Supporting information

Direct Selection Strategy for Isolating Aptamers with pH-sensitive Binding Activity

Chelsea K. L. Gordon[1,2], Michael Eisenstein[1,2], Hyongsok Tom Soh[1,2,3*]

1. Department of Radiology, School of Medicine, 2. Department of Electrical Engineering, Stanford University, Stanford, CA 94305, United States 3. Chan Zuckerberg Biohub, San Francisco, CA 94158, United States

* Corresponding author: tsoh@stanford.edu

METHODS

1. DNA sequences

Oligos were purchased from IDT with standard desalting. Primers and fluorescently labeled sequences were ordered with HPLC purification. The DNA sequences used in this study are shown in **Table S-1**.

2. Creation of monoclonal aptamer library

Forward primer (FP)-conjugated magnetic particles and monoclonal aptamer particles were made as described previously.[19] 500 µL of MyOne carboxylic acid magnetic particles (10^7 /µL) (Life Technologies) were washed once with 500 µL of 10 mM NaOH (Sigma-Aldrich) and three times with 1 mL nuclease-free water (Ambion), then resuspended in a 150 µL reaction of 200 mM NaCl (Teknova), 0.2 mM 5'-amino-modified FP (5'-amino-PEG18-ATA CCA GCT TAT TCA ATT-3'), 1 mM imidazole (Sigma-Aldrich), 50% dimethyl sulfoxide (DMSO) (ThermoFisher Scientific) and 250 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma-Aldrich). Particles were vortexed, sonicated and incubated overnight at room temperature

(RT) on a rotator. After FP coupling, the bead surfaces were passivated with PEG12. The remaining carboxyls on the particles were activated into amino-reactive NHS-ester in the presence of 250 mM EDC and 100 mM N-hydroxysuccinimide (NHS) in 100 mM 2-(N-morpholino)ethanesulfonic acid (MES; pH 4.7) (Sigma-Aldrich) for 30 minutes at RT, followed by conjugation with 20 mM amino-PEG12 (ThermoFisher Scientific) in MES buffer for one hour. The particles were washed four times with 500 µL of TE buffer [10 mM Tris-HCl, pH 7.5 (ThermoFisher Scientific); 0.1 mM EDTA (Ambion)], suspended in 500 µL of TE buffer and stored at 4°C. To test the coating of the FP particles, we incubated 1 µM Alexa Fluor 647-labeled FP complementary sequence with 0.2 µL FP particles in 100 µL of 10 mM Tris-HCl, pH 7.5 at room temperature for 20 minutes. The particles were washed twice with 100 µL 10 mM Tris-HCl, pH 7.5, vortexed, and sonicated for several seconds. The fluorescence intensity of the particles was measured using a BD Accuri C6 Flow Cytometer.

Monoclonal aptamer particles were generated by emulsion PCR. The oil phase was made up of 4.5% Span 80, 0.45% Tween 80, and 0.05% Triton X-100 in mineral oil, with all reagents purchased from Sigma-Aldrich. The aqueous phase consisted of 1x GoTaq PCR Master mix (Promega), 0.25 U/µL GoTaq polymerase (Promega), 25 mM $MgCl_2$, 0.2 mM of each dNTP (Promega), 40 nM FP, 3 µM fluorescently labeled reverse primer (RP), ~1 pM template DNA, and ~10^8 FP-conjugated magnetic beads in a total volume of 1 mL. For each reaction, 1 mL of aqueous phase was added to 7 mL of oil phase and emulsified at 620 rpm for 5 min in an IKA DT-20 tube using the IKA Ultra-Turrax device. The emulsion was pipetted into 100 µL reactions in a 96-well plate. The following PCR conditions were used: 95 °C, 2 min + [94 °C, 15s + 46 °C, 30s + 72 °C, 60s]*40 + 72 °C, 5 min.

After PCR, the emulsions were collected into an emulsion collection tray (Life Technologies) by centrifuging at 500 x g for 2 min. The emulsion was broken by adding 10 mL 2-butanol (Alfa Aesar) to the tray, and the sample was transferred to a 50 mL tube. The particles were centrifuged at 3,000 x g for 5 min. The oil phase was carefully removed, and the particles were resuspended in 1 mL of emulsion breaking buffer (100 mM NaCl, 1% Triton X-100, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) and transferred to a new 1.5 mL tube. The particles were centrifuged at 15,000 × g for 90 sec, and the supernatant was removed. The tube was placed on a magnetic separator (MPC-S, Life Technologies), and the remaining supernatant was removed. The particles were resuspended in 200 µL PBST [1x PBS (ThermoFisher Scientific), 0.01% Tween 20 (Sigma-Aldrich)] and moved to a new 1.5 mL tube. The coating of the particles was measured by adding 0.5 µL of the particle preparation to 99.5 µL 10 mM Tris-HCl, pH 7.5. The sample was analyzed using the Accuri C6 to confirm that 10–30% of the particles were coated with DNA, ensuring that close to all of the particles are monoclonal; this is based on the Poisson distribution, which predicts that most particles will be monoclonal when <35% of particles contain PCR products. The supernatant was removed from the remaining particles, and these were then resuspended in 200 µL 100 mM NaOH and incubated at RT for five minutes to generate single-stranded DNA. The particles were washed three times with PBST buffer using magnetic separation, then stored in 200 µL PBST at 4 °C.

3. Screen conditions

The selection buffer was PBSMCT, pH 7.4 [1x PBS, 2.5 mM $MgCl_2$ (Teknova), 1 mM $CaCl_2$ (Teknova), 0.01% Tween 20]. PBSMCT, pH 5.2 was prepared by titrating in 1M HCl (Sigma-Aldrich). Before each round of sorting, a binding assay was performed. The aptamer particles were annealed in selection buffer in the thermocycler using the following conditions:

95°C for 5 min, 5% ramp down, 4 °C for 5 min. The aptamer particles were incubated with 50-500 nM streptavidin Alexa Fluor 488 (SA-AF488) (ThermoFisher Scientific) in a final volume of 100 µL in both for 1 hour at room temperature on a rotator in the dark. Two samples were prepared at each concentration, one in pH 7.4 selection buffer and one in pH 5.2 selection buffer. After the incubation, the particles were washed once with selection buffer and resuspended in 100 µL selection buffer. The mean fluorescence intensity of each sample was measured using a BD Accuri C6 flow cytometer.

For each round of sorting, the aptamer particles were annealed in selection buffer in the thermocycler using the same conditions as above. For all three rounds, the aptamer particles were incubated with 200 nM SA-AF488 in pH 7.4 selection buffer in a final volume of 1 mL for either 1.5 hours at room temperature or overnight at 4 °C on a rotator in the dark. After incubation, the particles were washed once with 1 mL selection buffer and resuspended in 1 mL selection buffer, pH 7.4. The sample was sonicated for a few seconds and then measured on a flow cytometer (BD FACSAria III). Aptamer particles with high fluorescence intensity were collected in each round (515,000 events in the first round, 65,000 events in the second round, and 18,000 events in the third round). For the first round of positive selection at pH 7.4, all aptamer particles with fluorescence intensity above background were collected. The stringency was increased in the second round; the sort gate was shifted to the right, so that only the top 10% of aptamer particles were collected. In the first two rounds, after positive selection at pH 7.4, the particles were resuspended in 200 nM SA-AF488 in pH 5.2 selection buffer in a final volume of 250-500 µL, and incubated on a rotator in the dark at room temperature for 30 min. The particles were washed once and resuspended in 1 mL selection buffer, pH 5.2. The sample was sonicated and then measured on the flow cytometer, and all non-fluorescent beads were collected in order to eliminate

any aptamer particles that bound SA-AF488 at pH 5.2. 40,000 and 3,000 events were collected in the first and second rounds of negative selection at pH 5.2, respectively. The collected aptamer particles were then amplified to regenerate the aptamer pool for the following round of positive selection. In the third round of positive selection at pH 7.4, binding to the target was lower, so all particles with fluorescence above the background were collected. The collected particles were transferred to a 1.5 mL tube and placed on a magnetic rack for 5 min and the supernatant was removed. A pH 5.2 sort was not performed for the third round, and the aptamer particles were amplified immediately after screening.

4. Next generation sequencing of the enriched aptamer pools

The aptamer pools from all three rounds were indexed and prepared for sequencing according to the *16S Metagenomic Sequencing Library Preparation* guide by Illumina, using the Nextera XT Index Kit. Adaptor sequences were ordered from IDT. Three compatible sets of indexes were chosen based on guidelines from Illumina (N706+S503, N710+S503, N710+S504). Each index pair was added to one of the aptamer pools by PCR. NGS was performed using an Illumina MiSeq system at the Stanford Functional Genomics Facility. The resulting data was filtered to remove low-quality sequences (Q score < 20 for more than 10% of the bases) using Galaxy.[1] The FASTAptamer toolkit was used to calculate the copy number and enrichment of each sequence.[2] Cluster analysis was also performed to identify families of sequences (within an edit distance of 3).

5. Screening aptamer candidates

The ten candidate sequences shown in **Table S1** were ordered from IDT. Aptamer particles were generated for each sequence using bead PCR for use in fluorescence assays to measure

binding of each aptamer to streptavidin. Each 100 µL reaction contained 1 µL beads, a final concentration of 50 nM streptavidin-phycoerythrin (SA-PE) (Invitrogen), and either pH 7.4 or pH 5.2 selection buffer. A different streptavidin-fluorophore conjugate (SA-PE) was used to characterize the binding than was used during the screen (SA-AF488). This ensures the aptamer was not interacting with the Alexa Fluor 488. Each sample was incubated on a rotator at RT in the dark for 1 hour. Samples were washed once with 100 µL selection buffer using a magnetic rack and resuspended in 100 µL selection buffer, with the same pH as used during the incubation. Samples were vortexed and then sonicated for a few seconds. The fluorescence intensity of each sample was measured by analyzing 10,000 particles using a BD Accuri C6.

6. Binding affinity measurement of pH-switching aptamers

Binding curves were generated using bead-based fluorescence assays for aptamer candidates S3 and S8 and for the original aptamer, SBA29. Each 100 µL reaction contained 1 µL beads, varying volumes of SA-PE (final concentration 250 pM–1 µM), and either pH 7.4 or pH 5.2 selection buffer, with all reactions performed in duplicate or triplicate. Each sample was incubated on a rotator at RT in the dark for 1 hour. Samples were washed once with 100 µL selection buffer using a magnetic rack and resuspended in 100 µL selection buffer. The fluorescence intensity of each sample was measured by analyzing 10,000 particles using a BD Accuri C6. K_d was determined based on a saturation binding model (one site-total binding) using GraphPad Prism 7. Binding to forward primer-conjugated beads was measured using the same procedure, at concentrations of 0 nM and 50 nM SA-PE. The same procedure was also used to measure the binding of aptamer S8 to 50 nM SA-PE in selection buffers with a range of pH values. Selection buffers with pH values of pH 5.2, 5.6, 5.8, 6.2, 6.6, 6.9, and 7.4 were prepared by titrating in 1M HCl.

Microscale thermophoresis was performed by 2bind to measure the binding affinity of S8 and SBA29 to streptavidin at pH 7.4 and pH 5.2. For each experiment, a serial dilution of streptavidin was prepared (final concentration 61 pM–2 µM) and mixed with Cy5-labeled aptamer (final concentration held constant at 5 nM). The samples were analyzed on a Monolith NT.115 Pico at 25 °C, with 5% LED power and 60% laser power.

7. Mutation study to identify pH active motif for aptamer S8

The predicted structures were generated using mfold, with salt conditions similar to the selection buffer (137 mM Na^+, 2.5 nM Mg^{2+}). S8 point mutants were ordered from IDT with PAGE purification. Bead PCR was performed to create monoclonal aptamer particles for each sequence. Bead-based fluorescent measurements were performed as previously described to test the binding of each sequence to streptavidin. The binding of each sequence to 50 nM SA-PE was measured in triplicate. The total volume of each sample was 100 µL: 1 µL beads, a final concentration of 50 nM SA-PE, and either pH 7.4 or pH 5.2 selection buffer. Each sample was incubated on a rotator at room temperature in the dark for 1 hour. Samples were washed once with 100 µL selection buffer using a magnetic rack and resuspended in 100 µL selection buffer. Samples were vortexed and then sonicated for a few seconds. The fluorescence intensity of each sample was measured by analyzing 10,000 particles using a BD Accuri C6.

Forward primer	ATACCAGCTTATTCAATT
Reverse primer	AGATTGCACTTACTATCT
Library	ATACCAGCTTATTCAATT**ATTGACCGCTGTGTGAC GCAACACTCAAT**-N20-AGATAGTAAGTGCAATCT
Aptamer candidates	
S1	TATTATGTCTTTTTTGTTTT
S2	ATTCCCATCTCATGACGTCG
S3	ACCTTCTCAACGTTCTCTGT
S4	CCCCTCTATCCGTCCGTCTG
S5	GTCCTCGTCCCGCAGACTAA
S6	ATATGCAGAAGCCTCCCCGT
S7	CGTCTAAGTAGAGATGGTCT
S8	TCTTGGATCTCGCTGCACAC
S9	ACATTGCGAAATGGTTCCCG
S10	AAATTCTGGCACTCTACCGT
S8 mutants	
G61	TCTTGGATCTCGCGGCACAC
T62	TCTTGGATCTCGCTTCACAC
A61	TCTTGGATCTCGCAGCACAC
A62	TCTTGGATCTCGCTACACAC
C62	TCTTGGATCTCGCTCCACAC
A59	TCTTGGATCTCACTGCACAC
A65	TCTTGGATCTCGCTGCAAAC
A67	TCTTGGATCTCGCTGCACAA

Table S-1. Sequences of DNA sequences used in this study (5' to 3'). The SBA29 domain of the library is shown in bold. For the aptamer candidates and aptamer S8 mutants, primer binding

regions and the SBA29 domain are not shown. The mutated base for each S8 mutant sequence is shown in red.

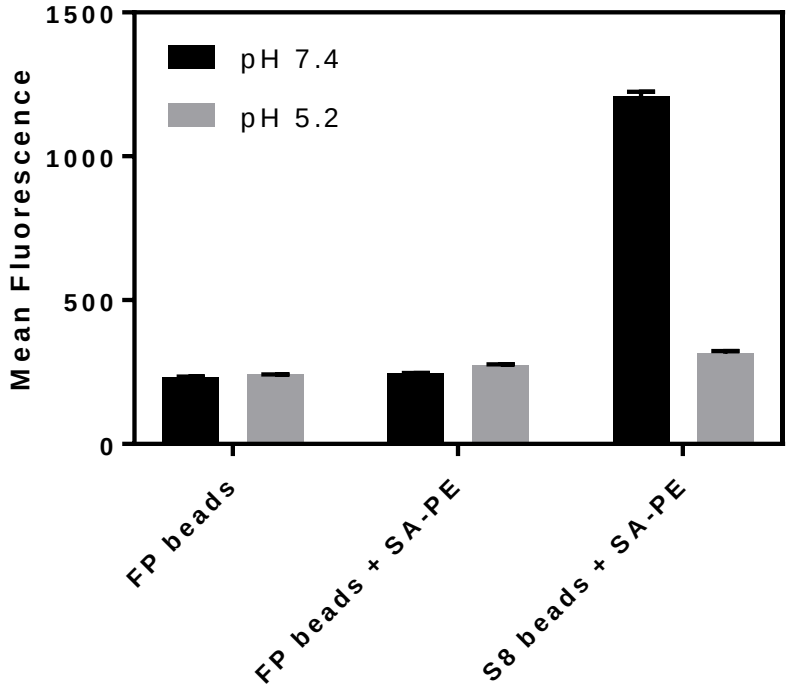

Figure S-1. Forward primer (FP)-coated beads show no difference in fluorescence in pH 7.4 and pH 5.2 selection buffer, either with or without 50 nM streptavidin-phycoerythrin (SA-PE). The mean + standard deviation for three experimental replicates is shown for each sample.

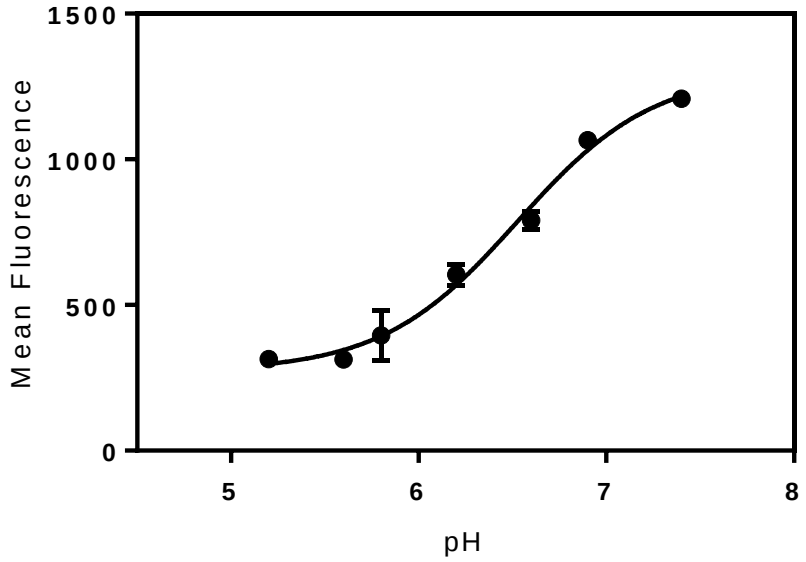

Figure S-2. Binding of aptamer S8 to 50 nM SA-PE over a range of pH values. Half-maximal binding occurs at pH 6.5. The mean ± standard deviation for three experimental replicates is shown for each pH.

References

1. Afgan E, Baker D, van den Beek M, Blankenberg D, Bouvier D, Cech M, Chilton J, Clements D, Coraor N, Eberhard C, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res*. 2016;44(W1):W3-W10. doi:10.1093/nar/gkw343.

2. Alam KK, Chang JL, Burke DH. FASTAptamer: A Bioinformatic Toolkit for High-throughput Sequence Analysis of Combinatorial Selections. *Mol Ther Nucleic Acids*. 2015;4:e230. doi: 10.1038/mtna.2015.4.