Single-Step Selection of Bivalent Aptamers Validated by Comparison with SELEX Using High-Throughput Sequencing

Robert Wilson*, Christian Bourne, Roy R. Chaudhuri**, Richard Gregory, John Kenny, Andrew Cossins

Centre for Genomic Research at the Institute of Integrative Biology, University Of Liverpool, Liverpool, United Kingdom

Abstract

The identification of nucleic acid aptamers would be advanced if they could be obtained after fewer rounds of selection and amplification. In this paper the identification of bivalent aptamers for thrombin by SELEX and single-step selection are compared using next generation sequencing and motif finding informatics. Results show that similar aptamers are identified by both methods. This is significant because it shows that next generation sequencing and motif finding informatics have the potential to simplify the selection of aptamers by avoiding multiple rounds of enzymatic transcription and amplification.

Introduction

Nucleic acid aptamers are high affinity binding molecules that have applications in diagnostics, therapy and separation science. They are normally identified by screening combinatorial (randomized) libraries of typically 10^12–10^16 oligonucleotides for nucleic acid sequences that bind to a chosen target molecule by a process called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) that consists of multiple cycles of selection and PCR amplification.[1–3] In the selection step oligonucleotides compete for binding sites on the target molecule and in the amplification step the remaining pool of oligonucleotides is enriched with sequences that bind. The stabilities and/or affinities of aptamers based on natural nucleotide bases can be improved by incorporation of chemically modified bases, but these are more difficult to amplify. Aptamers based on locked nucleic acids (LNAs) would be more stable in vivo because they are resistant to nuclease enzymes, but when Vester and colleagues carried out 7 cycles of partitioning and amplification with a library that contained LNA bases they found the ratio of these bases to natural bases decreased in every cycle of amplification.[4] Gold and colleagues have used SELEX to identify high affinity aptamers based on chemically modified DNA bases, but these bases must be transcribed onto natural bases for amplification and reverse-transcribed back onto modified bases for selection.[5–7] These complications would be avoided if it was possible to identify aptamers without multiple cycles of selection and amplification.

Several groups have developed alternatives to SELEX based on single-step methods of selection.[8–11] Krylov and colleagues used Non-SELEX to identify aptamers that inhibit an enzyme that promotes cell division.[8] A combinatorial library of single stranded DNA was incubated with the target substance and unbound sequences were eliminated by capillary electrophoresis (CE). Oligonucleotides that remained bound to the target substance and oligonucleotides that dissociated during CE were retained and subjected to a further round of CE. Oligonucleotides in the fraction that was collected after three rounds of CE were cloned and Sanger sequenced. Nitsche and colleagues used MonoLEX to identify aptamers that bound to virus particles.[9] A DNA library was applied to an affinity column with heat-inactivated virus particles attached to a solid support. The column was washed to eliminate weakly bound DNA and then physically cut into sections. DNA in the sections was amplified and pyrosequenced. While these methods show that aptamers can be identified by single-step selection they were not carried out in parallel with SELEX and therefore it is not known if they produce comparable results.

In this paper we describe the parallel selection of bivalent aptamers for thrombin by SELEX and single-step selection. Thrombin is a multifunctional serine protease that plays important roles in blood clotting.[12,13] The dominant structural feature of the 37 kDa protein is a deep negatively charged active site and adjacent hydrophobic pocket flanked at either-end by positively charged regions known as exosite I and II. Exosite I is the binding site for multiple macromolecular and low molecular weight ligands including fibrinogen, thrombomodulin, hirudin and heparin cofactor II, and exosite II is the binding site for heparin and platelet receptor GP Ib-IX-V. Interactions between all three sites mediate blood clotting. DNA aptamers that bind to both exosites are known.[14] Apt-15 (5’-GGTTGGTGTGGTTGG) binds to...
exosite I and inhibits the conversion of soluble fibrinogen to insoluble fibrin,[15] and Apt-29 (5’-AGTCCGTTGTAAGGCAGGTGGGGGTTGACT) binds to exosite II but only has a moderate effect on the conversion of fibrinogen.[16] Mayer and colleagues have shown that the inhibition of blood clotting is enhanced when APT-15 is connected to a higher affinity APT-29 by a 15 base poly-dA linker,[17] and Soh and colleagues have shown that linkers identified by selection are superior to designed linkers.[18]

Results

The structure of the DNA library screened for bivalent aptamers is shown in Figure 1. Individual oligonucleotides consist of a 30 base combinatorial (randomized) sequence bracketed by APT-15 and Apt-29, and primer sequences for PCR. The PCR primers used by us are the same as those used by Tassett and colleagues in the original selection of APT-29.[16] Selection was carried out with magnetic beads coated with thrombin conjugated to biotin polyethylene glycol (biotin-PEG). Before conjugation thrombin was characterized with antibodies and aptamers, and by MS, electrophoresis and western blotting as described previously.[14] The conjugation method was designed to minimize the number of biotin-PEG molecules per molecule of thrombin. Counter-selection was carried out with a mixture consisting of equal amounts of uncoated streptavidin beads, beads coated with biotin-PEG and beads coated with human serum albumin (HSA). HSA was characterized and biotinylated in the same way as thrombin.

The workflow for SELEX is shown in Figure S1. Five nanomoles of thermally conditioned library (3×10^15 oligonucleotides) was slow-tilt rotated with thrombin-coated beads in 50 ml of HEPES buffer containing 0.1 mg ml⁻¹ HSA and 1 µM poly(d-ribohalosinic-deoxyctydilic) acid (poly-IC) for one hour, and then the beads were magnetically precipitated and washed with buffer. DNA bound to the beads was amplified with a 5’-biotin antisense primer for the number of cycles required to produce an intense band at 189 bp, and extracted by preparative electrophoresis on 2% agarose as shown in Figure S4. Extracted products were quantified and profiled by micro-electrophoresis as shown in Figures S5 and S6. Next generation sequencing (NGS) was carried out on a 454 GS FLX platform using titanium chemistry according to manufacturer’s instructions. Reads were sorted by barcode; an average of 28,640 nested sequences was obtained from each cycle of single-step selection, and an average of 34,299 sequences from each round of SELEX. Sequences were processed as shown in Figure 3. Any sequences not 30 bases long were discarded (length filter) and duplicate sequences were discarded in the single-step method (unique filter), but not in SELEX. The remaining sequences were searched for motifs with MEME 4.9.0 downloaded onto a Linux machine and executed locally.[23] Figure 3a shows the three most abundant motifs in DNA still bound to the beads in the single-step method. The location of each motif in the 30 base sequence was plotted against its abundance (number of sequences with motif divided by total number of sequences) and then the consensus sequence with the motif located at the mean position was determined with Clustal Omega.[24] Figure 3 shows that an average of 28,640 nested sequences was obtained from each cycle of single-step selection, and an average of 34,299 sequences from each round of SELEX. Sequences were processed as shown in Figure 3. Any sequences not 30 bases long were discarded (length filter) and duplicate sequences were discarded in the single-step method (unique filter), but not in SELEX. The remaining sequences were searched for motifs with MEME 4.9.0 downloaded onto a Linux machine and executed locally.[23] Figure 3a shows the three most abundant motifs in DNA still bound to the beads in the single-step method. The location of each motif in the 30 base sequence was plotted against its abundance (number of sequences with motif divided by total number of sequences) and then the consensus sequence with the motif located at the mean position was determined with Clustal Omega.[24] Figure 3 shows that consensus sequences based on the two most abundant motifs (linkers 1 and 2) in the single-step method are very similar to consensus sequences based on the two most abundant motifs (linkers 4 and 5) in SELEX. The consensus sequence based on the third most abundant motif in the single-step method (linker 3) is a blend of linkers 1 and 2. Figure 4 shows how the abundance of motifs changed in each cycle of dissociation and each round of SELEX. In the single-step method most enrichment occurred in the last 4 cycles, but no peak was reached suggesting that further enrichment could be achieved by additional cycles. The initial increase of motifs 4 and 5 followed by a plateau is typical of SELEX.[25] and shows that enrichment was complete after three or four rounds. Linker 6 is different; it was first detected at low (0.01%) abundance in Round 4 and then increased more rapidly than any other motif in Round 5.

Lowest energy secondary structures corresponding to linker sequences bracketed by the aptamers and primer sequences were predicted with mfold (see Figures S7 and S8).[26] Table 1 shows the predicted amount of base-pairing between the aptamers and the scaffold (linker and primer sequences). The structure of the selected library contained a 9 base poly-dA linker (linkers 4 and 5) in SELEX. The consensus sequence based on the two most abundant motifs (linkers 1 and 2) in the single-step method are very similar to consensus sequences based on the two most abundant motifs (linkers 4 and 5) in SELEX. The consensus sequence based on the third most abundant motif in the single-step method (linker 3) is a blend of linkers 1 and 2. Figure 4 shows how the abundance of motifs changed in each cycle of dissociation and each round of SELEX. In the single-step method most enrichment occurred in the last 4 cycles, but no peak was reached suggesting that further enrichment could be achieved by additional cycles. The initial increase of motifs 4 and 5 followed by a plateau is typical of SELEX.[25] and shows that enrichment was complete after three or four rounds. Linker 6 is different; it was first detected at low (0.01%) abundance in Round 4 and then increased more rapidly than any other motif in Round 5.

Lowest energy secondary structures corresponding to linker sequences bracketed by the aptamers and primer sequences were predicted with mfold (see Figures S7 and S8).[26] Table 1 shows the predicted amount of base-pairing between the aptamers and the scaffold (linker and primer sequences). The structure of the
bivalent aptamer based on linker 2 is shown in Figure 5a. Structures based on linkers 1 and 3–5 are similar to structure 2, with double stranded regions formed by base-pairing between the linker and the primers. The structure of the bivalent aptamer based on linker 6 is different because there is base-pairing between the linker and APT-29. Bivalent aptamers with linkers 1–6 were investigated for their ability to inhibit thrombin-catalyzed conversion of fibrinogen to fibrin; APT-15, Apt-29 and argatroban (a small molecule inhibitor of thrombin) were also investigated. The increase in OD at 350 nm due to the thrombin-catalyzed conversion of fibrinogen to fibrin has a sigmoid profile where the duration of the initial lag-phase depends on the concentration of added the inhibitor. Inhibition curves obtained by plotting the duration of the lag-phase in the absence of the inhibitor divided by the duration in the presence of the inhibitor, against the log10 of the inhibitor concentration, are shown in Figures S9–S11, and affinity curves obtained by plotting absorbance at 450 nm against the log10 of the aptamer concentration are shown in Figures S13–S14. Table 1 shows that inhibitor concentrations required to produce half-maximal inhibition (IC50 values) and aptamer concentrations required to produce 50% maximal binding were similar for all bivalent aptamers. The weak affinity of APT-15 compared to its IC50 value is probably because the inhibition assays were carried out with pure aptamers, but selection was carried out in the presence of other oligonucleotides including some that would have been able to hybridize with the aptamers. All of the double-stranded regions in the bivalent aptamers stabilize APT-29 and we suggest that this protects it from disruption during selection. Thus the two main properties selected are absence of internal conflict between the linker and the aptamers, and cooperation between the linker and APT-29 to protect the latter during selection.

Our results show that single-step selection can produce similar results to SELEX. Further confirmation is provided by comparison with recent work by Soh and colleagues who started with a combinatorial library with the same general structure as in Figure 1, except that the combinatorial sequence had a length of 35 bases and the priming sequences were different.[18] After 5 rounds of high-stringency SELEX they Sanger-sequenced 10 clones. They were unable to identify any consensus motifs (we would have reached the same conclusion if we had used Sanger sequencing instead of NGS) but like us they observed base pairing between the linker and the primers. We investigated the highest affinity bivalent aptamer identified by them (TBV-08) and found that it had an IC50 value of 0.64 nM, which is almost identical to the bivalent aptamer based on linker 2 (Table 1). When SELEX
was introduced in 1992 the most effective way to identify selected aptamers was to clone them in bacteria and sequence DNA from individual clones using first generation Sanger sequencing, but it is now possible to sequence large numbers of DNA molecules in parallel using NGS. When combined with appropriate informatics this allows aptamers to be identified after fewer rounds of selection, [27–29] or even, as we have shown here, after a single selection step. The latter is important because single-step selection can eliminate the multiple cycles of transcription and reverse-transcription (see reference [30] for example) that are necessary when SELEX is used to select chemically modified aptamers, and replace them with a single transcription step. Importantly, this transcription step, from a chemically-modified template to a natural-base template, is reported to be significantly more efficient than reverse transcription from a natural-base template to a chemically-modified template.[31] Therefore we anticipate that in future our approach will make it much faster and easier to select aptamers with improved properties, including those based on chemically modified nucleotides.

Figure 3. Extraction of aptamer sequences from NGS reads. a) Informatics pipeline as applied to DNA retained on the beads in single-step selection. Key: numbers in rectangles show the number of sequences at each stage in the pipeline. b) Linkers 4–6 identified in round 5 of SELEX. doi:10.1371/journal.pone.0100572.g003

### Materials and Methods

#### Materials

Oligonucleotides (all HPLC purified), avidin, Type 1 Gel Loading Solution, 4-hydroxyazobenzene-2-carboxylic acid (HABA), human serum albumin (HSA), fibrinogen, salmon sperm DNA, poly(deoxyinosinic-deoxycytidilic) acid (poly-IC), 10× TBE buffer and tetramethylbenzidine (TMB) solution containing 0.006% H₂O₂ were from Sigma. All as-supplied oligonucleotides were characterized by UV/vis spectroscopy using ODs returned by OligoCalc [http://www.basic.northwestern.edu/biotools/OligoCalc.html][24] and PAGE. In general short oligos had concentrations that were in agreement with the supplier’s data sheet, but long oligos were typically 10% less concentrated. PAGE with silver-staining of aptamers showed single bands of the correct size. 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), NaCl, NaHCO₃, NaOH, NaHPO₄, KCl, MgCl₂, CaCl₂, sodium citrate, bovine serum albumin (BSA), water and Tween-20 (all molecular biology grade) were also from Sigma. Thrombin (3068 NIH units mg⁻¹) was from Enzyme Research Laboratories.
Swansea, UK. [14] MyOne streptavidin magnetic beads and Charge Switch magnetic beads were from Life Technologies. MyTaq HS DNA Polymerase and Reaction Buffer were from Bioline. The MinElute PCR Purification Kit was from Qiagen. EZ-Link NHS-PEG12-Biotin (biotin-PEG-NHS) and 7 k MWCO Zeba Spin Desalting Columns were from Thermo Scientific. 1.5 ml DNA Lo-Bind tubes were from Fisher Scientific. Streptavidin Peroxidase was from Abcam, Cambridge, UK. MiniPROTEAN TGX Precast gels were from Bio-Rad. The PlusOne DNA Silver Staining Kit was from GE Healthcare.

Equipment

UV/vis spectra were recorded on a Hewlett Packard 8452A Diode Array Spectrophotometer. Magnetic separations were

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Figure 4. Bar graphs showing enrichment of motifs 1–3 in each cycle of single-step selection, and motifs 4–6 in each round of SELEX.

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Figure 5. Bivalent Aptamer Structures. a) Predicted structure of bivalent aptamer based on Linker 2. b) Structures of truncated derivatives of bivalent aptamer based on linker 2; excised bases are shown as grey circles.

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carried out with either DynaMag-50 magnet or a DynaMag-2 magnet (both Life Technologies), and slow-tilt rotation was carried out with an MX2 sample mixer (Life Technologies). DNA of defined length was extracted from PCR products with a Pippin Prep preparative electrophoresis platform (Sage Science). Extracted DNA was characterized with a Qubit 2.0 Fluorometer (Life Technologies) and Bioanalyzer 2100 (Agilent). Thrombin affinity and inhibition assays were carried with a Benchmark microplate reader (Biorad).

### Biotin-PEG-Thrombin

The contents of each vial of as-supplied thrombin were dissolved in 47.5 μl of molecular grade water to give a 6.55 mg ml\(^{-1}\) solution (8.4×10\(^{-3}\) moles) in 0.1 mM sodium citrate buffer, pH 6.5, containing 0.4 M NaCl and 0.2% PEG-8000. A molar equivalent of biotin-PEG-NHS; MW = 941.09 in 5

### Magnetic Beads For Selection And Counter Selection

MyOne streptavidin magnetic beads (Invitrogen) were washed with 10 mM NaOH to a final concentration 0.5 mg ml\(^{-1}\) solution in 0.1 mM sodium citrate buffer, pH 6.5, containing 0.4 M NaCl and 0.2% PEG-8000. A molar equivalent of biotin-PEG-NHS; MW = 941.09 in 5 μl of dry DMSO was added to the solution with gentle mixing, followed immediately by 47.5 μl of 1 mM sodium bicarbonate solution. The bicarbonate solution increases the pH to 8.0 and initiates aminolysis of the NHS-ester when the solution is homogenous. After gentle mixing for 1 hour, biotinylated thrombin was purified on a Zeba spin-column with water as the eluant. The concentration of thrombin in the eluate was determined using an extinction coefficient of E\(_{280}\) 1% = 5.31.

### Biotin Assay

HABA was dissolved in 10 mM NaOH to a final concentration of 2.42 mg ml\(^{-1}\). Avidin was dissolved in 50 mM sodium phosphate buffer, pH 6.0, containing 0.15 M NaCl to a final concentration of 0.5 mg ml\(^{-1}\). A 50 μM solution of biotin was prepared in 50 mM sodium phosphate buffer. A calibrator solution was prepared by adding 61.32 μl of HABA solution to 2.4 ml of avidin solution. A calibration graph was prepared by measuring the absorbance at 500 nm 10 minutes after adding 2 μl increments of biotin solution to 100 μl of calibrator solution. The concentration of biotin in biotinylated thrombin was determined by measuring the decrease in absorbance at 500 nm 10 minutes after adding 5 μl of purified biotin-thrombin to 100 μl of calibrator solution.

### Single-Step Selection of Aptamers Compared with SELEX

A library solution was prepared by dissolving 5 nanomoles of library template \(\text{AGATGCCTGTCGAGCATGC} \text{AGTGTTGGTGGGGN} \) \(\text{AGTCCGTGGTAGGGCAGGGCA} \text{AGTGGTGGGAGTAGCTAAATCGTTTGTGACGGG} \) where \(\text{N} \) is a 30-mer combinatorial (randomized) sequence, underlined sequences are APT-15 (GGTTGGTGGTGGGG) and APT-29 (AGTCCGTGGTGGGGCA)\(_{15}\) and sequences in bold font are for PCR, in 0.5 ml of HEPES buffer. The library solution was thermally conditioned at 95°C for 10 minutes, cooled to 4°C, and allowed to attain room temperature. It was then added to 49.5 ml of selection buffer (HEPES buffer containing 0.1 mg ml\(^{-1}\) HSA and 1 μM poly-IC) containing 1 mg of uncoated streptavidin beads, 1 mg PEG beads and 1 mg beads HSA beads. The beads were rotated with the library solution at room temperature for 24 hours and then precipitated for one hour on a DynaMag-50 magnet. The supernatant was transferred to a second tube and placed on a DynaMag-50 magnet for one hour. The supernatant was then transferred to a third tube to which 2 mg of thrombin beads was added and rotated for 48 hours. The beads were precipitated with a DynaMag-50 magnet and transferred to a DNA Lo-Bind tube in 1 ml of HEPES buffer and immediately precipitated with a DynaMag-2 magnet.

### Table 1. IC\(_{50}\) values, affinities and base pairing between scaffold and linker in bivalent aptamers.

| Aptamer | IC\(_{50}\) (nM) | 50% Affinity (nM) | Base-pairing |
|---------|----------------|------------------|--------------|
|         |                |                  | With Apt-15  | With Apt-29 |
| Linker 1| 2.7            | 1.4              | 1            | 0           |
| Linker 2| 0.6            | 0.2              | 0            | 0           |
| Linker 3| 1.1            | 0.4              | 2            | 0           |
| Linker 4| 1.0            | 0.4              | 0            | 0           |
| Linker 5| 1.5            | 1.0              | 2            | 0           |
| Linker 6| 24.5           | ~20              | 2            | 11          |
| Apt-29  | >1000          | 0.7              | -            | -           |
| Apt-15  | 6.0            | >1000            | -            | -           |
| Argatroban| 1.8         | -                | -            | -           |
| I       | 0.6            | 1.0              | 0            | 0           |
| II      | 0.6            | 0.5              | 0            | 0           |
| III     | 0.3            | 0.5              | 0            | 0           |
| IV      | >1000          | >1000            | -            | -           |

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supernatant was discarded and the beads were washed with 3×1 ml of HEPES. The beads were then suspended in beads 1 ml of HEPES and immediately precipitated with the supernatant retained as Supernatant 1. Other supernatants were obtained in the same way at the times listed in Table 2. After removing Supernatant 9 the beads were suspended in 400 µl of water and retained.

Single-Step: Extraction Of DNA From Supernatants
1 ml of retained supernatant from a vial on the DynaMag-2 magnet was added to 200 µl of charge-switch purification buffer and 20 µl of charge-switch magnetic beads in a new Lo-Bind tube. After rotating for 15 minutes the beads were precipitated and the supernatants discarded. The beads were washed with 2×150 µl of charge-switch wash solution and re-suspended in 20 µl of 10 mM Tris buffer, pH 8.5.

Single-Step: PCR Amplification Of DNA From Supernatants
20 µl aliquots of Tris buffer from charge-switch beads were mixed with 50 µl of PCR solution I containing primers and Reaction Buffer, and 30 µl polymerase solution containing 5 units of DNA Polymerase in molecular grade water to give final concentrations of 1× Reaction Buffer (1 mM dNTPs, 3 mM MgCl₂) and 1 µM primers (sense primer: 5'-AGATGCCCTGTCTTCCAGATGCT; antisense primer: biotin-5'-CCCGTCGACAAGCGAGTGGTAC). The mixture was then amplified (60 s at 95°C; 20 cycles of 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 10 seconds; final extension at 72°C for 60 s). Products were characterized by electrophoresis.

Table 2. Times at which supernatants were collected in single-step selection, barcodes of sequencing primers (see Table 4) that were used to amplify DNA from these supernatants, and number of nested PCR cycles (n) used to attach primers (see Table 4 for sequences of sequencing primers).

| Supernatant | Time      | Sense Primer | Antisense Primer | n  |
|-------------|-----------|--------------|------------------|----|
| 1           | 0 minutes | MID 1        | MID 8            | 3  |
| 2           | 30 minutes| MID 2        | MID 10           | 3  |
| 3           | 90 minutes| MID 3        | MID 11           | 3  |
| 4           | 3 hours   | MID 4        | MID 13           | 3  |
| 5           | 6 hours   | MID 5        | MID 14           | 6  |
| 6           | 12 hours  | MID 1        | MID 8            | 6  |
| 7           | 24 hours  | MID 2        | MID 10           | 6  |
| 8           | 48 hours  | MID 3        | MID 11           | 6  |
| 9           | 96 hours  | MID 4        | MID 13           | 9  |
| Beads       | 96 hours  | MID 5        | MID 14           | 3  |

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Single-Step Selection of Aptamers Compared with SELEX

SELEX: First Round Selection
5 nanomoles of thermally conditioned library template in 0.5 ml of HEPES buffer was added to 49.5 ml of selection buffer containing 200 µg of thrombin beads and rotated for one hour at room temperature. The beads were then precipitated for one hour on a DynaMag-50 magnetic separator and washed with 4×1 ml HEPES buffer on a DynaMag-2 magnetic separator. The beads were then re-suspended in 340 µl of molecular grade water.

SELEX: Strand Separation Of PCR Products
8×100 µl of pooled PCR products were transferred into 4×30 µl of 10 mM Tris-HCl buffer, pH 8.0, with a MinElute PCR Purification Kit according to the supplier’s instructions. 119 bp DNA was extracted with a Pippin Prep by applying each 30 µl aliquot to one lane of a 2% agarose cassette (Sage Science). Extracted products were pooled and converted to single-stranded DNA (ssDNA) with streptavidin beads pre-conditioned in 20 mM NaOH as described previously.[19] The yield of single-stranded DNA was estimated using an OD of 1.0 for 0.786 µM solution.

SELEX: Second And Fourth Round Selection Steps
Thermally conditioned ssDNA was mixed with B (see Table 3) µg of thrombin beads in selection buffer; the amounts of beads in mg and concentrations of DNA are listed in Table 3. The mixture was rotated for one hour and then the beads were precipitated and washed with 4×1 ml HEPES on a DynaMag-2 magnetic separator. The beads were then re-suspended in 2B µl of molecular grade water.
**SELEX: Third And Fifth Round Selection Steps**

The method was identical to even numbered selection rounds except that single-stranded DNA was first rotated with a counter-selection mixture of HSA beads, PEG beads and streptavidin beads; the amounts of beads are listed in Table 3. After one hour counter-selection beads were removed by magnetic precipitation and the supernatant was mixed with thrombin beads and rotated for one hour.

**SELEX: PCR Amplification Of DNA Retained On Magnetic Beads In Second And Subsequent Selection Rounds**

PCR amplification was carried out in two stages. In the first stage 20 μl aliquots of thrombin beads in water from the selection step were mixed with 50 μl of PCR solution I, heated to 95°C for 15 minutes and cooled to 50°C for 5 minutes. Then 30 μl polymerase solution was added and the mixture was amplified (60 s at 95°C; 10 cycles of 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 10 seconds; final extension at 72°C for 60 s).

PCR products were pooled and in the second stage 20 μl aliquots were added to 50 μl of PCR solution I and 30 μl polymerase solution and amplified (95°C for 60 s; \( N \) cycles (Table 3); 95°C for 15 s, 60°C for 15 s, 72°C for 10 s; final extension at 72°C for 60 s).

**Preparation Of Samples For Sequencing**

20 μl aliquots of PCR I products were mixed with 50 μl of PCR solution II containing 454 sequencing primers (Table 4) and Reaction Buffer, and 30 μl polymerase solution containing 5 units of DNA Polymerase in molecular grade water to give final concentrations of 1× Reaction buffer and 1 μM primers. The mixture was then amplified (60 s at 95°C; \( n \) cycles (Tables 2 [single-step] and Table 5 [SELEX]) of 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 10 seconds; final extension at 72°C for 60 s). PCR products were transferred to 10 mM Tris buffer using a MinElute PCR Purification Kit and 189 bp DNA was isolated with a Pippin Prep running a 2% agarose cassette. Isolated DNA was transferred back into 20 μl of 10 mM Tris buffer using the MinElute kit, and characterized for size by micro-electrophoresis on a Bioanalyzer 2100, and concentration with a Qubit Fluorometer.

**Motif Finding Informatics**

Sequencing reads were sorted into sets by barcode and then processed by removing the 5’ and 3’ aptamer sequences using cutadapt.[32] Any trimmed sequences that were not 30 bases long were discarded. Duplicate sequences were also discarded in the single-step method, but not in SELEX. The remaining sequences were searched with MEME using the command line: meme./file.txt -dna -maxsize 600000 -mod zoops -nmotifs 10 -minw 4 -maxw 40, where file.txt is a Plain Text file containing the sequences to be searched in numbered fasta format. MEME is a computational tool for discovering motifs in a group of related DNA or protein sequences. The on-line version imposes a ceiling of <2000 on the maximum number of 30 base sequences that can be searched and therefore it was downloaded onto a Linux machine. There is no upper limit to the number of sequences that can be searched locally with the downloaded version of MEME, but the computational cost of analyzing a complete set of sequences (all sequences from one cycle of single step selection, or one round of SELEX) with MEME is prohibitive and therefore complete sets were divided into sub-sets of ≤10,000 sequences to derive intermediate motifs. Each search of a sub-set produced slightly different but highly similar intermediate motifs. Sequences with similar motifs were extracted and merged in a single file that

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**Table 3. SELEX: Concentrations of DNA, magnetic beads and number of additional PCR cycles.**

| Round | Volume/ml | DNA/nM | Beads/μg | Thrombin (μg) | HSA | PEG | Streptavidin |
|-------|-----------|--------|----------|--------------|-----|-----|-------------|
| 1     | 500       | 100    | 0        | 200          | 0   | 0   | 0           |
| 2     | 30        | 10     | 0        | 150          | 0   | 0   | 0           |
| 3     | 30        | 50     | 0        | 50           | 50  | 50  | 50          |
| 4     | 100       | 1      | 0        | 100          | 0   | 0   | 0           |
| 5     | 30        | 1      | 0        | 100          | 0   | 0   | 0           |

\( N \) cycles (Table 3): 95°C for 15 s, 60°C for 15 s, 72°C for 10 s; final extension at 72°C for 60 s.
was searched to identify final motifs. The position of the final motifs in the 30 base sequence was plotted against abundance (number of sequences with motifs divided by the total number of sequences in the set) and then sequences with the motif located at the Gaussian mean were extracted. The consensus of the extracted sequences was found with Clustal Omega, and secondary structures were predicted by mfold by applying the conditions: (number of sequences with motifs divided by the total number of motifs in the 30 base sequence was plotted against abundance was searched to identify final motifs. The position of the final motifs in the 30 base sequence was plotted against abundance (number of sequences with motifs divided by the total number of sequences in the set) and then sequences with the motif located at the Gaussian mean were extracted. The consensus of the extracted sequences was found with Clustal Omega, and secondary structures were predicted by mfold by applying the conditions:

## Thrombin Inhibition Assays

Thrombin inhibition assays were performed to determine the ability of the selected aptamers to inhibit thrombin activity. In a typical assay, 25 μl of thrombin in HEPES buffer was incubated with the aptamers in triplicate for one hour at 25°C. The enzyme reaction was stopped by addition of 50 μl of 1 M H2SO4 and the absorbance was measured at 450 nm.

## Supporting Information

**Figure S1** Scheme of SELEX. Key: size selection = preparative electrophoresis; PCR I = nested PCR with sequencing primers; QC = quality control (quantification and micro-electrophoresis); NGS = next generation sequencing.

**Figure S2** a) 15% poly-acrylamide gel developed at 100 V for two hours and stained with silver showing PCR products from SELEX; products produced by +3 cycles of PCR (red rectangle) were selected for preparative electrophoresis. Key: L = 20 bp ladder; white numerals = number PCR cycles where 10 is the product of the first stage PCR, and +3, +6, +9, +12 and +15 are the products of the second stage PCR. b) Results of preparative electrophoresis showing band centered on 119 bp that was extracted surrounded by white rectangles. Key: white numerals indicate lane numbers (lane 5 has calibrator DNA of lengths 20, 75, 150, 300 and 600 bp); screen E shows ethidium bromide fluorescence versus time; screen G shows a fluorescence image of the developed agarose gel.

### Table 4. Primers for nested PCR; 454 sequencing primers in bold type with MID sequences (barcodes) underlined.

| Barcode | Sequence |
|---------|----------|
| MID 1   | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 2   | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 3   | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 4   | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 5   | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 6   | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 7   | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 8   | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 9   | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 10  | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 11  | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 12  | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 13  | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |
| MID 14  | 5'-CGTATGGCTCCTCCTGCGCCATCAGAGCAGTGCGTTAGATGCCTGTCGAGCATGCT |

**Table 5.** SELEX: barcodes of sequencing primers and numbers of nested PCR cycles used to attach sequencing primers (see Table 4 for sequences of sequencing primers).

| Round | Sense Primer | Antisense Primer | n  |
|-------|--------------|------------------|----|
| 1     | MID 1        | MID 8            | 3  |
| 2     | MID 2        | MID 10           | 3  |
| 3     | MID 3        | MID 11           | 3  |
| 4     | MID 4        | MID 13           | 6  |
| 5     | MID 5        | MID 14           | 6  |

**Table 6.** Primers for SELEX.; 454 sequencing primers in bold type with MID sequences (barcodes) underlined.
Table 6. Sequences of aptamers investigated in affinity and inhibition assays; the sequences of APT-15 and APT-29 in bivalent aptamers are underlined.

| Bivalent Linker 1 | 5'-AGATGCCTGTCGAGCATGCTGGTTGGTGTGGTTGGTTGGCGTTCAAAGCAGTCTTGGGCTAAGTCCGTGGTAGGGCAGGTTGGGGTGACTGTAGCTAAACTGCTTTGTCGACGGG |
|-------------------|------------------------------------------------------------------------------------------------------------------|
| Bivalent Linker 2 | 5'-AGATGCCTGTCGAGCATGCTGGTTGGTGTGGTTGGTTGGTTGGCGTTCAAAGCAGTCTTGGGCTAAGTCCGTGGTAGGGCAGGTTGGGGTGACTGTAGCTAAACTGCTTTGTCGACGGG |
| Bivalent Linker 3 | 5'-AGATGCCTGTCGAGCATGCTGGTTGGTGTGGTTGGGTAGGGGCCCAGCATGACAGTTTTTGCCAAAGTCCGTGGTAGGGCAGGTTGGGGTGACTGTAGCTAAACTGCTTTGTCGACGGG |
| Bivalent Linker 4 | 5'-AGATGCCTGTCGAGCATGCTGGTTGGTGTGGTTGGGTTGTGGGGAATAAAAGCAGTTTAGCGTACAGTCCGTGGTAGGGCAGGTTGGGGTGACTGTAGCTAAACTGCTTTGTCGACGGG |
| Bivalent Linker 5 | 5'-AGATGCCTGTCGAGCATGCTGGTTGGTGTGGTTGGAAGTAGGTGGTTCAAAGCAGTGAAGGGTACAGTCCGTGGTAGGGCAGGTTGGGGTGACTGTAGCTAAACTGCTTTGTCGACGGG |
| Bivalent Linker 6 | 5'-AGATGCCTGTCGAGCATGCTGGTTGGTGTGGTTGGCGGGGGCCCTCTATAGTTGCGTTCTGGATGAGTCCGTGGTAGGGCAGGTTGGGGTGACTGTAGCTAAACTGCTTTGTCGACGGG |
| TBV-08           | 5'-AGCAGCACAGAGGTCAGATGGGTTGGTGTGGTTGGTGAGACCTTGCATGCGACTTGGTGAGCACGTGAGAAGTCCGTGGTAGGGCAGGTTGGGGTGACTCCTATGCGTGCTACCGTGAA |
| Apt-15           | 5'-GGTTGGTGTGGTTGGAGTCCGTGGTAGGGCAGGTTGGGGTGACTGTAGCTAAACTGCTTTGTCGACGGG |
| Apt-29           | 5'-AGTCCGTGGTAGGGCAGGTTGGGGTGACT |
| Truncate I       | 5'-GGTTGGTGTGGTTGGTTGGTTGGCGTTCAAAGCAGTCTTGGGCTAAGTCCGTGGTAGGGCAGGTTGGGGTGACT |
| Truncate II      | 5'-AGATGCCTGTCGAGCATGCTGGTTGGTGTGGTTGGTTGGCGTTCAAAGCAGTCTTGGGCTAAGTCCGTGGTAGGGCAGGTTGGGGTGACT |
| Truncate III     | 5'-GGTTGGTGTGGTTGGTTGGTTGGCGTTCAAAGCAGTCTTGGGCTAAGTCCGTGGTAGGGCAGGTTGGGGTGACT |
| Truncate IV      | 5'-TTGGTTGGCGTTCAAAGCAGTCTTGGGCTAAGTCCGTGGTAGGGCAGGTTGGGGTGACT |

Figure S3 15% poly-acrylamide gel developed at 200 V for one hour and stained with silver of DNA amplified from supernatants and retained on beads in single-step selection. Key: Lanes 1–9 show PCR products from supernatants 1–9; Lane 10 shows PCR products from DNA retained on beads.

Figure S4 a) Scheme of nested PCR for attachment of 454 sequencing primers. b) 12% poly-acrylamide gel developed at 200 V for 45 minutes and stained with silver showing PCR trials to find number of cycles required to produce a band at 198 bp with minimal non-specific products. In the example shown products produced by 6 cycles of PCR enclosed in red rectangle were selected for preparative electrophoresis. Key: L = 20 bp ladder; white numerals = number of nested PCR cycles. c) Preparative electrophoresis on lanes 1–4 with extraction of a band centered on 189 bp (enclosed in white rectangle). Key: white numerals indicate lane numbers (lane 5 has calibrator DNA of lengths 20, 75, 150, 300 and 600 bp); screen E shows ethidium bromide fluorescence versus time; screen G shows a fluoresce image of the developed agarose gel.

Figure S5 Quality control of samples from single-step selection before sequencing. Numbers in brackets are sample concentrations in ng μl⁻¹ in a volume of 20 μl. Peaks at 35 and 10,350 bp in micro-electrophoresis profiles are internal calibrators.

Figure S6 Quality control of samples from SELEX before sequencing. Numbers in brackets are sample concentrations in ng μl⁻¹ in a volume of 20 μl. Peaks at 35 and 10,350 bp in micro-electrophoresis profiles are internal calibrators.

Figure S7 Predicted lowest energy secondary structures of bivalent aptamers based on linkers 1–3. Nucleotides of Apt-15 are shown in red font and nucleotides of Apt-29 are shown in blue font.

Figure S8 Predicted lowest energy secondary structures of bivalent aptamers based on linkers 4–6. Nucleotides of Apt-15 are shown in red font and nucleotides of Apt-29 are shown in blue font.

Figure S9 Inhibition curves of Apt-15 and Apt-29.

Figure S10 Inhibition curves of bivalent aptamers.

Figure S11 Inhibition curves of argatroban and bivalent aptamer TBV-08.

Figure S12 Inhibition curves of truncated derivatives of the bivalent aptamer based on linker 2.

Figure S13 Affinity curves of Apt-29 and Apt-15.

Figure S14 Affinity curves of bivalent aptamers.

Figure S15 Affinity curves of truncated derivatives of the bivalent aptamer based on linker 2.
Author Contributions
Conceived and designed the experiments: RW. Performed the experiments: RW CB JK. Analyzed the data: RW RC RG. Contributed<br>References
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