Supplementary Data

Selecting Aptamers for a Glycoprotein through the Incorporation of the Boronic Acid Moiety

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Materials and Methods

General selection procedures: Our goal is to harvest boronic acid-modified DNA for binding to certain glycoproteins using a modified SELEX approach. For the aptamer selection work, fibrinogen was immobilized to magnetic beads using amidation chemistry. During the whole SELEX process, we first prepared a random pool of ssDNA. In order to minimize the exposure of the boronic acid moiety to high temperature for an extended period of time, the ssDNA pool was first PCR-amplified. The incorporation of boronic acid-modified nucleotide (B-TTP) was performed in the last round of polymerase reaction using a single primer. We then exposed this library to fibrinogen immobilized on beads, isolated the DNA associated with the fibrinogen, then re-amplified in order to enrich those aptamers with high affinity. Radioactive ATP was used to incorporate a radio-tracer for binding detection. Throughout the process, the percentage of radioactivity retained by immobilized fibrinogen was monitored. After the 4^{th} round, we started noticing significant enrichment of radioactivity in the fibrinogen-bound fraction. After the 8^{th} round, there was over 20% of radioactivity retained by the immobilized fibrinogen. After the 13^{th} round, nearly 80% of the radioactivity was retained by immobilized fibrinogen indicating a high percentage of specific binders for the target glycoprotein. In order to minimize the selection of non-specific binders and those that would only bind to the protein portion of the glycoprotein, several counter-selection steps were built into the process. After the 6^{th} round, we counter-selected the library against the beads alone. The aim was to remove those that have non-specific binding to the magnetic bead matrix. After 13^{th} round, we incubated the DNA pool without boronic acid modification with immobilized fibrinogen. In this step, we only collected whatever remained unbound to the immobilized fibrinogen. Since the aim of our work is to select a library that recognizes the glycosylation site of the glycoprotein, we were only interested in those that use the boronic acid moiety for recognition. We hypothesize that the involvement of one or more boronic acid moiety would allow the selection to gravitate toward glycosylation recognition. By removing the pool of DNA that can recognize fibrinogen without using the boronic acid moiety, the aim was to enhance the chance of selecting aptamers that specifically recognize the carbohydrate portion of the glycoprotein.

Reagents: All samples and buffers were prepared using de-ionized water obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA). The BioMag Carboxyl beads were purchased from Bangs Laboratories, Inc. (Fishers, IN). *Taq* DNA polymerase was purchased from Roche (Mannheim, Germany). Radiolabelled α-^{32}P-dATP was purchased from PerkinElmer (Waltham, MA). Recombinant neuraminidase (sialidase) was purchased from New England Biolabs (Ipswich, MA). Human fibrinogen, solvents, buffer salts and other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), Fisher (Pittsburgh, PA) and Acros (Morris Plains, NJ) and used without further purification.

Immobilization of fibrinogen to beads: 25 μL of BioMag Carboxyl beads were washed with 0.5 mL of coupling buffer (0.01M K_2HPO_4, 0.15 M NaCl at pH 5.5) four times. The supernatant was aspirated to leave the beads as a wet cake on the container wall. 0.5 mL of Coupling Agent (20 mg EDCI in 20 mL of water) was added to beads and the mixture was shaken briefly for 30 min. Fifty mg of fibrinogen in 22 mL of coupling buffer was added and the mixture was shaken overnight. The beads were separated by magnetic separator and washed by 5 mL of wash buffer (0.01M Tris, 0.15 M NaCl, 0.1% w/v BSA, 0.1% NaN_3 and 0.001 EDTA at pH 7.4) three times. The immobilized beads were stored at 2-8°C as a suspension in wash buffer. During this phase, the immobilization efficiency was monitored by using the Kaiser test.[1]

Release of glycan from fibrinogen: This procedure involves hydrazinolysis to release the glycan from fibrinogen.[2] To 1 mg of fibrinogen in an absolutely dry reaction vial was added 100 μL of dry hydrazine. The vial was then sealed and incubated for 12 hr at 85 °C. Hydrazine was then removed in vacuum by repeated addition of anhydrous toluene. The vial was placed on ice. Then 200 μL of 0.2 M sodium acetate was added. This was followed by the addition of 20 μL of acetic anhydride. After reaction on ice for 10 min, a second aliquot of 20 μL acetic anhydride was added and the vial was incubated for an additional 50 min at room

temperature. The product was desalted using DOWEX AG50X12 and separated using Whatman 1 Chr chromatography paper for 60 hr with 4:1:1 butanol/ethanol/water. The paper containing glycan was eluted with 1.5 mL of water. Then the glycan sample was pushed through a 0.45-μm 13-mm hydrophilic Millex-LCR filter and dried by a vacuum-freeze evaporator.

Periodation of fibrinogen:[3,4] To a solution of 100 mg of fibrinogen in 100 mL of 0.2 mM sodium periodate was added 100 mL of 10 mM sodium acetate. Then the mixture was incubated for 30 min on ice-bath. 10 mL of 10 mM glycerol was then added and the mixture was incubated for 30 min at room temperature to consume the excess periodate. The resulting fibrinogen was purified by IWT TMD-8 ion-exchange resin (Sigma-Aldrich, St. Louis, MO) and concentrated using a freeze dryer.

Desialylation of fibrinogen:[5] The saccharide moiety in fibrinogen was desialylated by using neuraminidase-mediated α2→3, α2→6 and α2→8 linked *N*-acetyl-neuraminic acid hydrolysis. In brief, 500 units of recombinant neuraminidase were incubated with 0.2 nmol of fibrinogen in a 20 μL reaction buffer for 20 hr at 37 °C. The reaction was then stopped by heating for 5 minutes at 100 °C. The resulting desialylated fibrinogen was purified by Microcon YM-100 spin column (Millipore, Billerica, MA) and lyophilized.

SELEX: A DNA template containing 50 randomized positions (5'-CCTTCGTTGTCTGCCTTCGT-50N-ACCCTTCAGAATTCGCACCA-3'), flanked by primer regions complementary to primers 5'-CCTTCGTTGTCTGCCTTCGT-3' and 5'-TGGTGCGAATTCTGAAGGGT-3', was synthesized. The starting dsDNA library was constructed by a 25-round PCR amplification using *Taq* polymerase from DNA template in the presence of four standard nucleotides (dNTPs) using a Effendorf thermal cycler. The PCR product was then concentrated by a YM-30 spin column (Millipore, Billerica, MA). The ssDNA pool was then prepared by one-round PCR using the above dsDNA product using [α-^{32}P] dATP, dATP, dCTP, dGTP and B-TTP. The DNA pool was incubated with the fibrinogen-immobilized BioMag carboxyl beads for one hour in binding buffer (300 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.6). The incubated beads were then separated by using a magnetic separator and washed with binding buffer for six times and then fibrinogen-containing (10 μg/mL) binding buffer for three times. Aliquots (20 μL) were taken from every washing, and radioactivity in each aliquot was determined using a Beckman LS 6500 liquid scintillation counter. The fractions from the fibrinogen washings were combined, extracted with phenol, precipitated in ethanol, and amplified by PCR for the next round of SELEX using the same protocol.

Binding Assays: Dissociation constants in solution were determined by equilibrium filtration.[6,7] Using this technique, the bound and unbound ligand (DNA) partition between two compartments separated by a membrane. DNA was first amplified into dsDNA using two primers by PCR (25 cycles of 0.5 min at 94 °C, 0.5 min at 46 °C, and 0.5 min at 72 °C, followed by 5 min at 72 °C). The dsDNA product was then split into ssDNA using one primer and α-^{32}P-dATP by one-round PCR. The ^{32}P-labeled ssDNA ligand and the protein at various concentrations in the 100 μL of binding buffer were incubated for 15 min at 25 °C prior to loading into the Microcon YM-100 unit (Millipore, Billerica, MA). The solution was centrifuged at 13,000 *g* for 10 sec to saturate the membrane, and the filtrate was transferred back to the unit. The solution was centrifuged for another 20 sec, and the filtrate (about 10 μL) was collected. Aliquots (10 μL) were taken from both the remaining solution and the filtrate, and radioactivity in each aliquot was determined by using a Beckman LS 6500 liquid scintillation counter. All binding assays were at least duplicated. The equilibrium dissociation constants (K_d) of the ligand–protein interaction were obtained by fitting the dependence of bound fractions of specific binding on the concentration of the aptamers to the equation $Y = B \max X /(K_d + X)$, using SigmaPlot program.

Molecular Cloning and Sequencing. Clones of the ssDNA pool were prepared after 13 rounds of SELEX selection. An aliquot of the ssDNA solution was PCR amplified. The PCR reagent mixture and cycling conditions were similar to those described above and only 20 PCR cycles were performed. Final extension was

carried out for 15 min at 72 °C. The PCR product was ligated into the pCR® 4-TOPO vector (Sigma, St. Louis, MO) at room temperature for 30 min. This ligation product was transferred into One Shot TOP10 Chemically Competent *E. coli* on ice for 30 min and heat-shocked at 42 °C for 30 sec and the transformation liquid was spread on a pre-warmed LB plate and incubated overnight at 37 °C. Hundreds of colonies were raised. Twenty of them were picked up at random and cultured overnight in LB medium containing 100 μg/ml ampicillin. Plasmids from these colonies were isolated and purified using the PureLink™ HQ Mini Plasmid Purification Kit (Sigma, St. Louis, MO), and sequenced using the T3 promoter primer by the DNA/Protein Core Facility at Georgia State University (Atlanta, GA).

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                10        20        30        40        50        60        70        80        90
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
114A(87J)       CCTTCGTTGTCTGCCTTCGTACGCGCGCATAGTCCGAGTAGTATGACGCATATGTGCTACTGAGTCCTACACCCTTCAGAATTCGCACCA
114B(85M)       CCTTCGTTGTCTGCCTTCGTGCGCGCATAGACCGAGTGGCATGACGCCTATCTCGTGATAGAGGACTCCGACCCTTCAGAATTCGCACCA
114C            CCTTCGTTGTCTGCCTTCGTTAGACTATCACGGATGGACGTATCCTGTGCGTATGACGCATGAAGCACTAACCCTTCAGAATTCGCACCA
114D(87O)       CCTTCGTTGTCTGCCTTCGTGCTATGTCTGAGCAGTGCGTATGGTACCTCGTATCAGCCATATGACGCAAACCCTTCAGAATTCGCACCA
114E            CCTTCGTTGTCTGCCTTCGTATATGACGCATGCCTAGACCTCCCTATGATAGCCTGGATCGTACGTACGTACCCTTCAGAATTCGCACCA
114F(85C)       CCTTCGTTGTCTGCCTTCGTAGTCGACTCTGACGCATGGACGTATCCTGTGCGTATGCATTATGAAGCACACCCTTCAGAATTCGCACCA
114G(85Q)       CCTTCGTTGTCTGCCTTCGTAGCCCTTGCACCTATGAGGTATGATCTTCGTTGGACGCAGTTACTACGCCACCCTTCAGAATTCGCACCA
114H(85A)       CCTTCGTTGTCTGCCTTCGTAGCGGATCGAATTACGCGTTAACGGCAACCGATAACGGGACCGATTGCACACCCTTCAGAATTCGCACCA
114I(87A)       CCTTCGTTGTCTGCCTTCGTAGGACCGCAGACATCGACGCAGGGAAATTCCGCAAGTCCAGCCAAATGCCACCCTTCAGAATTCGCACCA
114J            CCTTCGTTGTCTGCCTTCGTGAGCAGCGTAGCTCTAAGCCAGACTAGTAACGTATCCTGATATGACGCATACCCTTCAGAATTCGCACCA
114K(87T)       CCTTCGTTGTCTGCCTTCGTCGTGACCAGGACATATGAGGCATAGCGCTTGACTCTACCGCTGCTAGCACACCCTTCAGAATTCGCACCA
114L(87Q)       CCTTCGTTGTCTGCCTTCGTTATGACGCAACTGTGCACAATGCGACTCAGGACGTGTACGAGCGAGTGTAACCCTTCAGAATTCGCACCA
114M(85P)       CCTTCGTTGTCTGCCTTCGTCCGTGTCCCGCTATGATGCTACTTGCATTCGCGGAATTGAACCGTCGCGCACCCTTCAGAATTCGCACCA
114N(85E)       CCTTCGTTGTCTGCCTTCGTGAGCGGAGTCAGACGCACGCTCGTACCTGTGCGCAAGCACTATGACGGACACCCTTCAGAATTCGCACCA
114O(85B)       CCTTCGTTGTCTGCCTTCGTAGGACCGCAGACATCGACGCAGGGAAATTCCGCAAGTCCAGCCAAATGCCACCCTTCAGAATTCGCACCA
114P            CCTTCGTTGTCTGCCTTCGTTCAGGCTGCTATATGACGCATATCGACAGACGAGTCAGTAGCTGCACACAACCCTTCAGAATTCGCACCA
114Q(85T)       CCTTCGTTGTCTGCCTTCGTCAGCTACTGGGCTATCTGGACTTGGCAATCTCGCTTGCAGCATTGAGCGCACCCTTCAGAATTCGCACCA
114R(76C)       CCTTCGTTGTCTGCCTTCGTACGAACGCTGACATCGACGGTCGGCAATTCCGCAAGTCCAGCCTAATGACACCCTTCAGAATTCGCACCA
114S            CCTTCGTTGTCTGCCTTCGGCATCACTACGGTCGAGATACATAGTCGCTATGACGCATCAGTCTTACGCTACCCTTCAGAATTCGCACCA
114T(76D)       CCTTCGTTGTCTGCCTTCGTAGCGCTAGGACGTAAGATGCATGCCCTAGATTCGAAGCTGATGCCATGAGACCCTTCAGAATTCGCACCA
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Figure S1. The sequences for 20 aptamers

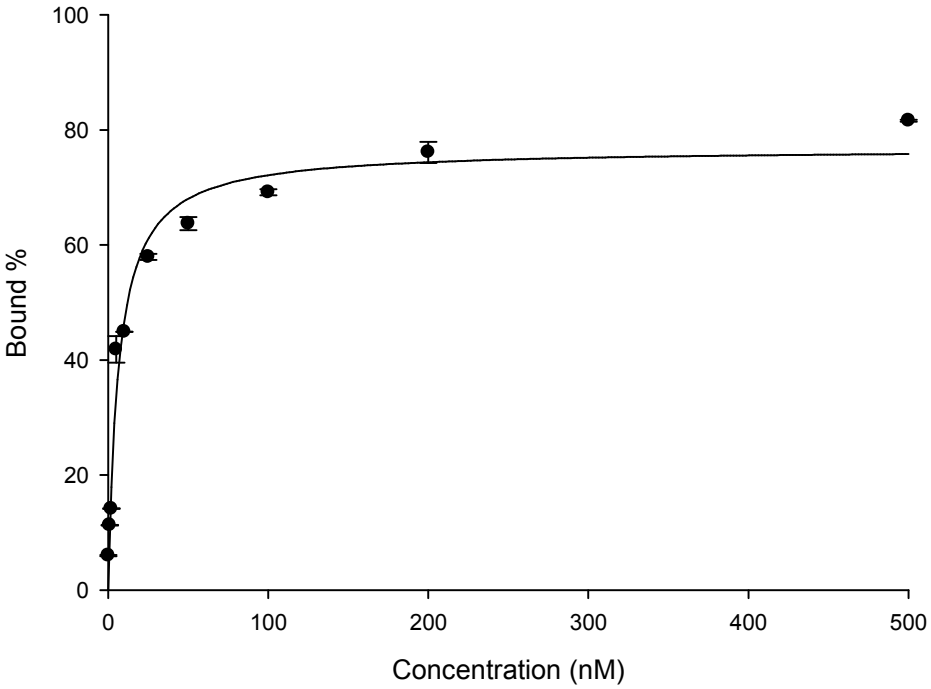

Figure S2. Binding curve of B-TTP-**85B** aptamer with fibrinogen

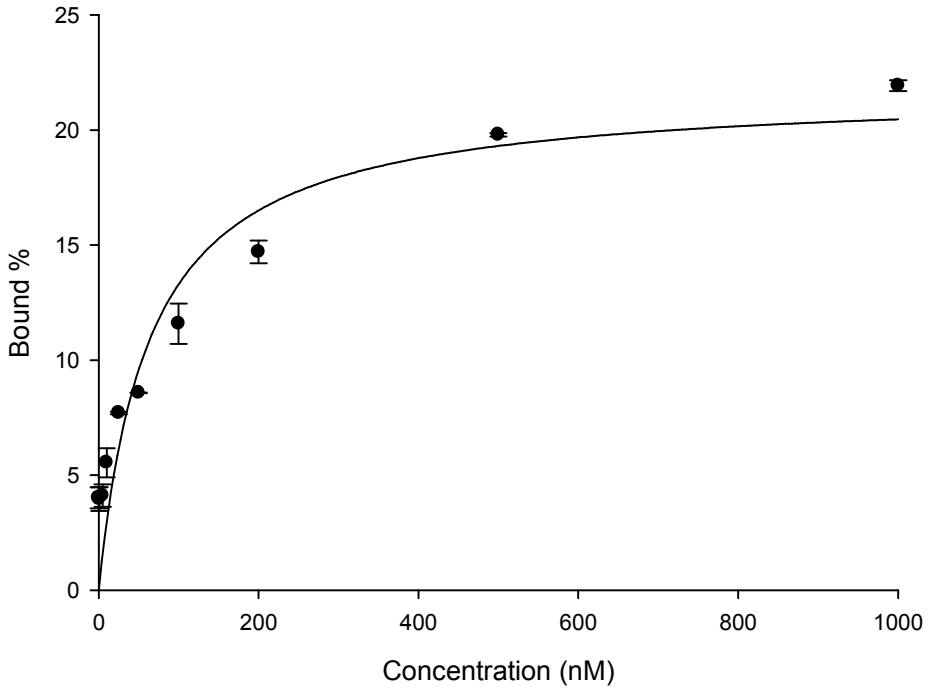

Figure S3. Binding curve of TTP-**85B** aptamer with fibrinogen

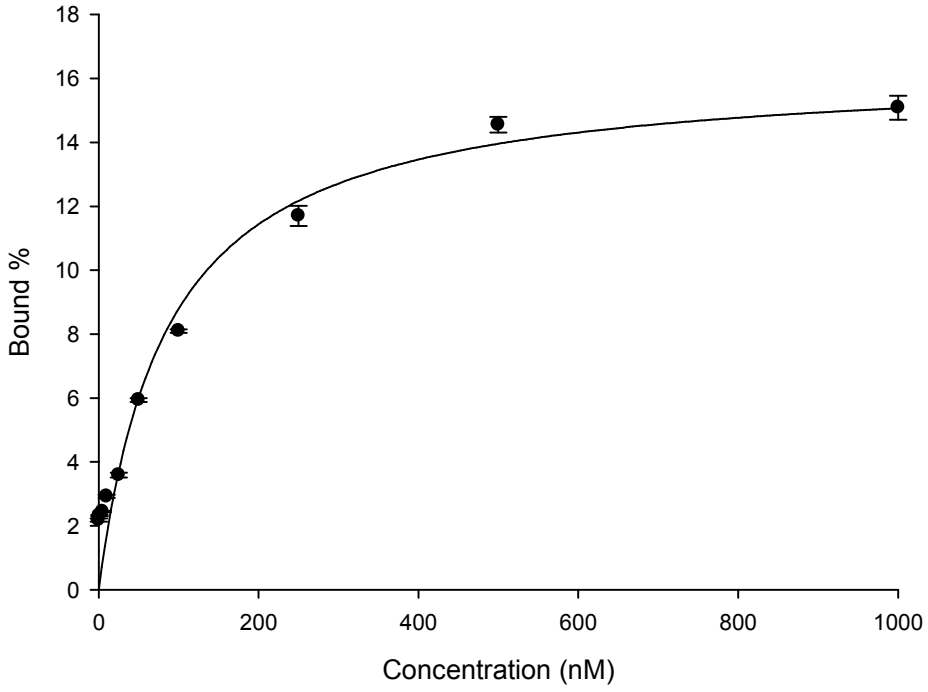

Figure S4. Binding curve of B-TTP-**85B** aptamer with deglycosylated fibrinogen

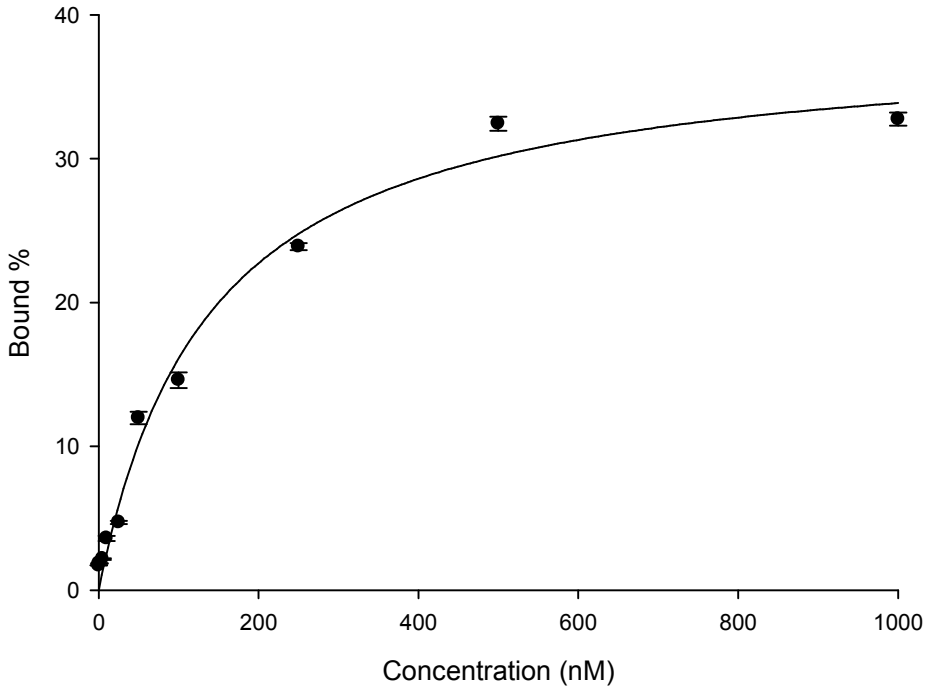

Figure S5. Binding curve of TTP-**85B** aptamer with deglycosylated fibrinogen

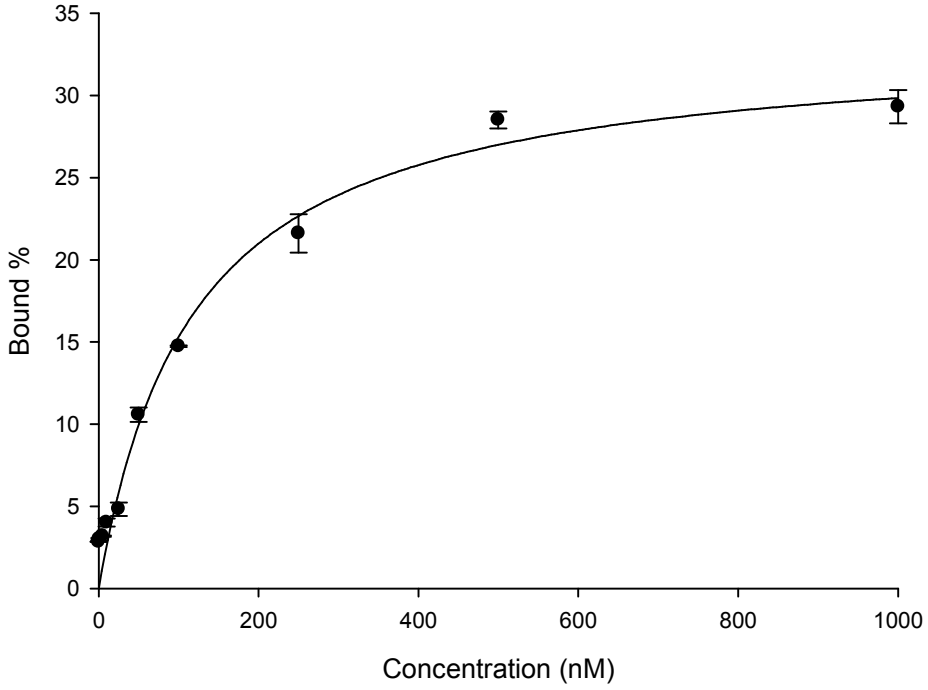

Figure S6. Binding curve of B-TTP-**85B** aptamer with per-iodated fibrinogen

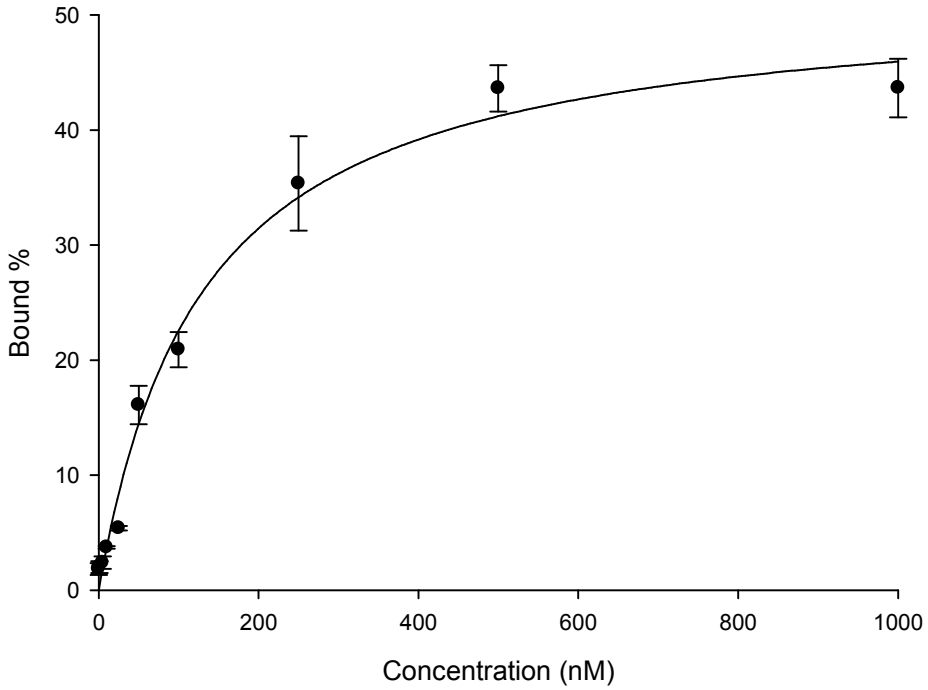

Figure S7. Binding curve of TTP-**85B** aptamer with per-iodated fibrinogen

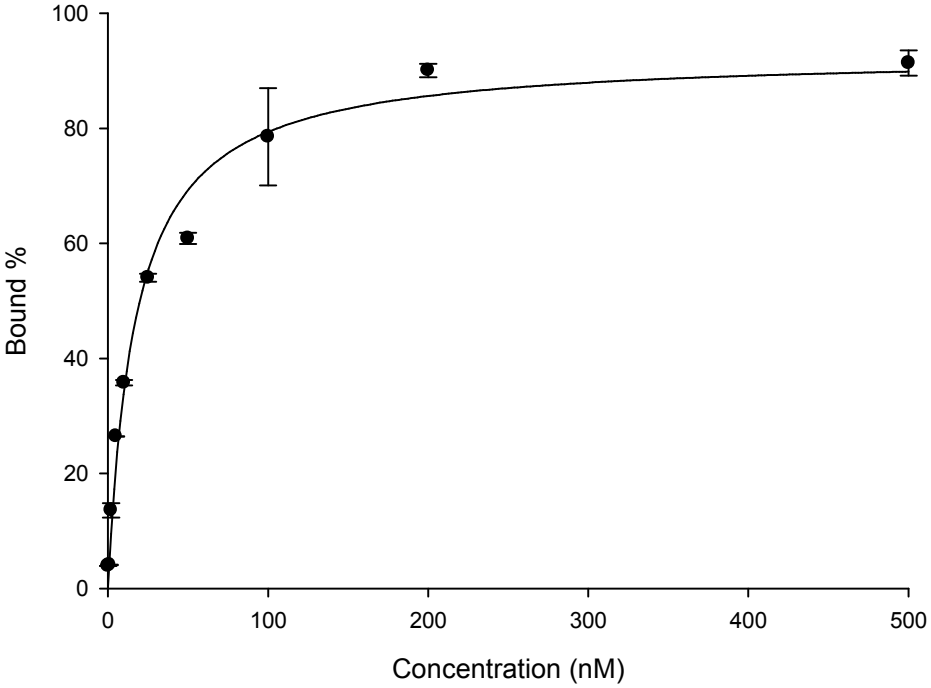

Figure S8. Binding curve of B-TTP-**85C** aptamer with fibrinogen

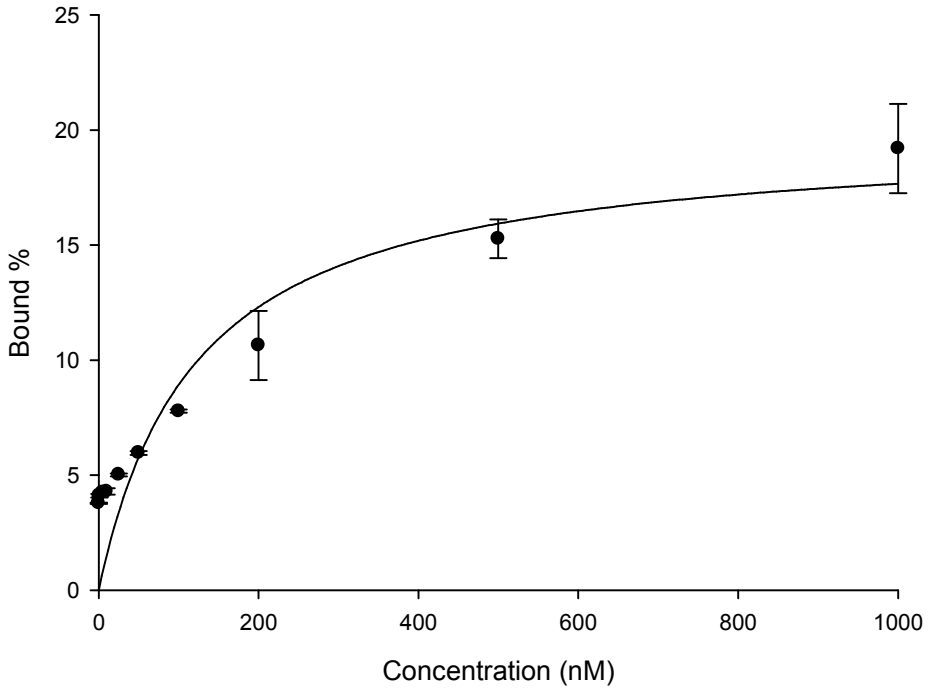

Figure S9. Binding curve of TTP-**85C** aptamer with fibrinogen

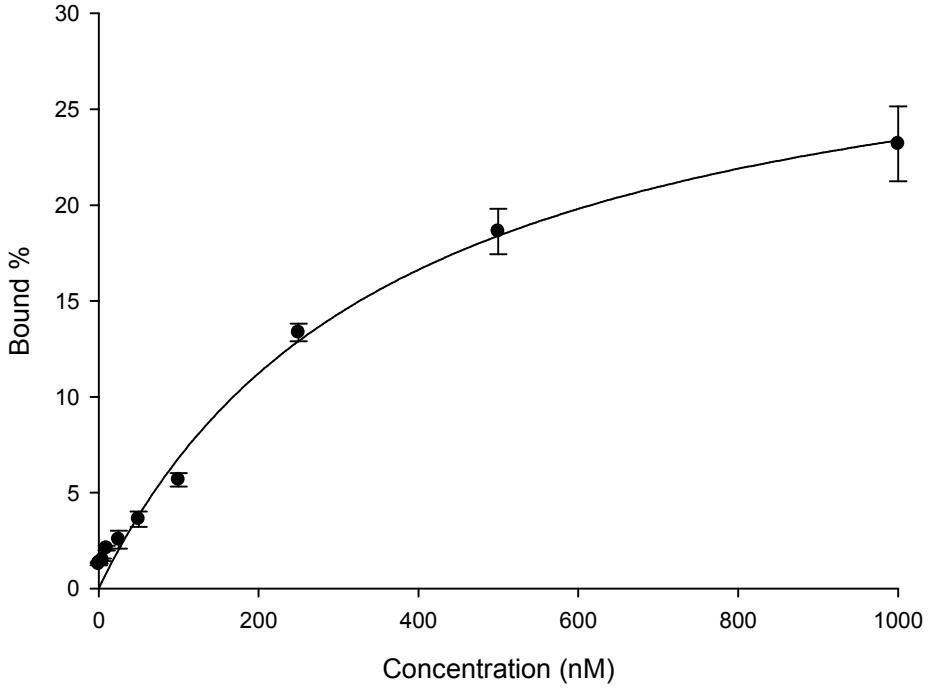

Figure S10. Binding curve of B-TTP-**85C** aptamer with deglycosylated fibrinogen

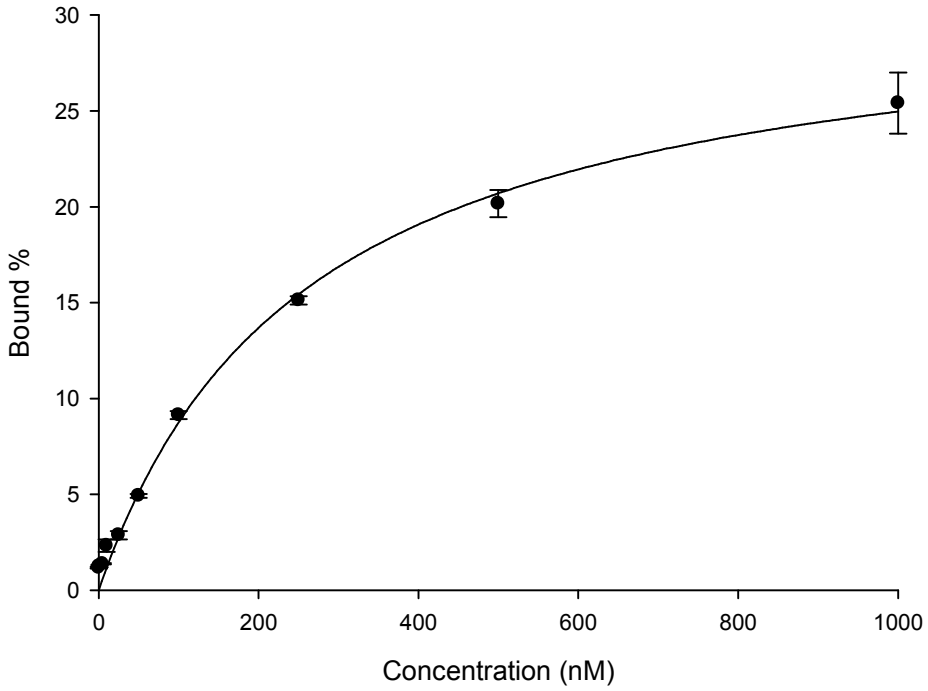

Figure S11. Binding curve of TTP-**85C** aptamer with deglycosylated fibrinogen

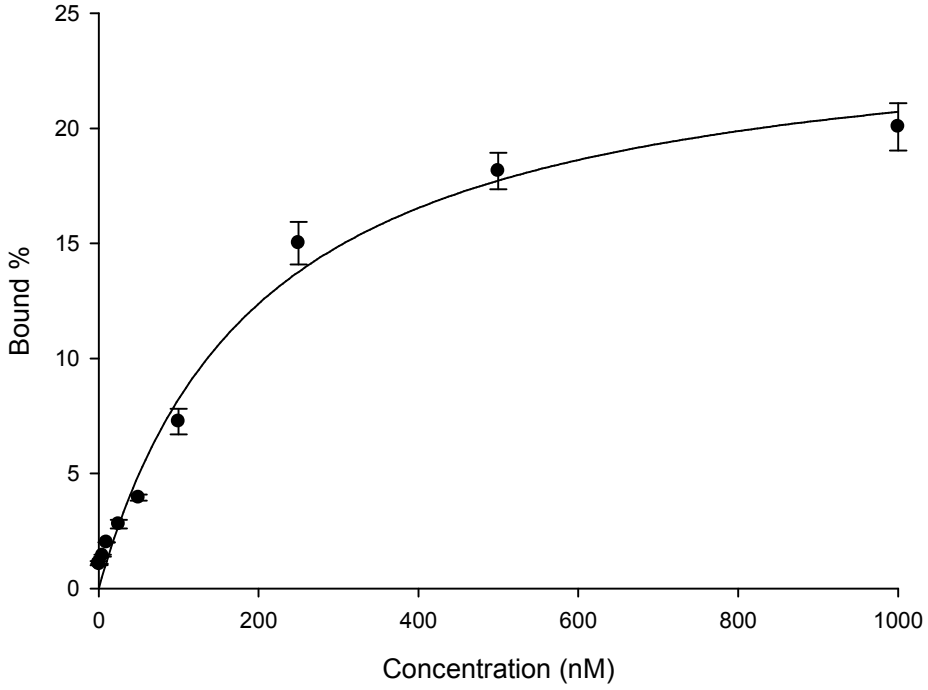

Figure S12. Binding curve of B-TTP-**85C** aptamer with per-iodated fibrinogen

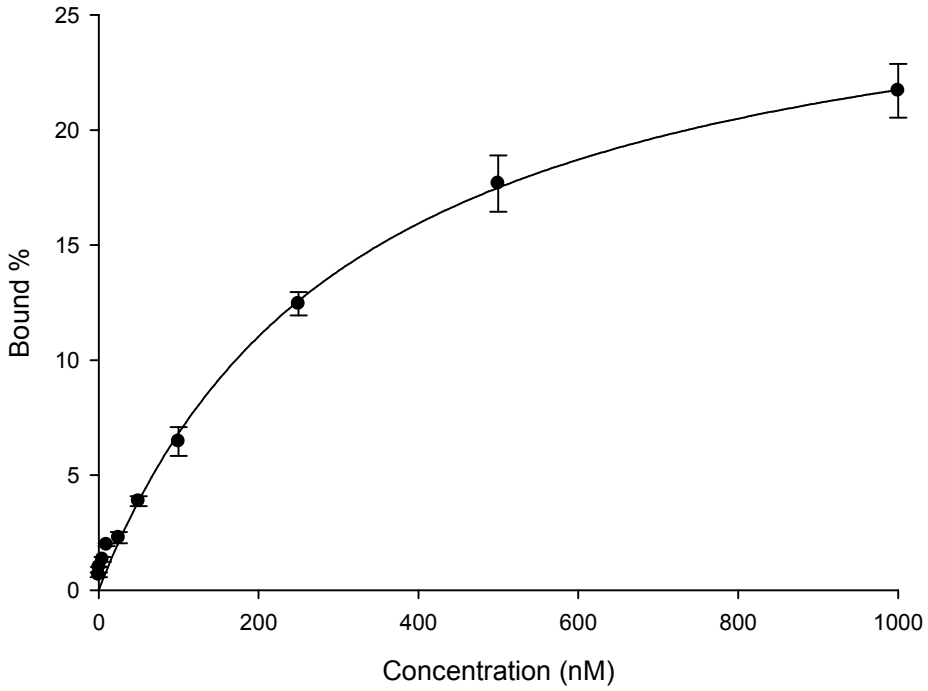

Figure S13. Binding curve of **TTP-85C** aptamer with per-iodated fibrinogen

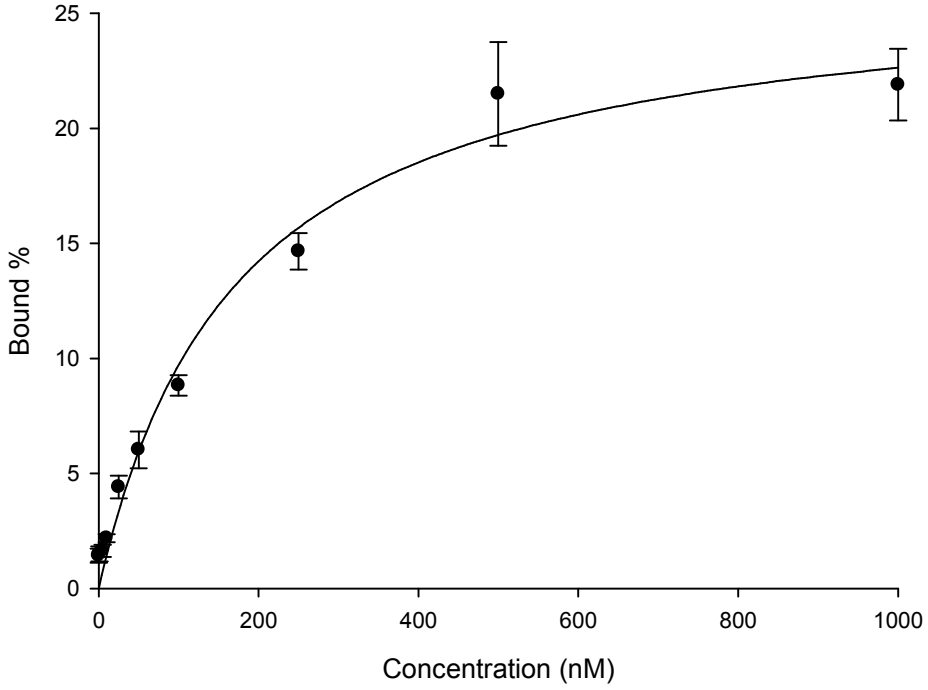

Figure S14. Binding curve of peroxidated B-TTP-**85A** aptamer with fibrinogen

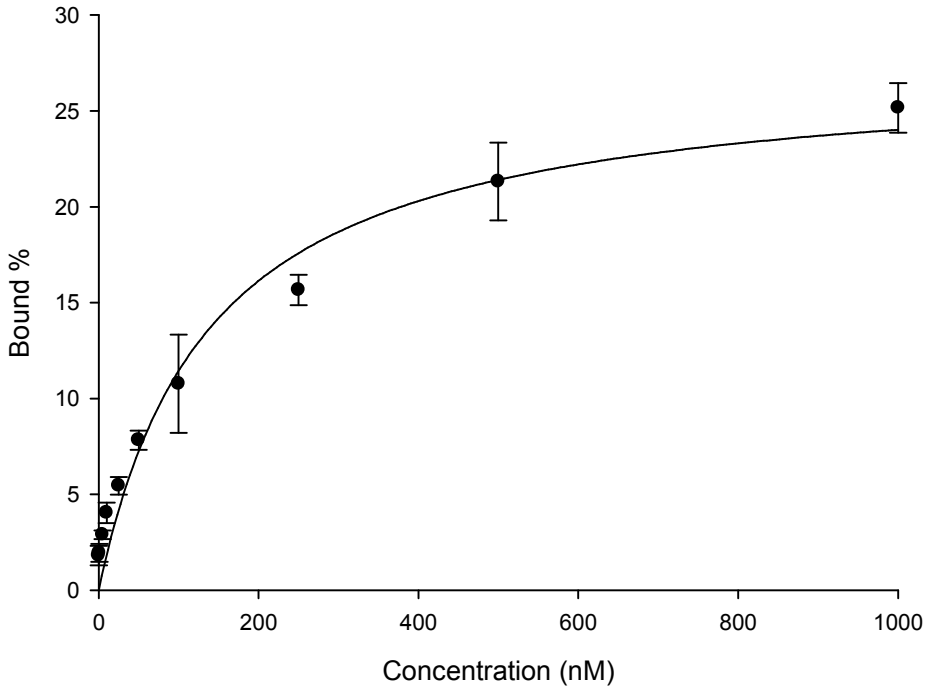

Figure S15. Binding curve of peroxidated TTP-**85A** aptamer with fibrinogen

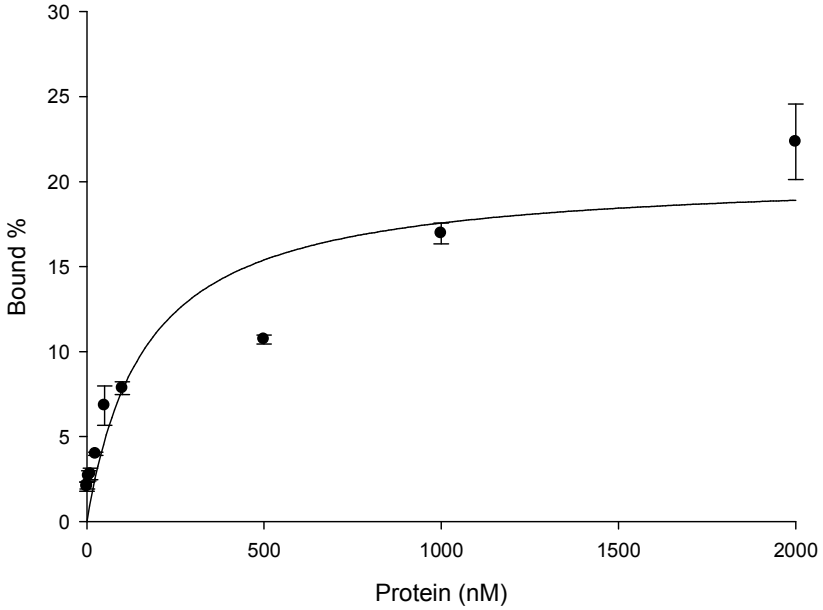

Figure S16. Binding curve of B-TTP-**85A** aptamer with desialylated fibrinogen

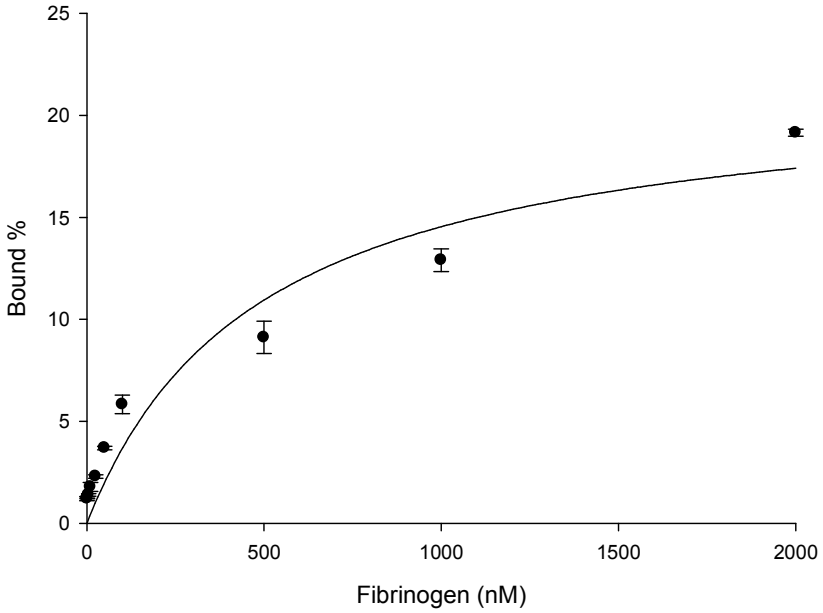

Figure S17. Binding curve of B-TTP-**85A** aptamer with fibrinogen in the presence of 50 mM fructose

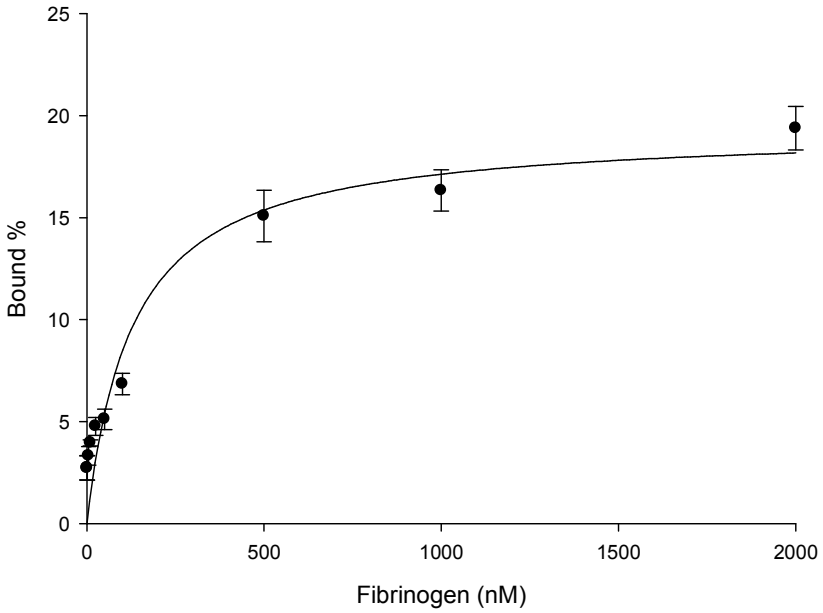

Figure S18. Binding curve of B-TTP-**85A** aptamer with fibrinogen in the presence of 1 mM 4-IQBA

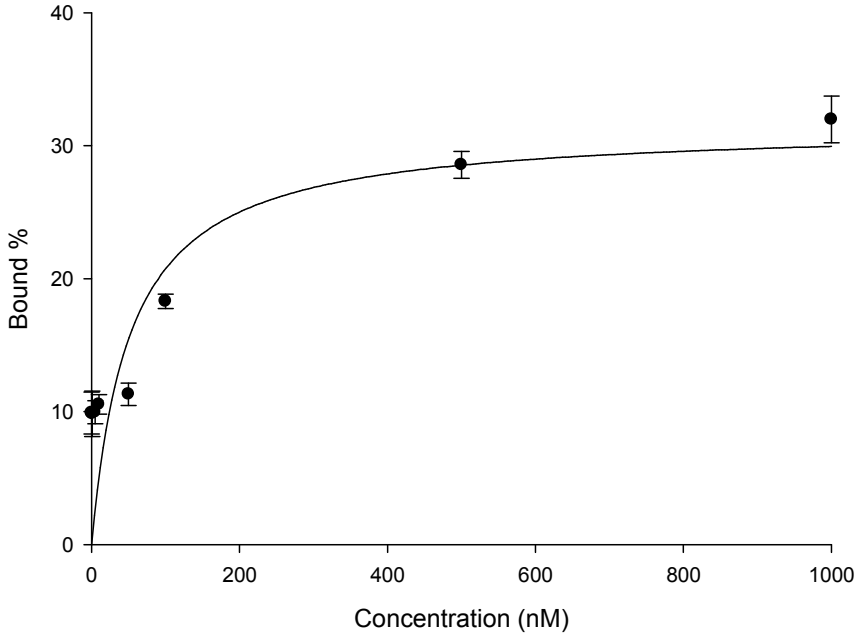

Figure S19. Binding curve of B-TTP-**85A** aptamer with fibrinogen in the presence of 100 nM glycan from fibrinogen

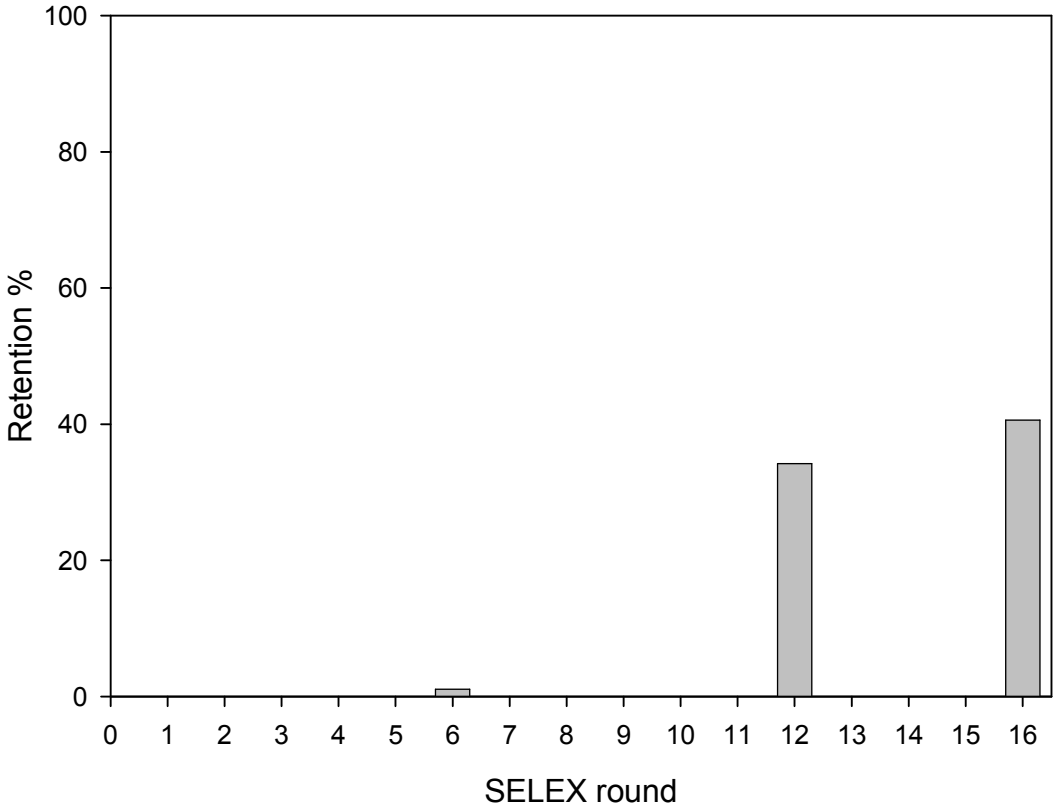

Figure 20. Retention of radioactive DNA on fibrinogen-immobilized beads during 16 rounds of TTP-based selection

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                10        20        30        40        50        60        70        80        90
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121A  CCTTCGTTGTCTGCCTTCGTCGATCGGCATTACGCCATTGGTTGGTGTGGTTGCGATTATGCGCTAATTCACCCTTCAGAATTCGCACCA
121B  CCTTCGTTGTCTGCCTTCGTTAATCGGCTCGATTGATTCGAGGTGACGGTCGTTGGACGTGACGTTACGCACCCTTCAGAATTCGCACCA
121C  CCTTCGTTGTCTGCCTTCGTCGTATTATCGTAGCCCGTGATGCTGACAACGTGACGTGAACGGATTGACGACCCTTCAGAATTCGCACCA
121D  CCTTCGTTGTCTGCCTTCGTTATGGAACGTGATCGTGCCGTGATGCCCTGATGCTGAGCGGATGCCGAATACCCTTCAGAATTCGCACCA
121E  CCTTCGTTGTCTGCCTTCGTCTCTTATCTTAGGCTAGGCTGATGCTGGACCAGTGACGGCTTAGCTGATGACCCTTCAGAATTCGCACCA
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121G  CCTTCGTTGTCTGCCTTCGTCGTCGTAGTGATTAACGTAGAATGCCAAAGCTGATCGTGCAATGCTGTACACCCTTCAGAATTCGCACCA
121H  CCTTCGTTGTCTGCCTTCGTATCCCGGATCGATTAATCGTGATCGTAGGCTTATGCAAGCTGCCTAGCCGACCCTTCAGAATTCGCACCA
121I  CCTTCGTTGTCTGCCTTCGTTAATCTTAGCTGGGATCGTAGCTGATTAGCTGCTAGTAGCGTAGCTGACGACCCTTCAGAATTCGCACCA
121J  CCTTCGTTGTCTGCCTTCGTATCTCGGTAGCTGATCGCTGATGTCTGATCTGAAGCTCGATCGGGCTAGCACCCTTCAGAATTCGCACCA
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Figure S21. The sequences for 10 aptamers from TTP-based SELEX

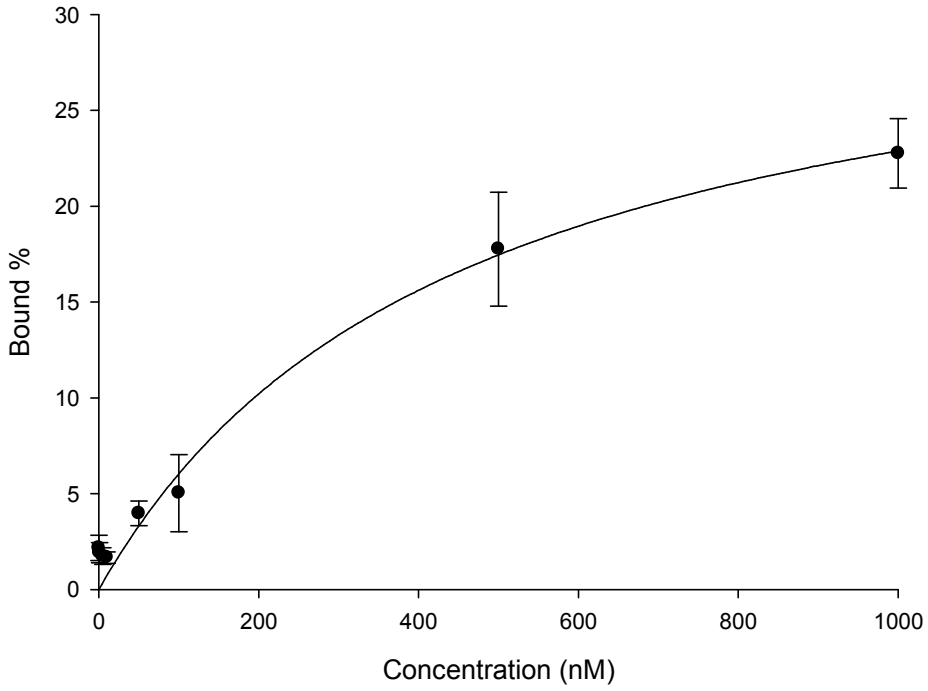


Figure S22. Binding curve of TTP-**121A** aptamer with fibrinogen

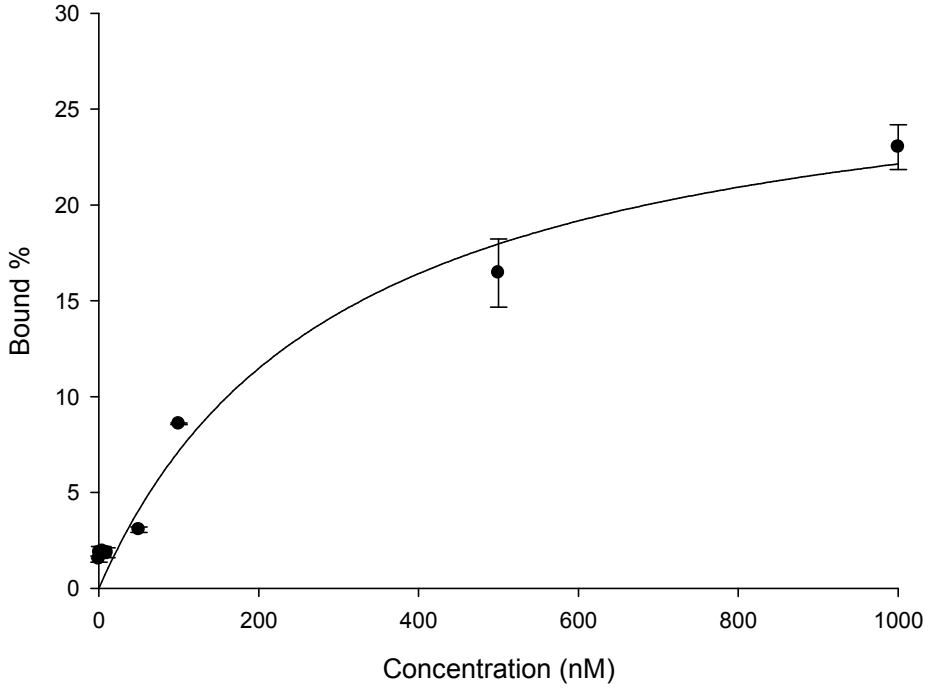


Figure S23. Binding curve of B-TTP-**121A** aptamer with fibrinogen

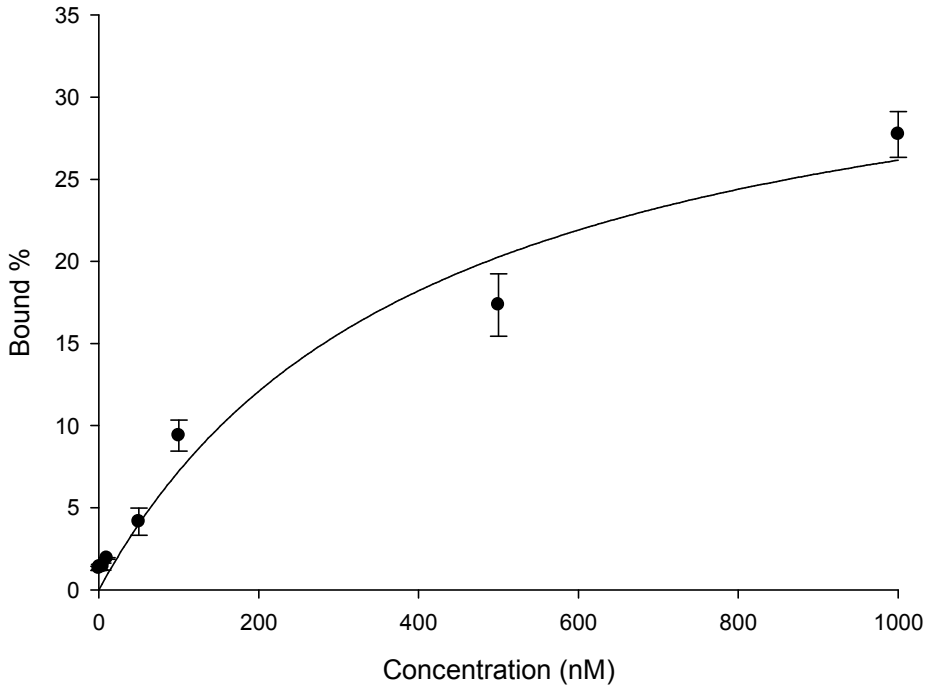


Figure S24. Binding curve of TTP-**121A** aptamer with deglycosylated fibrinogen

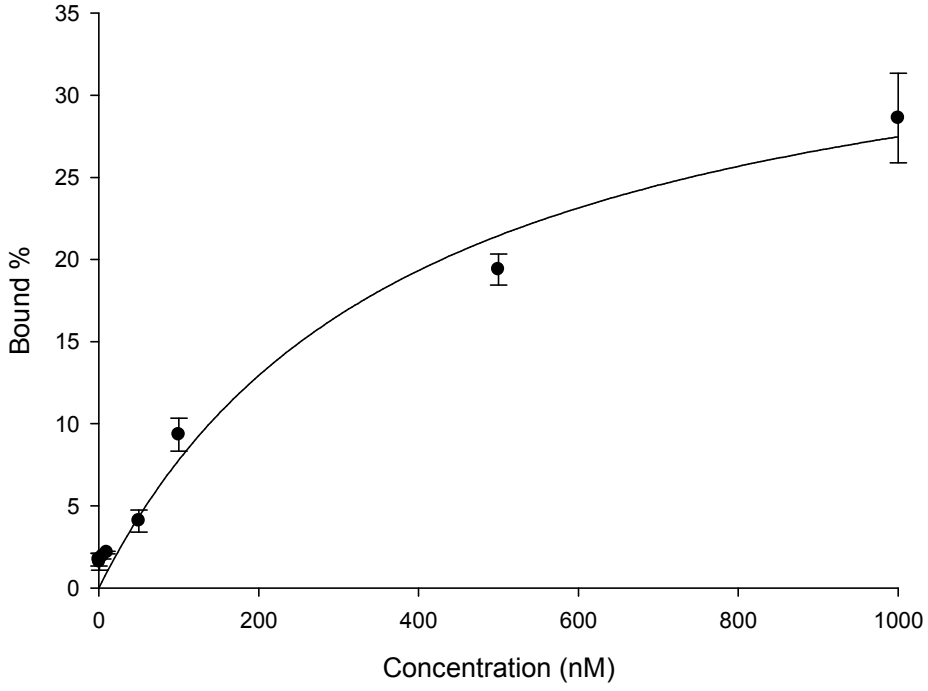
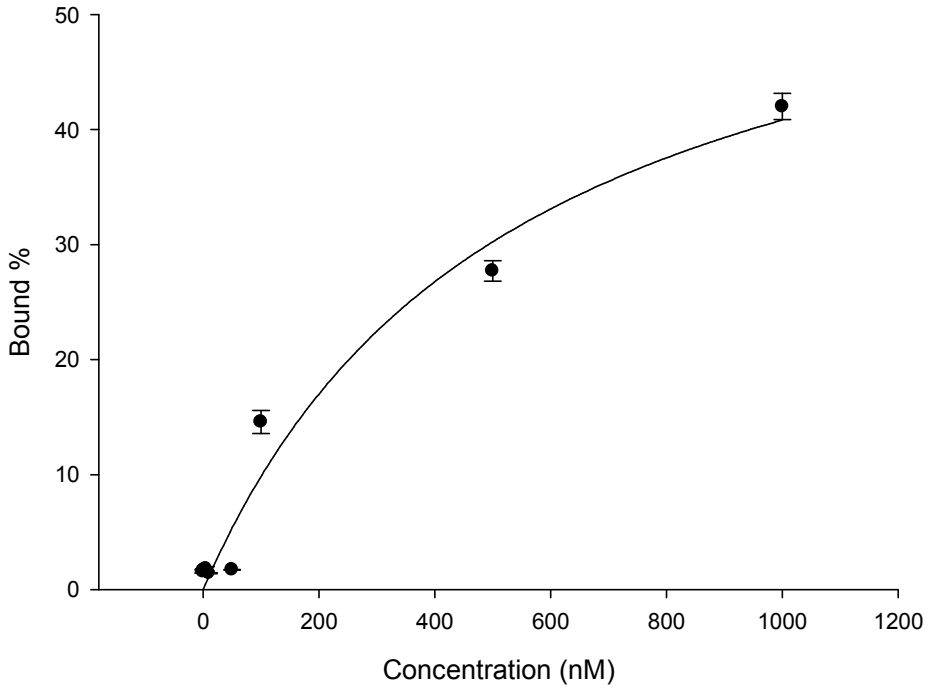
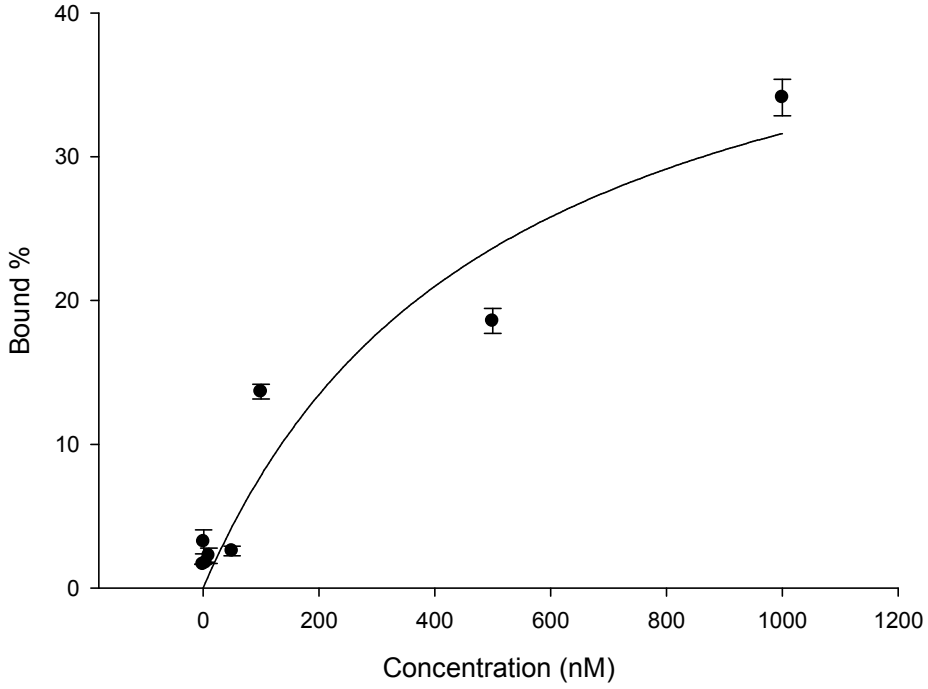


Figure S25. Binding curve of B-TTP-**121A** aptamer with deglycosylated fibrinogen



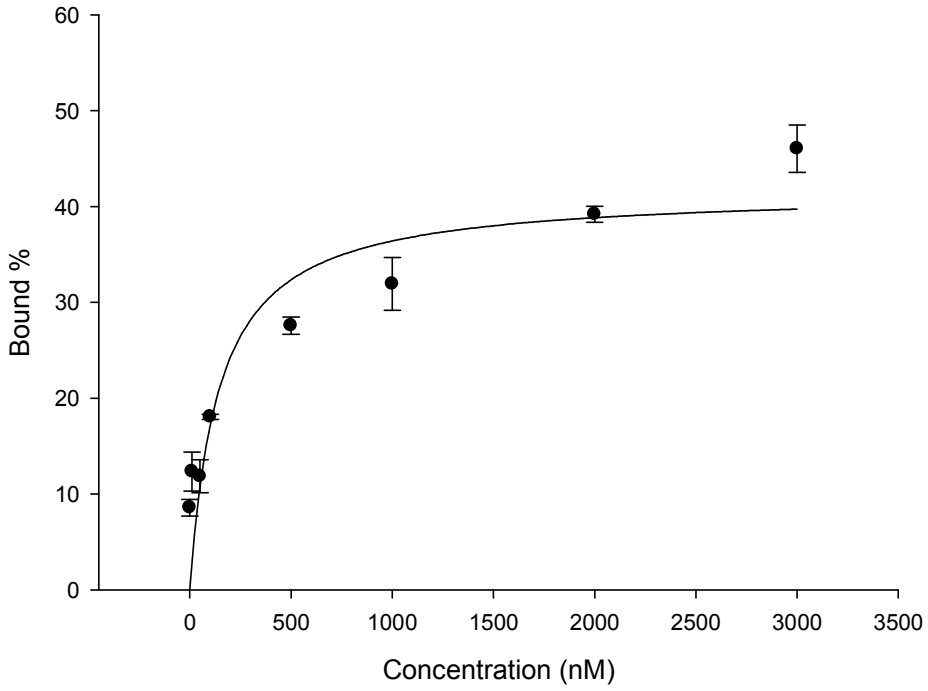
Kd = 540 ± 160 nM

Figure S26. Binding curve of TTP-**121B** aptamer with fibrinogen



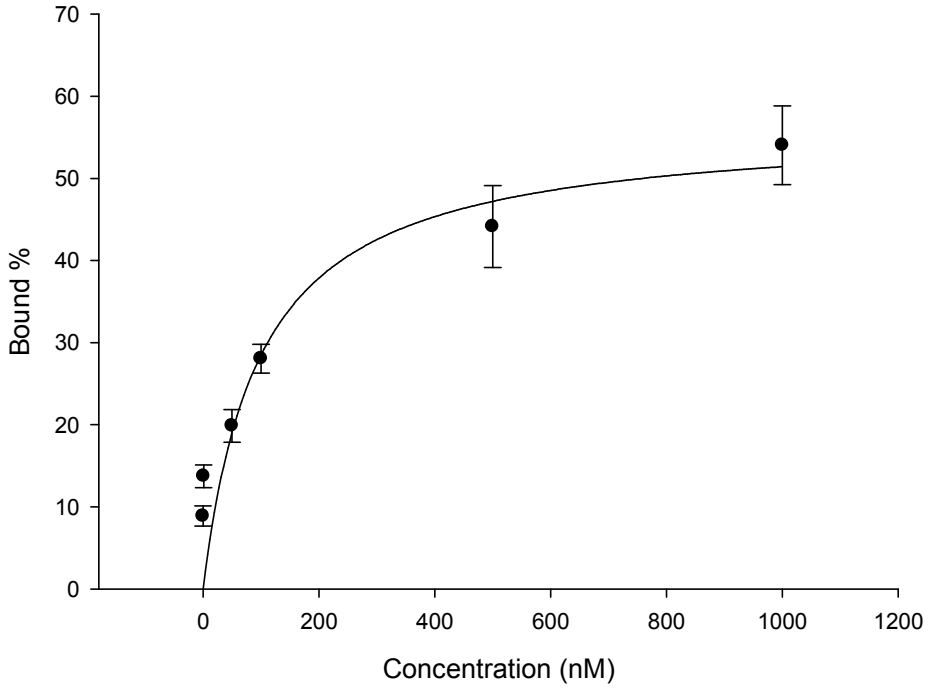
Kd = 500 ± 150 nM

Figure S27. Binding curve of B-TTP-**121B** aptamer with fibrinogen



Kd = 250 ± 50 nM

Figure S28. Binding curve of TTP-**121B** aptamer with deglycosylated fibrinogen



Kd = 200 ± 40 nM

Figure S29. Binding curve of B-TTP-**121B** aptamer with deglycosylated fibrinogen

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