of selection cycles required to achieve this metric could be reduced using the pre-blocking protocol to increase the number and sequence diversity of high-affinity aptamers retained. Three rounds of aptamer selection were performed on Library 2 using thrombin as the target, with the use of Library 2 in this case also serving to confirm that the performance of the protocol is not sensitive to the flanking sequences employed. Here, Library 2 was screened in its basic un-supplemented form to confirm that the new protocol provides rapid enrichment of a target aptamer from a standard SELEX library. In parallel, three selection rounds were also performed in the absence of the blocking agents (i.e., conventional SELEX) as a reference. Retained aptamer fractions were subjected to PCR amplification up to the cycle number after which by-product formation was detected in a pilot PCR run and subsequent gel electrophoresis. Before proceeding to the next round, the bulk affinity of eluted and amplified material was measured using a new qPCR-based binding assay. That binding assay was initially validated on the Library-1 form of TBA29-L1 by serially diluting and then incubating TBA29-L1 with a constant molar amount of human α-thrombin immobilized on paramagnetic beads. Following successive wash steps to remove unbound aptamers, bound TBA29-L1 was eluted in 50 mM NaOH at 85°C, neutralized with HCl, and then amplified by qPCR to permit absolute quantification of total amount of bound TBA29-L1 using a standard curve. Bound aptamer fractions (θ) were calculated and plotted against the equilibrium solution concentration (C), the results of which were nonlinearly fitted to the Langmuir equation to determine the dissociation constant. Due to the low number of α-thrombin binding sites present in the system, C could be taken as the incubation concentration without error. The isotherm results for this validation assay are presented in Figure 7, with the regressed binding affinity ($K_D = 5.76 \pm 1.17$ nM; Fig. 7A) matching within experimental error that value measured using ITC (5.49 ± 1.46 nM; Fig. 7B). The advantages of the qPCR-based assay described here are that its cost and completion time are at least an order of magnitude less than the SPR or ITC method more commonly used to determine bulk affinities.

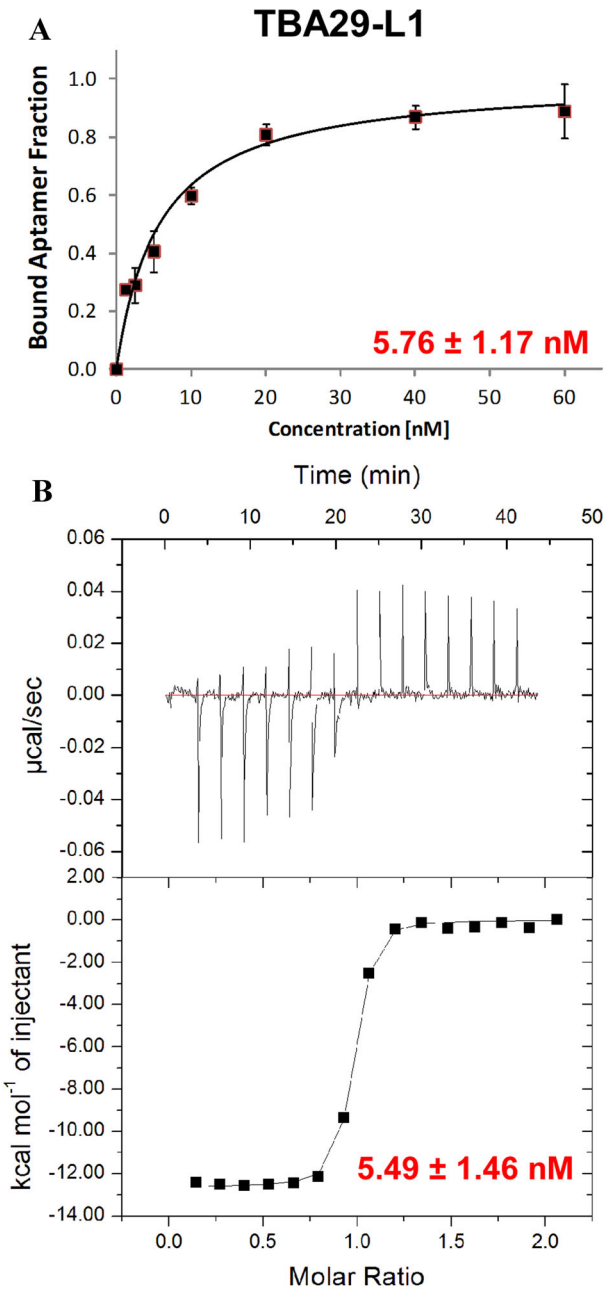

Figure 7. Validation of the qPCR-based affinity-binding assay for the SELEX Library-1 version of the 29-mer thrombin benchmark aptamer. **A**: Quantified bound fractions θ of TBA29-L1 were amplified by qPCR and plotted against the corresponding incubation concentration *C*. The equilibrium dissociation constant K_D was then estimated through data regression to the Langmuir isotherm equation. Amplification reactions were conducted in duplicates, in 20 µL volumes, with the average value of C_q used to determine the bound fractions. **B**: ITC results for titrating the same aptamer in a solution of thrombin at physiological conditions (top). The resulting fit to a single binding site model (bottom) yields the same binding affinity as measured by the qPCR-based assay.

Using this assay, mean binding affinities of retained Library-2 aptamer pools against thrombin were measured after each round of SELEX conducted either in the absence (conventional) or the presence of fixed-region blocking

Round 1		Round 2		Round 3	
Unblocked	Blocked	Unblocked	Blocked	Unblocked	Blocked
N.D.	N.D.	N.D.	344.3 ± 68.2 nM	278.2 ± 80.9 nM	99.6 ± 13.1 nM

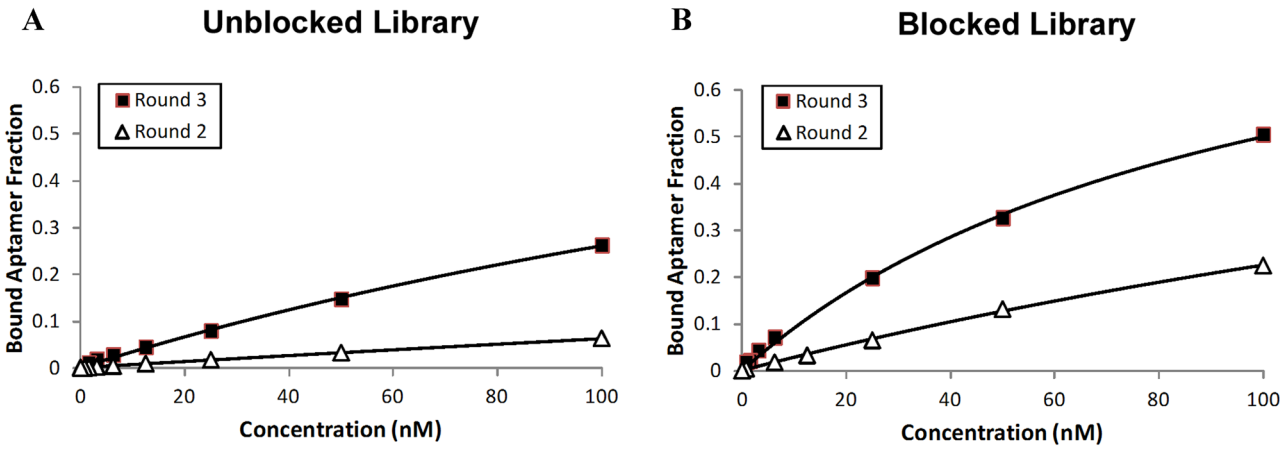

Figure 8. Changes in the bulk affinity of the retained aptamer pool in successive rounds of SELEX screenings of Library-2 against thrombin using either the conventional SELEX method or the proposed pre-blocking method. Three rounds of each SELEX method were conducted in parallel and average affinities of retained aptamer pools were measured by qPCR after each round. **A**: When the fixed regions of the library are left unblocked, the average affinity of the enriched aptamer pool falls below the assay threshold after the second round (Table; Round 2, Unblocked), and lies in the μM K_D range at the end of the third round (Table; Round 3, Unblocked). **B**: Pre-blocking the fixed regions of the library before every selection round results in greater enrichment of high-affinity aptamers.

agents (Fig. 8). For the conventional method, the mean affinity of retained aptamers after the first selection round fell below the detection limit of the assay, indicating relatively modest enrichment of high-affinity binders in the retained pool. A modest enrichment of tight-binding aptamers is consistent with results obtained after two rounds of selection, where binding of the retained aptamer pool could be detected, but not at levels that allowed statistically significant regression of a mean K_D. The third round of selection results in further enrichment of high-affinity members such that the measured mean affinity for the retained pool is 278.2 ± 80.9 nM (Fig. 8A), indicating that the conventional SELEX method is incrementally progressing toward the isolation of a pool of high-affinity aptamers. This result is consistent with the 8–20 rounds of selection that are typically required to identify suitable aptamers using this standard SELEX approach (Tahiri-Alaoui et al., 2002).

When 5′-Comp-L2 and 3′-Comp-L2 are applied to the Library-2 pool before each SELEX round, a significantly greater enrichment of tight-binding aptamers is observed. After the second round of selection, the mean affinity of the retained aptamer pool (344.3 ± 68.2 nM) is comparable to that reached after three rounds of the conventional SELEX protocol. Significant further enhancement of the mean binding affinity to 99.6 ± 13.1 nM is then observed after the third round (Fig. 8B).

Discussion and Conclusions

The findings presented here provide an improved understanding of the conditions required for successful enrichment of tight-binding aptamers within a SELEX library. We have shown that the conventional SELEX protocol, and by extension most of the recent improvements to it (Ahmad et al., 2011; Berezovski et al., 2006; Cho et al., 2013; Schütze et al., 2011), do not account for or prevent fixed-region interference effects that can lead to a complete loss of high-affinity aptamers to the wash fraction of the first or subsequent selection rounds. For example, despite the nM affinity of TBA15 to thrombin, our results show that a standard library version of this high-affinity aptamer (TBA15-L1) is falsely screened out of the binding pool in the early rounds of standard SELEX due to disruption of aptamer fold and a concomitant suppression of binding affinity. Although the severity of these interference effects could be lessened through careful sequence analyses to address self-association within the fixed regions, that strategy will not alleviate losses in aptamer fold and function due to complementarity between fixed and random region sequences.

We have addressed this problem through the introduction of a complementary oligonucleotide to each fixed region of an aptamer library. The results obtained collectively show that this concept serves to improve the retention and enrichment

of tight-binding aptamers for subsequent rounds of selection by eliminating folding or binding interferences caused by the single-stranded fixed regions of library members. As a result, pools of aptamers retained after successive rounds of selection contain a greater diversity and proportion of high-affinity library members than is observed when the pre-blocking agents are not employed in an otherwise identical selection process. The proposed method may therefore provide a more efficient route to high-affinity aptamer enrichment, particularly when applied to the many variants of the SELEX protocol that do not address fixed-region interference effects fully or in any way.

The method described may further serve to complement currently employed SELEX protocols through its ability to isolate and fully preserve aptamer folds and functions within the random region of the library. Confining candidate sequences to that region of a library should ease the difficult downstream analyses involved in deciphering the minimal binding sequence of newly discovered aptamers. Indeed, challenges in maintaining binding affinity of newly discovered aptamers after truncating the fixed regions are well documented, with complete loss in binding affinity having been reported for a number of aptamers selected against various proteins (Niebel et al., 2013), cells (Dua et al., 2013; Hamula et al., 2008) and small molecules (Wochner et al., 2008) alike. A particularly salient example is provided by Cho et al. (2013), who recently reported that three of the four most highly represented sequences in a final retained aptamer pool exhibited high affinities when analyzed as full-length library sequences, while much weaker affinities were measured when their corresponding core-region sequences were analyzed. This is important because the elegant high-throughput Agilent^{TM} arrays they pioneered and used to analyze the binding properties of selected library members were applicable to the 40-nt core-region sequence but not to the full 80-nt sequence of each retained library member due to limitations in the length of DNA that can be reliably prepared by available surface-initiated synthesis methods.

Along with the strengths and unique attributes noted above, the method has specific limitations when compared to other the SELEX-type methods currently employed. Namely, it precludes all aptamers in a library that form their structures from sequences within both the core and flanking regions. Though the contribution of such aptamers to the total pool of aptamers within a library is thought to be small (see above), it is not negligible, and some of the high-affinity aptamers discovered to date adopt such structures. In addition, the blocking strategy utilized here can create sites for those proteins known to bind duplex DNA. Thus the method is not likely to be suitable for library screening of those protein families (e.g., transcription factors, nucleases, histones, etc.). We therefore view the proposed method as being complementary to other effective SELEX methods by offering a unique approach to aptamer selection.

Toward this last point, a key virtue of the proposed method lies in its ability to be easily incorporated into many of the most widely used SELEX protocols, including those operating on patterned libraries containing universal nucleotides in the core region (Choi et al., 2010). Little to no changes to the selection conditions or the library design are required and the approach may therefore offer a certain level of standardization to a SELEX-type process. Moreover, although the results provided here were reported for DNA aptamer libraries, the technique should be applicable to RNA-based libraries. Based on these findings, we argue that complementing current SELEX-type protocols to include a selection path utilizing blocking oligonucleotides that minimize flanking-region mediated interference effects can improve aptamer discovery efforts by isolating consensus sequences of aptamers to the random region of the library, thereby preventing the complete and undesirable loss of these potential tight-binding library members.

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