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Precipitation SELEX: Identification of DNA Aptamers for Calcium Phosphate Materials Synthesis

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Supporting Information

S1. Materials and Methods

Reagents. Sodium phosphate dibasic (>99.0%) and calcium chloride dehydrate (>99.5%) were sourced from Sigma-Aldrich. Tris/boric acid/EDTA buffer (TBE, 10x concentrated, electrophoresis grade) was sourced from Bio-Rad Laboratories. Sodium chloride (>99.5%) was sourced from Fluka. HotStarTaq MasterMix from Qiagen (No. 203443) was used for amplification. GelStar nucleic acid gel stain (10,000x) was sourced from Lonza Rockland Inc. Synthetic ssDNA strands were purchased from Integrated DNA Technologies and used after reconstitution in Tris buffer (10 mM trizma hydrochloride, 1 mM magnesium chloride, pH 7.3, filtered to 0.22μm). Synthesis and purity were confirmed by the vendor using mass spectrometry. Concentration was confirmed by UV absorbance. The following ssDNA sequence was used for the initial library: 5'-TCC CAC GCA TTC TCC ACA TCN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN CCT TTC TGT CCT TCC GTC AC-3'. Forward primer 5'-TCC CAC GCA TTC TCC ACA TC-3' and reverse primer 5'-GTG ACG GAA GGA CAG AAA GG-3' were sourced from Integrated DNA Technologies. Phosphorylated reverse primer 5'-Phos-GTG ACG GAA GGA CAG AAA GG-3' was sourced from Integrated DNA Technologies with HPLC purification. Lambda Exonuclease and buffer (B0262S, 10x, pH 9.4) was sourced from New England BioLabs.

Precipitation SELEX. In preparation for precipitation, 5 pmol single-stranded DNA library (ssDNA) was annealed in a Bio-Rad T100™ Thermocycler (95°C for 2 min, 25°C for 30 s). Then, 200 μL phosphate buffer (8 mM, pH 7.40) and 200 μL calcium chloride solution (20 mM) were combined with sodium chloride, sterile water, and ssDNA in a 1.5 mL microcentrifuge tube for a total volume of 1.0 mL. The solution was allowed to react on a tube inverter for two hours. The tube was centrifuged at 13.4K rpm for 10 minutes and the supernatant was removed. A pellet of mineral and ssDNA remained and was washed in DI water 3-5 times. The pellet was dissolved in glycine buffer (0.1M, pH 2.26), mixed, and neutralized with 24.3μL of sodium hydroxide (1.0M). This precipitation protocol was repeated with increasing sodium chloride solution concentration ranging from 10 mM to 137 mM or with decreasing DNA library concentrations.

PCR Amplification. Amplification reactions (50 μL) were performed using a Bio-Rad Thermocycler (95°C for 15 min, 95°C for 30 s, 55°C for 30 s, 72°C for 10 s, steps 2-3 repeated 30x, 72°C for 10 min) with 25 μL of PCR MasterMix, 7 μL of sterile water, 4 μL of 5 μM forward primer, 4 μL of 5 μM phosphorylated reverse primer, and 10 μL precipitation sample in a PCR tube. After 24, 26, 28, and 30 cycles of PCR, 5 μL of sample was removed for analysis by 3% Bio-Rad PCR certified agarose gel electrophoresis. Samples (5 μL) were prepared with 1 μL loading dye and run alongside a 20 base-pair (bp) molecular weight marker (Bio-Rad). The gel was run with a Bio-Rad PowerPak source at 200V for 35 minutes in 1X TBE buffer solution. The gel was visualized via GelStar stain and imaged under trans-UV illumination with a Bio-Rad Universal Hood II 720BR. The minimal number of cycles with the most robust amplification of the 80 bp product was selected for further PCR. Thereafter, nine PCR reactions (50 μL each) were prepared as described above and run in order to amplify up the collected DNA aptamers. Any DNA that became degraded in the selection process would not be PCR amplified and would not be carried forward in the selection.

ssDNA Generation. Two tubes of PCR products (100 μL) were combined with 1 μL of Lambda Exonuclease and 10 μL of 10X lambda exonuclease buffer. The reaction was performed in a Bio-Rad Thermocycler at 35°C for 120 min, followed by 75°C for 10 min. The singled stranded digestion products were analyzed by agarose gel electrophoresis in the same manner as described above, and following visualization the band

corresponding to the 80-bp products were excised with a sterile scalpel and added to pre-weighed 1.5mL microcentrifuge tubes. A Promega Wizard® SV Gel and PCR Clean-up System (A9281) was then used to purify the ssDNA library.

Quantification. A Thermo Scientific NanoDrop Lite spectrophotometer was used with ssDNA library standard to measure the concentration of ssDNA library in the purified sample. The sample was used in the mineralization step of each subsequent round.

Cloning. The TOPO® TA Cloning® Kit (Life Technologies) was used to clone representative DNA aptamers. To do this the ssDNA was amplified and converted to dsDNA by PCR. A reaction of 25 µL Taq PCR MasterMix, 7 µL of sterile water, 4 µL of 5 µM forward primer, 4 µL of 5 µM reverse primer, and 10 µL of DNA from a precipitation reaction were combined in a PCR tube for a total volume of 50 µL. PCR was run in a Bio-Rad Thermal Cycler for 30 rounds (95°C for 15 min, 95°C for 30 s, 55°C for 30 s, 72°C for 10 s, steps 2-3 repeated 30x, 72°C for 30 min). PCR product (10 nM equivalent) was cloned as per the manufacturer's recommendations (Life Technologies). Next this cloned plasmids were transformed into One Shot® competent *E. coli* cells (Life Technologies). To do this the cells were thawed on ice and incubated with the plasmids for 30 minutes on ice and then immediately moved to a 42˚C water bath for 30 seconds before moving back to the ice bath. SOC medium (250 µL) was added to the *E. coli* cells and the reaction incubated for 60 minutes in a shaker at 200 rpm and 37˚C. These cells were plated on kanamycin (50 µg/mL) selective agar plates at 37˚C overnight.

Plasmid Purification and Sequencing. The Zuppy Plasmid miniprep Kit was used to isolate plasmids from single colonies grown overnight in 50 µg/mL kanamycin LB broth (Novagen). Following pelleting and resuspension in 600 µL sterile water, lysis buffer (100 µL) was added to each tube and allowed to react for exactly two minutes before 350 µL cold neutralization buffer was added. The tubes were centrifuged at 13.4K rpm for 5 minutes and the supernatant was removed with a microliter pipette and added to a plasmid prep column. The column was allowed to incubate for 1 minute at room temperature. The column was centrifuged at 13K rpm for 15 seconds. Endo wash buffer (400 µL) was added and the columns were centrifuged at 13K rpm for one minute. The collection tube was emptied and 350 µL of wash buffer was added before centrifugation for an additional minute. Elution buffer (25 µL) was used to elute the plasmids and DNA was quantified using a NanoDrop as described above. Further, plasmid integrity was evaluated by gel electrophoresis in a 1% agarose gel. Plasmids were submitted for sequencing (Dana-Farber/Harvard Cancer Center DNA Resource Core) with the M13R primer.

Mineralization Analysis. In preparation for mineralization, ssDNA was annealed in a Bio-Rad T100™ Thermocycler (95°C for 2 min, 25°C for 30 s). For experiments that included high ionic strength, the ssDNA was annealed in an equally high ionic strength solution. Then, 40 µL phosphate buffer (8 mM, pH 7.40), 10 µL of universal indicator (1:10 dilution from stock, Bogan Universal Indicator, Carolina Biological), the appropriate amount of ssDNA (all DNA aptamers were initially tested at 5, 50 and 500 nM concentrations), NaCl, and DI water to yield 160 µL were pre-mixed and added to a 96 well-plate. Calcium chloride (40 µL, 20 mM) was added to initiate the reaction. A positive control (using double the amount phosphate solution), a no-calcium control (DI water substituted for calcium chloride), and a no-DNA control were run on every plate for consistency, comparison, and blanking. Each sample was always tested in triplicate on the same plate. Each sample was blank subtracted (subtracting the no-calcium control) and standardized relative to the no-DNA control. A Molecular Devices SpectraMax 190 plate reader was used to measure

absorbance at 405 or 420 nm every 1-10 minutes for 30-240 min total. Non-linear polynomial fitting was done in Excel to highlight the data trends.

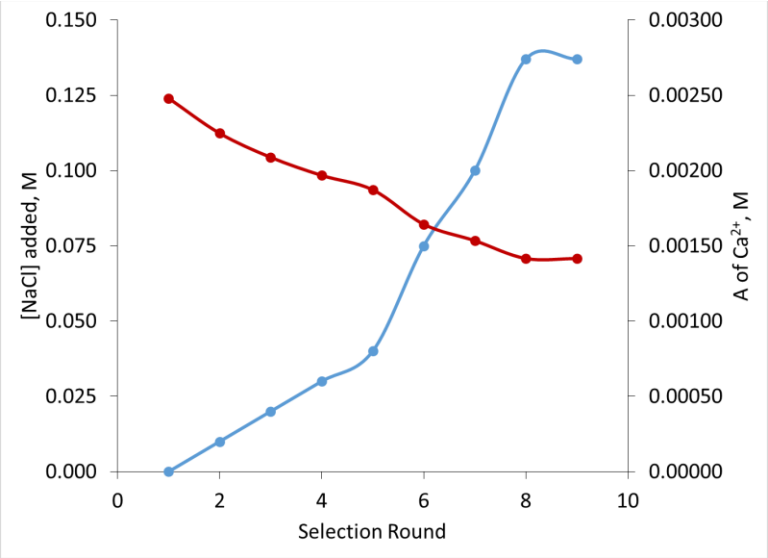
Analysis of DNA Entrapment. Mineralization experiments were prepared as described above, mimicking the first round of SELEX. A control that also included the initial library DNA was run with phosphate buffer at pH 6.0 instead of 7.4 to mimic conditions where no mineral pellet would form. The mineralized pellet, or lack thereof, was washed with DI water, dissolved in glycine buffer, and neutralized as above. A 10 μL sample was used in PCR as described above, completing 16 rounds amplification. Gel electrophoresis was used to analyze the sample, as described above and shown in Figure 1. This demonstrates that DNA that is not trapped in mineral is washed out and removed from the reaction vessel.

TEM Analysis. For TEM analysis, mineralization experiments proceeded exactly as described above in "Mineralization Analysis" except that the universal indicator was not included in the reaction. Samples reacted for 240 min on the benchtop instead of in the plate reader. After reaction, 200 μL of solution was added to an Amicon spin filter (10,000 MWCO) and centrifuged at 13,400 rpm for 5 min. The collected mineral was washed with DI water and recentrifuged 3 time. After the final wash, DI water was added to cover the filter which was then vortexed to mix, inverted in a collection tube, and centrifuged to collect the sample off of the filter. A 10 μL sample was added to a FormVar/Carbon 200 mesh copper grid (Ted Pella, Inc) for 2 min and the solution was wicked away using filter paper. The sample was analyzed on a JEOL JEM-1200EX Electron Microscope.

FT-IR Analysis. For FT-IR analysis, mineralization experiments proceeded exactly as described above in "Mineralization Analysis" except that the universal indicator was not included in the reaction and that the reaction was scaled up to a 5 mL total reaction volume to produce enough mineral for analysis. Samples reacted for 240 min on the benchtop instead of in the plate reader. After reaction, samples were collected by vacuum filtration on MCE filters, washed with DI water, and allowed to dry in air. A Thermo Nicolet Avatar 370 with Smart iTR diamond plate instrument was used. Spectra were taken at 1 cm^{-1} resolution for 32 or 64 scans.

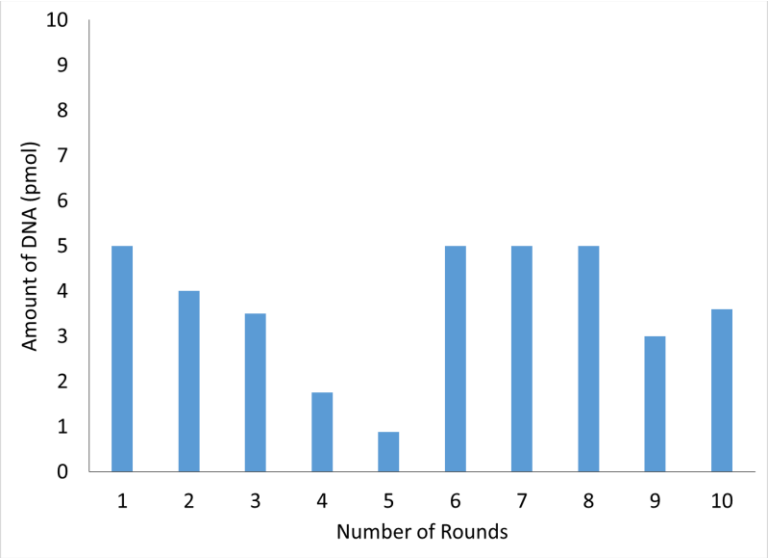
DNA Melt Curve Analysis. The stability of DNA G-quadruplex structures was analyzed by looking at melting curve profiles to determine Tm. The 40-base DNA sequence shown in Figure 2B was prepared at 100 nM concentrations in 5 mM Tris buffer at pH 7.4 and with 50 mM added salt. Experiments were conducted with added NaCl and KCl. Gel star dye (Lonza) was diluted 1:50,000 and added to each sample in a PCR 96 well plate. Fluorescence was measured on an Applied Biosystems StepOnePlus real-time PCR instrument while increasing the temperature from 25 ^{o}C to 95 ^{o}C. The Gel star dye decreases in fluorescence ability as it unbinds from DNA when the DNA strand denatures.

S2. Activity stringency profile for SELEX evolution



Stringency was increased in consecutive rounds of selection by increasing the molarity of added NaCl from 10 mM to 137 mM (shown in blue). This resulted in a decrease in activity of both Ca^{2+} and PO_4^{3-} ions. The activity of Ca^{2+} at each round is shown here (in red).

S3. Complementary selection using a change in library concentration

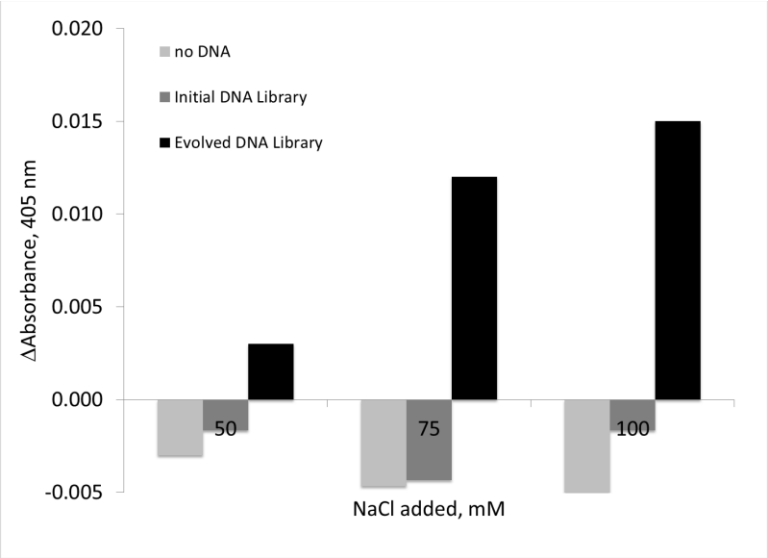


Selection was completed according to the methods described in Supporting Information, **S1**. This selection did not include the addition of NaCl and the ionic strength of solution remained constant. For the first five rounds of selection, the starting amount of DNA library was decreased from 5 pmol down to less than 1 pmol. After the 5th round, the amount of DNA used was increased for the remainder of the experiment.

Sequencing analysis of the evolved library showed enrichment in %G to 41 $\pm$ 9%, or 64% enrichment. Only 68% of the strands were capable of forming a G-quadruplex and overall the stability of the G-quadruplex structures was decreased. This is shown in the decreased prevalence of 3-G (GGG) repeats and an increased prevalence of 2-G (GG) repeats. Also, G-scores from Kearse, et al were lower. This may indicate that the increased ionic strength in the complementary selection aided in selecting more stable and more active G-quadruplex structures.

Specific strands from this selection have been tested in mineralization experiments at various concentrations of DNA and with/without small amounts of added salt. Initial experiments show mineralization enhancement for some strands in the absence of added salt (data not shown).

S4. Mineralization data for evolved and unevolved libraries



When no DNA was present in solution, the lowered activity of precursor ions in high ionic strength solution inhibits mineralization in a 30 min mineralization experiment. When the initial, unevolved library is used in solution at 25 nM, mineralization is similarly inhibited. When the evolved library, after 9 rounds of selection, is used in solution at 25 nM, mineralization is observed and appears to be enhanced at higher added NaCl concentrations. Mineralization was monitored according to the reaction described and shown in Eq (1).

S5. Target sequences identified through SELEX and histogram of %G. These sequences were identified from the selection using increasing concentrations of salt as an applied evolutionary stress. The starred (*) sequences are those shown in **Figure 2**. The histogram shows the range of %G found in entire sequenced pool, ranging from 25-57.5%. No sequences were less than 25% G. The histogram shows a non-Gaussian distribution apparently weighted towards the higher %G values. Melting curves of the 40-base DNA aptamer show that the DNA has a higher Tm and is therefore stabilized by the presence of K^+, which supports the hypothesis that G-quadruplex structures do form. Tm of this strand in Na^+ is 55.1 $\pm$ 0.5 ^oC and in K^+ is 58.1 $\pm$ 0.9 ^oC. Representative melting curves are shown below.

5’-GCGGACGACCTGGGTGCTTGCTCACGGTGTCGGGAGGCGG-3’

GATGGCCGCGGGCGAGGTGAGTGGCTCGGGTGGTCGTGCGGG

CGGGACGGTGTGAGCTTAGTTGCAGAGTGCGGTGGTGGTGG

GCGGTGGGGGGAGAGGGGGCGTGGTTCGGGATAGATCGGTCGC

*CAGGTGGGCGCGCTGTCGTGGGTGCTCGGGTGCGGTTGGG

*TCCCACGCATTCTCCACATCGGGGGCGGGGCGAGGGTGGATCGGGCACAGGCTTGGGTGGCCTTTCTGTCCTTCCGTCAC

TCCCACGCATTCTCCACATCGCGGCCACTGTGTGGCGTATGCTCACGAAGTCGTGGGTGGCCTTTCTGTCCTTCCGTCAC

TCCCACGCATTCTCCACATCGGGGAGTGGCGAAGAGTGTGGGTACGCCTGGGTCGCGGGCCCTTTCTGTCCTTCCGTCAC

TCCCACGCATTCTCCACATCCAGCGCGGAGTGACCGGTTATGCGNACTCGCCAGGCGGGTGGCCCTTTCTGTCCTTCCGTCAC

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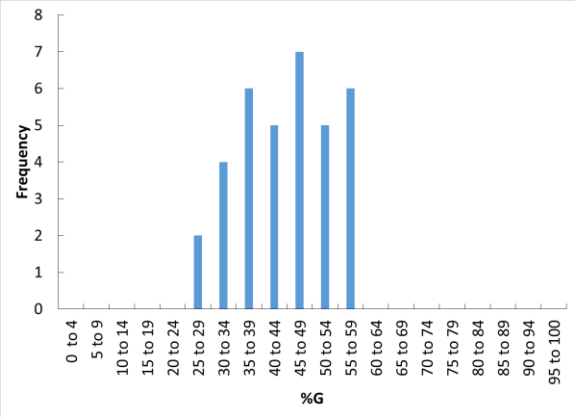
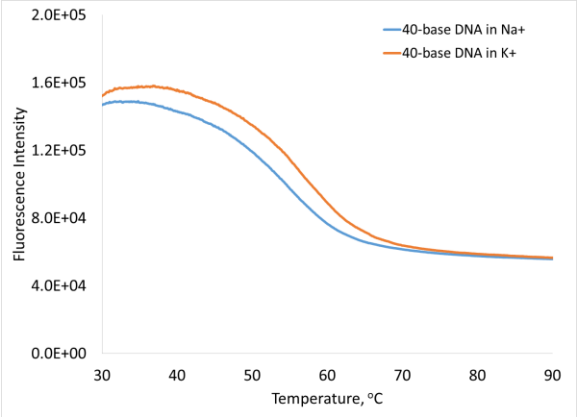
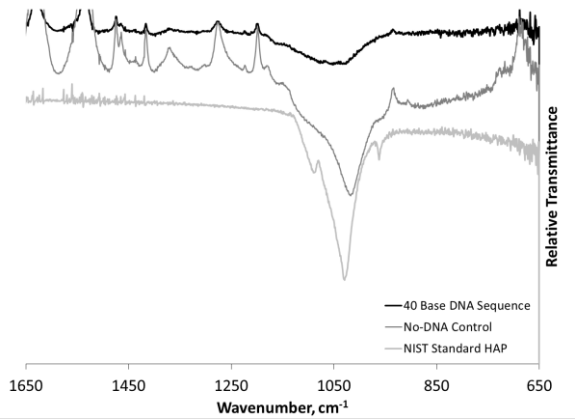


Histogram of %G



Tm of 40-base DNA

S6. TEM and FT-IR characterization of mineralized sample



FT-IR spectra of mineral produced from reactions involving the 40 base DNA sequence shown in Figure 2 suggest an amorphous material, consistent with TEM and SAED. The broad peak centered at ~1050 cm^{-1} is indicative of calcium phosphate, but does not suggest a crystalline phase. A control reaction in the absence of a DNA aptamer template produced calcium phosphate mineral with a sharper and more defined phosphate peak. This indicates a greater degree of crystallinity and lower amorphous character, but this material does not appear to approach the crystallinity observed in a NIST standard of hydroxyapatite (HAP), included for comparison. Peak at 1198, 1269, 1416, 1473, 1526, and 1618 cm^{-1} are artifacts from an MCE background.