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Selection of DNA Aptamers for Root Exudate L-Serine Using Multiple Selection Strategies

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ABSTRACT: Agricultural biosensing can aid decisions about crop health and maintenance, because crops release root exudates that can inform about their status. L-Serine has been found to be indicative of nitrogen uptake in wheat and canola. The development of a biosensor for L-serine could allow farmers to monitor crop nutrient demands more precisely. The development of robust L-serine-binding DNA aptamers is described. Because small molecules can be challenging targets for Systematic Evolution of Ligands by EXponential enrichment (SELEX), three separate DNA libraries were used for SELEX experiments. A L-homocysteine aptamer was randomized to create a starting library for a L-serine selection (randomized SELEX). The final selection rounds of the L-homocysteine selection were also used as a starting library for L-serine (redirected SELEX). Finally, an original DNA library was used (original SELEX). All three SELEX experiments produced L-serine-binding aptamers with micromolar affinity, with Red.1 aptamer having a K_d of 7.9 ± 3.6 μM. Truncation improved the binding affinity to 5.2 ± 2.7 μM, and from this sequence, a Spiegelmer with improved nuclease resistance was created with a K_d of 2.0 ± 0.8 μM. This L-serine-binding Spiegelmer has the affinity and stability to be incorporated into aptamer-based biosensors for agricultural applications.

KEYWORDS: *exudate, amino acids, aptamers, biosensing, precision agriculture, Spiegelmer*

INTRODUCTION

Precision agriculture uses technological innovations to improve the efficiency of farming, allowing producers to respond to the specific needs of their crops. Data from global positioning systems, remote sensing, computer modeling, and chemical analysis of soil samples are used to inform decision making about crop management. Although more general parameters, such as soil temperature, moisture, and drainage, are typically measured rather than the specific biochemical composition of soils, the presence of specific biomarkers can inform about crop health.[1] Gene expression biomarkers have been found to indicate nitrogen and water status, and protein biomarkers have been identified that indicate abiotic stress, for example.[2−4] Plants have been estimated to release between 5 and 25% of net fixed carbon as root exudates, ranging from low-molecular-weight organic anions and amino acids to higher molecular weight compounds, such as polysaccharides and proteins.[5] Although the function of many of these exudates is unknown, many specific root exudates have been suggested to mediate interactions affecting plant and microbe growth.[6,7] The development of new biosensors capable of binding these exudates at low concentrations and in complex environments could allow farmers to monitor and respond to the specific needs of their crops.

Aptamers are single-stranded synthetic oligonucleotides that fold into unique shapes and bind their targets with high affinity and specificity. They have a low cost of production and labeling and no batch-to-batch variation and have been incorporated into a wide range of biosensor and responsive material applications.[8,9] Aptamers are selected from a large oligonucleotide library through an iterative process of target incubation, partitioning, and amplification called Systematic Evolution of Ligands by EXponential enrichment (SELEX), resulting in a population of sequences binding the target of interest. Aptamers have been selected for a wide range of target types, including many root exudate molecules and environmental targets.[1,8] The amino acid L-serine has been identified as a root exudate from wheat and canola whose presence in the soil increases during periods of nitrogen uptake by the crop.[10] An aptamer targeting L-serine could be used for biosensor development to inform on fertilizer application or incorporated into smart materials for on-demand fertilizer release.[10]

Small molecules can be challenging targets for aptamer selection as a result of their limited number of functional groups and have been found to have lower affinities for their targets on average when compared to proteins and whole cells.[11] Nevertheless, several aptamers for amino acids have been discovered, with K_d values generally ranging from high micromolar to low millimolar, shown in Table 1.

Partitioning of unbound sequences from target-bound sequences is a crucial step of the SELEX process to isolate and amplify target-binding sequences. This step is especially challenging for small-molecule targets because the aptamer binding a small molecule does not cause a large change in mass. Typically, the target or oligonucleotide library must be

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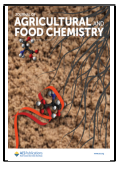

Table 1. Apparent Dissociation Constants for DNA and RNA Aptamers Selected for Amino Acid Targets

amino acid target	DNA/RNA	apparent K_d	reference
L-arginine	DNA	2.5 mM	Harada et al.[12]
	RNA	56−76 μM	Famulok[13]
	RNA	330 nM	Geiger et al.[14]
D-arginine	RNA	410 μM	Famulok[13]
L- and D-glutamic acid	DNA	580−810 μM	Ohsawa et al.[15]
L-histidine	RNA	8−54 μM	Majerfeld et al.[16]
isoleucine	RNA	0.9 mM	Legiewicz et al.[17]
L-isoleucine	RNA	200−500 μM	Majerfeld et al.[18]
	RNA	1−7 mM	Lozupone et al.[19]
L-phenylalanine	RNA	34−55 μM	Illangasekare et al.[20]
L-tryptophan	DNA	1.757 μM	Yang et al.[21]
D-tryptophan	RNA	18 μM	Famulok et al.[22]
L-tyrosine	RNA	35 μM	Mannironi et al.[23]
L-valine	RNA	2.9 mM	Majerfeld et al.[24]

immobilized during selections, introducing the additional challenge of non-specific binding to solid supports. The resulting aptamers could show affinity for the target only in its immobilized form, binding some functional groups provided by the solid support in addition to the target molecule. For example, the aptamer selected for the small molecule dye sulforhodamine B showed a higher binding affinity to the agarose-bound dye compared to the dye free in solution, suggesting that the aptamer had some affinity for agarose.[25] This partial binding of the aptamer to the solid support could be a challenge for downstream applications if the target needed to be analyzed in solution. Interference from the solid support during SELEX can be minimized with the application of control rounds, wherein the DNA library is exposed to the support material prior to its incubation with the immobilized target (referred to herein as "negative selection"), reducing the propagation of sequences with affinity for the solid support.

The success of small-molecule selections can also be improved with the application of high-throughput sequencing (HTS) technologies. In high-throughput SELEX (HT-SELEX), part of each selection library is used for sequencing, while the rest is used as the starting library for the next SELEX round.[26] Sequencing libraries throughout the selection allows for the presence of specific sequences to be tracked, facilitating the identification of target-binding sequences. By sequencing a control library containing sequences with affinity for the solid support, these sequences can be computationally removed, leaving the sequences with affinity for the target.

Because SELEX is a time-consuming process, some groups have generated new aptamers from existing aptamer sequences for similar targets. For example, a citrulline-binding RNA aptamer was randomized 30% and used to generate an L-arginine-binding aptamer.[13] A dopamine-binding RNA aptamer was also randomized to generate a L-tyrosine-binding aptamer.[23] A L-homocysteine-binding DNA aptamer had been developed that had undergone several control rounds, such as negative selections and counter selections against similar amino acids, which would be beneficial for a L-serine aptamer selection.[27] A L-homocysteine aptamer generated from this selection, which showed some affinity to L-serine, was randomized to create a starting library for a L-serine selection (randomized SELEX). The final selection rounds of the L-homocysteine selection were also used as a starting library for a L-serine selection to see if additional L-serine binding sequences could be found (redirected SELEX). Finally, an original DNA library, which had never been used in a selection, was used for a L-serine selection (original SELEX). The development of each of the three starting libraries for L-serine SELEX is outlined in Figure 1.

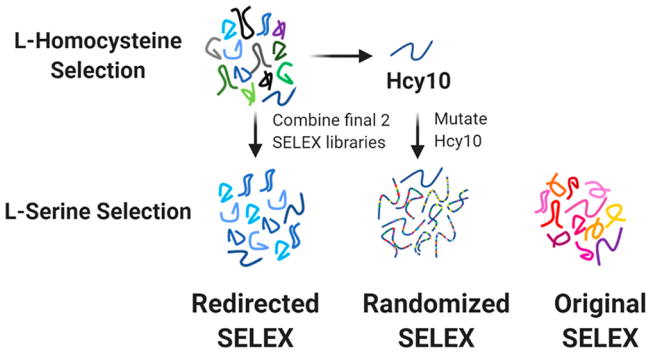

Figure 1. Development of three DNA libraries for L-serine aptamer selection. The final two SELEX rounds from a L-homocysteine selection were combined for the redirected SELEX library. The Hcy10 aptamer obtained from the L-homocysteine selection was mutated 15% to create the randomized SELEX library.[27] The original SELEX library was synthesized with no bias.

Transitioning aptamers from proof-of-concept studies to real world applications can be challenging because complex environments can affect aptamer target binding, long DNA sequences can be expensive to produce, and nucleases can quickly degrade oligonucleotide aptamers. These problems can be mitigated by modifying the aptamer selection and characterization techniques to reflect downstream applications. Incorporating complex matrices and interfering molecules into the selection and ensuring target/aptamer immobilization does not hinder target binding in solution could help the transition of aptamers into more robust biosensing platforms. A full-length aptamer contains nucleotides involved in target binding, nucleotides that provide structural support for the binding site, and nucleotides that are not involved in target binding.[28] A longer aptamer results in a larger production cost for any downstream applications. Truncating non-essential nucleotides to create a minimal aptamer or "minimer" reduces the production cost and can sometimes increase the affinity of the aptamer for the target.[29,30] Because oligonucleotide aptamers could be subject to quick degradation by nucleases when applied in biological systems, several strategies to

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increase aptamer stability have been successfully applied. Aptamer selections have been successful using nucleotides with 2′-amino, 2′-fluoro, and 2′-*O*-methyl modifications for example, conferring nuclease resistance to the resulting aptamers.[31] Increased stability has also been achieved through capping 5′ and 3′ ends of an aptamer with biotin or inverted bases or by substituting sulfur for oxygen in the phosphodiester backbone.[32,33] Spiegelmers, a registered trademark of NOXXON Pharma, can also be used when nuclease resistance is required, because they are mirror-image aptamers that are not recognized by natural nucleases.[34] Spiegelmers are composed of L-(deoxy)ribose nucleotides, which do not occur in nature. Naturally occurring nucleases are stereoselective for D-oligonucleotides, allowing Spiegelmers to avoid nuclease degradation.[34] Modifications such as these could be useful in preventing the degradation of aptamers used in agricultural applications.

Generating affinity ligands for specific crop biomarkers, such as root exudates, could allow for new biosensor platforms to be developed that can quickly inform about crop health and development, helping farmers make decisions in real time. In this work, DNA aptamers for the root exudate L-serine were selected from an original DNA library, from a redirected L-homocysteine selection, and from a randomized L-homocysteine aptamer library. The development of a minimer and corresponding Spiegelmer, which increased both the affinity to L-serine and nuclease resistance, is described. These robust L-serine-binding aptamers can be incorporated into intelligent fertilizer systems for on-demand fertilizer release.[10]

MATERIALS AND METHODS

Chemicals and Reagents. For DNA synthesis, all amidites, modifiers, and reagents were purchased from Glen Research (Sterling, VA, U.S.A.), while 1000 Å CPG 1 μmol scale columns were purchased from BioAutomation (Irving, TX, U.S.A.). Synthesis-grade acetonitrile was obtained from VWR (Radnor, PA, U.S.A.), and 28% ammonium hydroxide solution was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). For DNA purification, polyacrylamide gel electrophoresis (PAGE) reagents, acrylamide/bis-acrylamide 40% solution, Tris, boric acid, ethylenediaminetetraacetic acid (EDTA), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), were obtained from BioShop Canada (Burlington, Ontario, Canada), while formamide and 3 kDa cutoff Amicon Ultra centrifugal filter columns were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

For SELEX and affinity experiments, NHS-activated Sepharose 4 Fast Flow, ninhydrin, and buffer components, sodium bicarbonate ($NaHCO_3$), sodium acetate (CH_3COONa), sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), magnesium chloride ($MgCl_2$), and HCl, were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Additional buffer components, sodium chloride (NaCl), Tris, and urea, as well as target molecules, L-serine, L-glycine, and L-threonine, were obtained from BioShop Canada (Burlington, Ontario, Canada). Costar Spin-X cellulose acetate centrifuge filters were purchased from Corning Incorporated (Corning, NY, U.S.A.). Reagents for polymerase chain reaction (PCR) (25 mM $MgCl_2$, 10 mM dNTP mix, and Taq DNA polymerase) were purchased from BioShop Canada (Burlington, Ontario, Canada). Red.1 minimer sequences with 5′-cyanine 5 labels were ordered from IDT.

For Spiegelmer synthesis, the following L-amidites: β-L-deoxy adenosine (*n*-bz) CED phosphoramidite, β-L-deoxy cytidine (*n*-acetyl) CED phosphoramidite, β-L-deoxy guanosine (*n*-ibu) CED phosphoramidite, β-L-thymidine CED phosphoramidite, and β-L deoxy cytidine (*n*-Ac) 3′-lcaa CPG 1000A support columns, were obtained from ChemGenes. For the nuclease resistance study, DNase I was obtained from BioShop Canada (Burlington, Ontario, Canada).

The soil used to obtain soil solutions was a sandy loam with a pH of 6.6 in water. The soil belongs to the Manotick series of the Haplorthods Great Soil Group. Soil solutions were obtained after placing 50 g of moist soil into a Vivacell 70 (Sartorius Stedim Biotech, Goettingen, Germany), which was subsequently centrifuged at 2000*g* for 10 min at 4 °C. Each collected solution sample was placed into a 10 mL glass vial and kept frozen at −18 °C until chemical analysis.

DNA Synthesis. *SELEX Libraries.* The SELEX libraries and corresponding primers were synthesized using a MerMade 6 oligonucleotide synthesizer (BioAutomation Corporation, Plano, TX, U.S.A.), using standard phosphoramidite chemistry, and purified using 12% denaturing PAGE. The Original Pool SELEX library was 80 bases long, with the sequence 5′-AGCAGCACAGAGGTCAGATG-N40-CCTATGCGTGCTACCGTGAA-3′, where N represents an equimolar mixture of all four phosphoramidites. Forward and reverse PCR primers for this library were 5′-6-FAM-AGCAGCACAGAGGTCAGATG-3′ and 5′-poly-dA_{20}-HEG-TTCACGGTAGCACGCATAGG-3′, respectively.

The L-homocysteine aptamer (Hcy10) selected by McKeague et al. was used as a template for the randomized SELEX starting library.[27] The Hcy10 aptamer sequence, 5′-ATACCAGCTTATTCAATT**GTGGAAAGCCGAATGTGATTAGGGACCAGTGGAGAAGTAGTACGGACTGACCTCGCGTGTA**AGATAGTAAGTGCAATCT-3′ was synthesized, with the region shown in bold having 15% randomization. For this randomized region, 15% of the amidite volume used contained an equimolar mixture of the three other amidites. The forward and reverse PCR primers for this library were 5′-6-FAM-ATACCAGCTTATTCAATT-3′ and 5′-dA_{20}-HEG-AGATTGCACTTACTATCT-3′, respectively.

The redirected SELEX library was created by combining the final two selection rounds (rounds 7 and 8) of the L-homocysteine selection by McKeague et al.[27] The forward and reverse primers used in PCR were the same as for the randomized SELEX library.

Aptamer Candidate Sequences. Aptamer candidates were synthesized with 5′-6-FAM labels using a MerMade 6 oligonucleotide synthesizer (BioAutomation Corporation, Plano, TX, U.S.A.), using standard phosphoramidite chemistry, purified using denaturing PAGE, and confirmed by electrospray ionization/liquid chromatography/mass spectrometry (ESI/LC/MS, Novatia, Newtown, PA, U.S.A.).

Spiegelmer Synthesis. Using L-amidites and standard DNA synthesis reagents, the following Spiegelmer was synthesized on a Mermade 6 oligonucleotide synthesizer: 5′-Cy5-AGATAGTAAGTGCAATCTAGATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCATC-3′. The Spiegelmer sequence was purified using 12% denaturing PAGE and confirmed by ESI/LC/MS (Novatia, Newtown, PA, U.S.A.).

Column Preparation. L-Serine was coupled to NHS-activated Sepharose 4 Fast Flow resin through the amine group on L-serine. All solutions used for coupling were cooled to 4 °C, and prior to coupling, the Sepharose resin was washed with 10−15 column volumes of 1 mM HCl. For coupling, 115 mM L-serine was dissolved in coupling buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl at pH 8.3) and incubated with Sepharose resin at a ratio of 0.5:1 coupling solution to resin for 4 h, shaking at room temperature. The coupling solution was removed and used to determine coupling efficiency. Sepharose was incubated with 0.1 M Tris−HCl on a shaker for 3 h at room temperature to block any unreacted groups. After blocking, Sepharose was washed using 3× medium volumes of 0.1 M Tris−HCl at pH 8−9, followed by a wash with 0.1 M acetate and 0.5 M NaCl at pH 4−5. These buffers were used alternately for a total of eight column washes. The column was then transferred to SELEX buffer (50 mM Tris−HCl, 500 mM NaCl, and 5 mM $MgCl_2$ at pH 7.4) and stored at 4 °C until use. This procedure was repeated to generate L-glycine and Tris-conjugated Sepharose as controls.

To confirm that L-serine had successfully coupled to the Sepharose column, the ninhydrin test was used with the pre- and post-coupling samples. A range of L-serine concentrations (70−230 mM) were prepared in coupling buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl at pH 8.3). A ninhydrin solution was prepared by adding 0.1 g of ninhydrin

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to 10 mL of glacial acetic acid. The ninhydrin solution was slightly heated until dissolved. Each concentration of L-serine and the pre- and post-coupling solutions were mixed 1:1 with ninhydrin solution. Coupling buffer mixed with ninhydrin was used as a control. The solutions were left at room temperature for 5−10 min before examining their color change at 570 nm.

SELEX Conditions. Before each round of selection, the DNA pool in SELEX buffer (50 mM Tris−HCl, 500 mM NaCl, and 5 mM $MgCl_2$ at pH 7.4) was denatured at 90 °C for 5 min, cooled at 4 °C for 10 min, and brought to room temperature for 15 min. A 5 μL sample of the DNA starting pool was set aside for fluorescence analysis. The column beads were placed in 0.22 μm Costar Spin-X cellulose acetate centrifuge filters and washed 3 times with SELEX buffer prior to beginning. The DNA libraries were incubated with the negative column (Tris-coupled Sepharose) for 30 min of shaking at room temperature in SELEX buffer. The beads were centrifuged at 14000*g* for 1 min. The DNA solution that passed through the negative column was added to the positive column (L-serine-coupled Sepharose) and incubated for 1 h of shaking at room temperature. The column was centrifuged at 5000*g* for 1 min, collecting the flow through. Four additional washes using SELEX buffer were also collected. The fourth wash was centrifuged at 14000*g* for 1 min. DNA was eluted from the column by incubating the column with 100 mM L-serine for 10 min and centrifuging at 14000*g* to collect the filtrate. This was repeated for five elutions. An additional five elutions were collected using 7 M urea at 90 °C to ensure that most DNA was removed from the column. In the penultimate selection round for each SELEX experiment, counter selections against L-glycine and L-threonine were performed. Prior to eluting desired sequences using L-serine, an elution was performed using a solution of L-glycine and L-threonine (100 mM each), followed by one wash with SELEX buffer. The elution and wash of this counter selection were kept for HTS. The target incubation step in the final round of each SELEX experiment was performed using a 1:1 mixture of soil solution and SELEX buffer. To increase stringency throughout the selections, the target incubation time was gradually reduced to 20 min and the bead volume was reduced to increase competition. See Table S2 of the Supporting Information for a summary of experimental details.

PCR To Amplify SELEX Rounds. PCR reactions were prepared to a volume of 100 μL by combining 50 μL of buffer (0.2 M Tris−HCl at pH 9, 0.1 M KCl, 2% Triton X-100), 37 μL of deionized water (diH_2O), 8 μL of 25 mM $MgCl_2$, 2 μL of dNTP mix, 0.5 μL of fluorescently labeled forward primer, 0.5 μL of polyA reverse primer, 1 μL of Taq DNA polymerase, and 1 μL of the DNA library to be amplified (on the order of 1 pmol). Positive and negative control reactions were run by substituting 1 μL of control pool or diH_2O in place of the SELEX pool, respectively. PCR was run on a Mastercycler epgradient (Eppendorf, Hauppauge, NY, U.S.A.). The PCR conditions for the original pool SELEX were denaturation at 94 °C for 5 min, 25 cycles (melting at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 74 °C for 20 s), and final extension at 72 °C for 5 min. For the randomized pool and redirected pool SELEX, the PCR conditions were denaturation at 94 °C for 10 min, 25 cycles (melting at 94 °C for 1 min, annealing at 47 °C for 1 min, and extension at 72 °C for 1 min), and final extension at 72 °C for 10 min. After PCR, the reactions were dried down on a Savant AE2010 SpeedVac. They were resuspended in a minimum volume of diH_2O, mixed 1:1 with formamide, purified using 12% denaturing PAGE, and used as the starting pool for the subsequent SELEX round.

HTS. Illumina adapter sequences were ordered from IDT and added to the DNA libraries by PCR amplification. For the original pool SELEX, a master mix was made containing 350 μL of buffer (0.2 M Tris−HCl at pH 9, 0.1 M KCl, and 2% Triton X-100), 260 μL of diH_2O, 56 μL of $MgCl_2$, 14 μL of dNTPs, 10.5 μL of forward primer, 10.5 μL of reverse primer, and 7 μL of Taq. The master mix was mixed well, and 95 μL was removed for a PCR control. To the rest of the master mix, template was added (6 μL of the original pool or 36 μL of a SELEX round library) and divided into six reactions. The PCR conditions were as previously described, using a gradient annealing temperature from 54.1 to 56.4 °C and a total of 20 cycles. For randomized SELEX, the same master mix was used, with 20 μL of template added (6 μL of the round 1 pool) and divided into six reactions. The cycle used was as previously described with a gradient of annealing temperatures from 49 to 59 °C. For the redirected SELEX, a master mix was made containing 350 μL of buffer, 266 μL of diH_2O, 56 μL of $MgCl_2$, 14 μL of dNTPs, 15 μL of forward primer, 15 μL of reverse primer, and 7 μL of Taq. After removal of 95 μL for a PCR control, 20−28 μL of each template was added and divided into six reactions. The cycle used was as previously described with gradient annealing temperatures from 49 to 59 °C for 20 cycles. After each PCR, like reactions were combined and dried down on a Savant AE2010 SpeedVac. The DNA libraries with Illumina adapters were purified and extracted from denaturing PAGE gels as previously described, using 8% gels to accommodate the longer sequence length. After purification and extraction from the 8% denaturing PAGE gels, the DNA libraries were diluted to 5 ng/μL, and 40 ng of each library was sent to Genome Quebec, as shown in Table S3 of the Supporting Information. Genome Quebec performed HiSeq 2000 PE100 sequencing and provided sequencing data in the form of .fastq files to be analyzed with HTS software.

HTS Analysis Using AptaCluster. The analysis of sequencing files was performed using AptaCluster software.[26] A configuration file was made for each SELEX experiment, outlining the selection-specific parameters, such as template length, primer sequences, and number of selection rounds. The quality control parameters, locality sensitive hashing options, and cluster relation settings were not changed from the default settings of the program. For the randomized Hcy10 pool SELEX, the lsh_sequence_similarity parameter was reduced from the default 6 to 4, because this library was expected to contain much more sequence similarity than the other selection libraries. The configuration files for each selection experiment were run individually in AptaCluster, and the data was examined using AptaGUI.

L-Serine-Conjugated Bead Binding Assays. 5′-Fluorescein-modified aptamer candidate sequences were diluted in modified phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 5 mM $MgCl_2$, and 490 mM NaCl at pH 7.4) from 2 μM to 50 nM. The DNA was heated to 90 °C for 5 min, cooled at 4 °C for 10 min, and left at room temperature for 15 min prior to testing. L-Serine-coupled Sepharose beads were diluted 1:1 in PBS buffer, and 20 μL of the bead slurry was added to 0.22 μm Costar Spin-X cellulose acetate centrifuge filters for each aptamer concentration tested. The beads were washed with 100 μL of PBS and centrifuged at 13000*g* for 1 min. A total of 100 μL of each aptamer concentration was incubated with L-serine-conjugated Sepharose for 45 min at room temperature with shaking. Unbound DNA was removed by centrifuging at 5000*g* for 1 min, followed by three 100 μL washes using PBS. To elute bound aptamer, 100 μL of 7 M urea was added to the beads, incubated at 90 °C for 10 min, and centrifuged at 13000*g* for 1 min. This was repeated 3 times per test concentration. The fluorescence intensity of the elutions was measured using a Fluorolog fluorescence spectrophotometer (Horiba Jobin Yvon, Edison, NJ, U.S.A.) with an excitation wavelength of 490 nm, emission wavelength of 520 nm, and slit widths of 5 nm. The amount of DNA eluted at each concentration was calculated by comparing to a standard curve of fluorescence intensity at known concentrations.

Microscale Thermophoresis (MST). A range of L-serine concentrations (from 7.6 nM to 250 μM in PBS) was incubated with 20 nM of labeled aptamer and analyzed by 2bind (Regensburg, Germany) on a Monolith NT at 25 °C, with 15% light-emitting diode (LED) power and 80% laser power.

Examination of Nuclease Resistance. A total of 10 μL of the random region minimer or Spiegelmer (50 μM each in diH_2O) was mixed with 90 μL of DNase I reaction buffer (10 mM Tris−HCl, 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$ at pH 7.0) for each time point to be tested. To each DNA solution, 1 μL of DNase I was added and the tubes were incubated at 37 °C. For the random region minimer, DNase I was incubated with DNA for 0, 30, 60, and 90 s. For the Spiegelmer, DNase I was incubated with DNA for 0, 12, 36, and 60 h. The reaction was stopped by adding 1 μL of 0.5 M EDTA and

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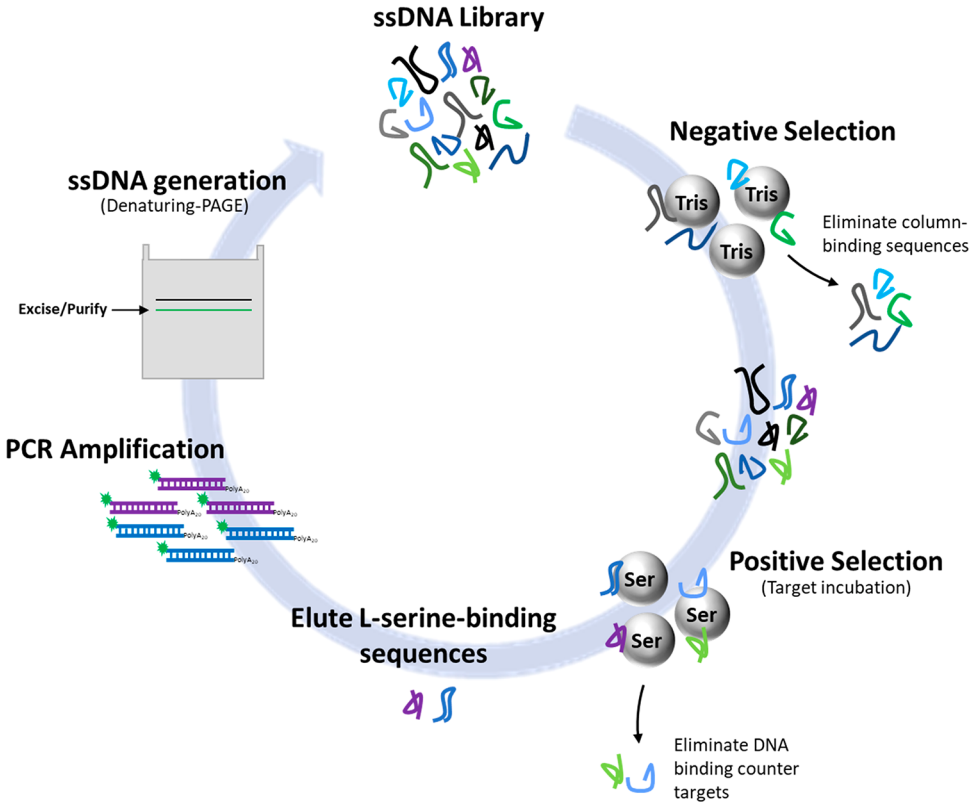

Figure 2. L-Serine SELEX procedure. A ssDNA library was added to Tris-conjugated Sepharose for a "negative selection". Sequences that did not bind Tris−Sepharose were added to L-serine-conjugated Sepharose for a "positive selection". L-Serine-binding sequences were eluted with the concentrated target followed by urea. For counter selections, DNA was eluted with L-glycine and L-threonine prior to L-serine elution. Target-binding sequences were PCR-amplified and purified using 12% denaturing PAGE.

incubating tubes at 90 °C for 10 min. The reactions were then analyzed using 19% denaturing PAGE.

Illustrations were created with BioRender (www.biorender.com).

RESULTS AND DISCUSSION

Library Design. During the evaluation of L-homocysteine-binding aptamers, one aptamer, Hcy10, showed moderate affinity for L-serine (high micromolar range).[27] Further investigation of Hcy10 as a potential L-serine-binding aptamer showed binding to immobilized L-serine but no binding to L-serine in solution (see Figure S1 of the Supporting Information). Because the desired downstream applications for L-serine-binding aptamers required L-serine-binding in solution, this Hcy10 aptamer would not provide the necessary binding. In an effort to make use of the partial L-serine affinity of Hcy10, two strategies were adopted using the L-homocysteine selection to find a L-serine-binding aptamer. In the first strategy, the 61-base random region of the Hcy10 aptamer was randomized by 15% to find beneficial base substitutions, increasing the affinity of the aptamer for L-serine. This selection strategy was called "randomized SELEX". Aptamers have been successfully identified using this randomization strategy for L-arginine and L-tyrosine for example, from L-citrulline- and dopamine-binding aptamers, respectively.[13,23] In the second strategy, the final selection rounds from the L-homocysteine selection were combined and used as the starting library for a L-serine selection. This selection strategy was called "redirected SELEX". The purpose of this redirected SELEX was to identify other aptamer candidates within the L-homocysteine selection that may have had some affinity for L-serine and enrich those sequences further. These sequences would have withstood counter selections against Sepharose beads as well as against structurally similar molecules L-cysteine and L-methionine, which would be beneficial for a L-serine binding aptamer as well.[27] A final library was created called "original SELEX", which had not been used previously in any selection experiments to contain no selective bias. The DNA template length used in the randomized SELEX and redirected SELEX experiments was 97 bases with a 61-base random region, because this was the template length used in the L-homocysteine selection.[27] Although no statistically significant relationship between the template length and affinity has been found, a shorter template would sample more sequence space and reduce cost.[11] For the original SELEX, a shorter 80-base template with a 40-base random region was used.

Monitoring SELEX Progression. Using a fluorescein-modified forward primer, the DNA libraries were fluorescently tagged during PCR to allow for the progress of the selection to be monitored. When the fluorescence of the eluted DNA was measured at each SELEX round, the proportion of the DNA library binding L-serine could be estimated relative to the whole SELEX library. Because L-serine is a small molecule and was conjugated to Sepharose resin for these selections, non-specific binding was a concern. To mitigate non-specific binding to the Sepharose resin, a "negative selection" step, in which each SELEX library was exposed to Sepharose in the absence of the target, was conducted prior to each "positive selection" step, where the DNA libraries were exposed to L-serine-bound Sepharose, shown in Figure 2. The stringency of the selections was increased by decreasing the amount of the

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target, L-serine, and decreasing the incubation time of the libraries with the target (Table S2 of the Supporting Information). "Counter selections", in which the SELEX libraries were exposed to structurally similar molecules L-glycine and L-threonine, were conducted in the penultimate selection rounds, with the goal of increasing the selectivity of resulting aptamer candidates. The final round for each selection experiment was conducted in a 1:1 mixture of soil solution and SELEX buffer to expose the libraries to anticipated confounding molecules present in soil.

The proportion of each SELEX library bound to L-serine-conjugated Sepharose or Tris-conjugated Sepharose can be seen in Figure 3. Monitoring the proportion of DNA binding was used throughout the SELEX experiments to ensure that a proportion of DNA was able to bind the target through all

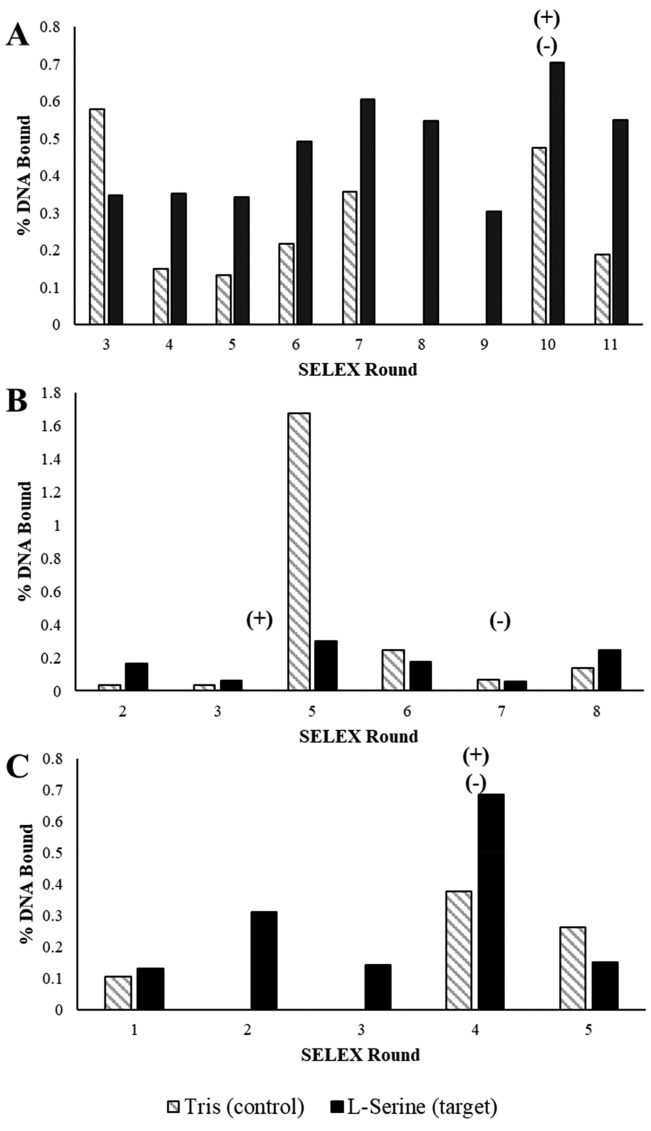

Figure 3. Percent recovery of the ssDNA bound to L-serine-immobilized Sepharose (positive selection) and control Tris-blocked Sepharose (negative selection) after each selection round for (A) original SELEX, (B) randomized SELEX, and (C) redirected SELEX. The selective pressure was increased (+) by reducing the amount of target, and counter selections were performed against L-glycine and L-threonine (−) prior to the positive selection. The final rounds were completed in soil extract.

conditions of increased stringency, such as decreased target concentration, decreased incubation time, exposure to structurally similar molecules L-glycine and L-threonine, and exposure to soil solution. This information also showed the proportion of the SELEX libraries that were binding to the Sepharose resin rather than the target, L-serine. The proportion of the DNA libraries binding to the Sepharose resin in the negative selection rounds differed between SELEX strategies. For the original SELEX, a higher proportion of the library bound the Sepharose resin compared to L-serine-bound Sepharose, indicating a high degree of non-specific binding. This non-specific binding decreased over the course of the selection, showing the importance of including a negative selection step throughout the selection. For the randomized and redirected SELEX experiments, the non-specific binding was much lower at the beginning of the selections, with more of the DNA library binding to L-serine−Sepharose than the control Sepharose resin. Because these SELEX libraries were derived from a previous selection experiment for L-homocysteine, it is likely that the negative control rounds performed during the L-homocysteine selections were able to reduce the propagation of DNA sequences non-specifically binding Sepharose. All three SELEX experiments produced DNA sequences showing binding to L-serine Sepharose, which withstood exposure to counter selection steps against L-glycine and L-threonine as well as exposure to soil solution. The proportion of DNA eluted with affinity for L-serine in each SELEX experiment was low, under 1% of the library. HTS was employed for each SELEX experiment to maximize the chances of identifying L-serine-binding sequences in such a small proportion of the libraries.

HTS. From each of the three SELEX experiments, four DNA libraries were sent for HTS: the starting library, the last selection round completed in SELEX buffer, the final selection round completed in soil solution, and the control library. Sequencing the starting library provided the initial sequence diversity from which enrichment could be measured. The final selection round performed in buffer was analyzed to find aptamer candidates that remained after counter selections against L-glycine and L-threonine. The sequences identified from the final selection rounds made it through these counter selections as well as selection performed in soil solution. A control library containing DNA sequences binding the unwanted targets, such as Tris, the Sepharose matrix, and counter targets L-glycine and L-threonine, was sent for each selection. The sequencing and analysis of these control rounds allowed for the computational deletion of non-specific binding sequences, facilitating the identification of L-serine-binding DNA sequences.

Sequencing Analysis Using AptaCluster. AptaCluster software provided a global overview of each selection experiment over the SELEX rounds sequenced. Figure 4 shows the progression of each SELEX experiment. The "enriched" fraction describes the sequences with a count higher than 1, while "singletons" describe the fraction of sequences with a count of 1. The "unique fraction" describes the number of unique sequences in the DNA library relative to the library size. In a successful SELEX experiment, the unique fraction of the library would decrease as the selection stringency increased, eliminating sequences without affinity for L-serine. The enriched fraction would increase as more L-serine-binding sequences were amplified.

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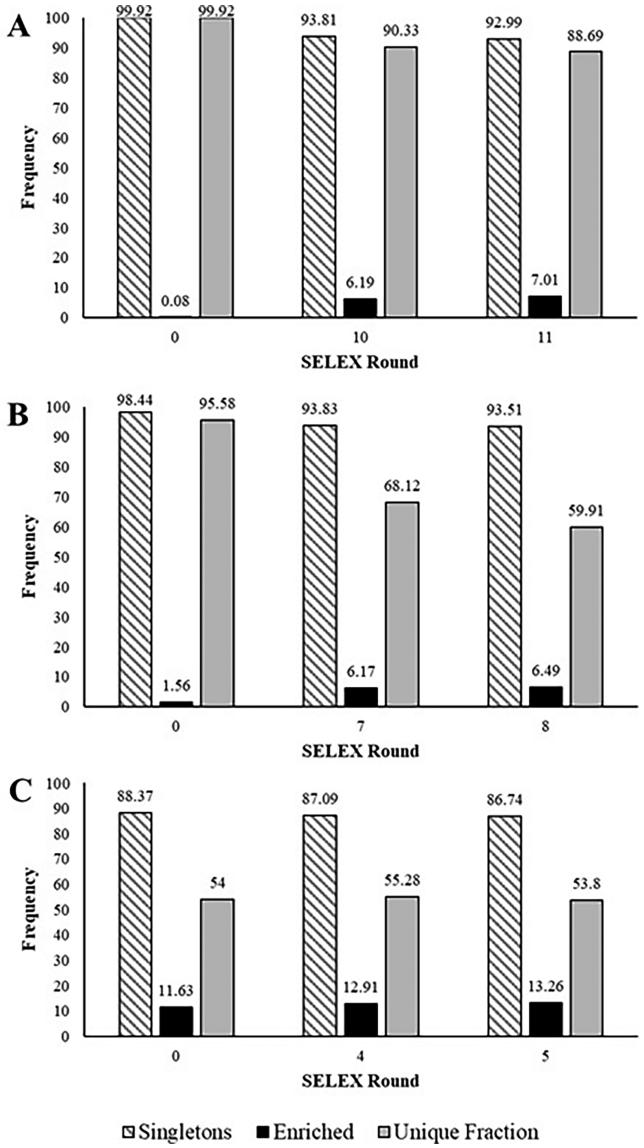

Figure 4. Overview of sequence convergence after Illumina HTS and analysis using AptaCluster and AptaGUI software for (A) original SELEX, (B) randomized SELEX, and (C) redirected SELEX.

In the original SELEX experiment, the unique fraction of the starting pool was 99.92%, which indicated a diverse starting library with few repeated sequences. After 11 selection rounds, the unique fraction of the library decreased to 88.69%. The enriched fraction increased from nearly zero (0.08%) in the starting library to 7.01% in the final SELEX round. Both the decreasing unique fraction and increasing enriched fraction of the libraries suggested that L-serine-binding sequences were becoming amplified and enriched, while non-binding sequences were being removed from the library. In the randomized SELEX experiment, the unique fraction of the starting pool was 95.58%, which indicated that this library was less diverse than the original SELEX library. This lower sequence diversity was attributed to the way this library was synthesized, because all of the sequences were generated by randomizing the Hcy10 aptamer by 15%. By the final round of the randomized SELEX experiment, the unique fraction had decreased to 59.91%, which was the largest drop seen for any of the selections, indicating the largest drop in sequence diversity. In the redirected SELEX experiment, the unique fraction of the starting library was 54.00%, which was the lowest of all of the selection strategies. The redirected library was created by combining the final two SELEX rounds from a L-homocysteine selection. During the L-homocysteine selection experiment, the enrichment of the library increased over eight SELEX rounds, which lowered the sequence diversity of the pool used as the starting library in the redirected SELEX. Over five additional selection rounds for L-serine, the unique fraction of the pool increased to 55.28% and ended at 53.80% in the final round, only slightly less diverse than the starting library. This plateau in sequence diversity suggested that the starting pool was so enriched for L-homocysteine-binding sequences that five additional rounds of selection for L-serine were not sufficient to have an appreciable effect on the sequence diversity of the library. Despite this plateau in the unique fraction of the redirected SELEX library, the enriched fraction did show a small increase from 11.63 to 13.26% over the five selection rounds, indicating that L-serine-binding sequences may still have been enriched.

Identification of Aptamer Candidate Sequences. AptaCluster software sorted DNA sequences by count, which was the frequency of each sequence that appeared in the library, or by enrichment, which was the fold change in the frequency of each sequence with respect to the library size.[26] Sequences with the highest count may represent the best aptamer candidates; however, they may also represent sequences that amplified more efficiently during PCR. To avoid this PCR bias, DNA sequences with the highest enrichment values compared to the control library were selected as aptamer candidates. From each SELEX experiment, the sequence with highest enrichment in the final round was chosen, because this round was performed in a soil solution. The goal of using soil solution was to produce aptamer candidates capable of binding L-serine in conditions mimicking soil. In the event that this final round was too stringent and caused the loss of strong L-serine-binding sequences, the sequence with the highest enrichment in the final buffer round was also selected from each SELEX experiment. The selected aptamer candidate sequences are shown in Table 2, and their respective enrichment and count values are shown in Table S1 of the Supporting Information.

Identification of Conserved Sequence Motifs. Because three separate SELEX experiments were performed using three different DNA libraries for a common target, L-serine, an examination of any common sequence motifs present in the resulting aptamer candidates could help elucidate possible binding motifs. Shared sequence motifs were identified using a pairwise sequence alignment tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/), and all sequences were manually screened for common motifs. These conserved sequence motifs are shown in Table 2.

Upon examination of the sequences obtained from the original SELEX, the reverse primer-binding site (5′-CCTATGCGTGCTACCGTGAA-3′) was found within the central sequence of the aptamer candidate Ser.1, immediately following the forward primer-binding site, shown in Table 2. The reverse primer-binding site from the randomized and redirected SELEX experiments (5′-AGATAGTAAGTGCAATCT-3′) was also found immediately following the forward primer-binding sites in aptamer candidates Ran.1 and Red.1. This site was also found in candidate Red.2, although slightly shifted toward the 3′ end. Prior to these SELEX experiments,

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Table 2. Conserved Sequence Motifs (>10 Nucleotides) in Each L-Serine-Binding Aptamer Candidate and Hcy10[a]

Aptamer Candidate	Selection Experiment	Sequence
Ser.1	Original SELEX	**AGCAGCACAGAGGTCAGATG**CCTATGCGTGCTACCGTGAAACCGATCGG AAGAGCACACG**CCTATGCGTGCTACCGTGAA**
Ser.2	Original SELEX	**AGCAGCACAGAGGTCAGATG**CGATCTGGATATTATTTTTGATACCCCTTT GGGGAGACAT**CCTATGCGTGCTACCGTGAA**
Ran.1	Randomized SELEX	**ATACCAGCTTATTCAATT**AGATAGTAAGTGCAATCTTGATCGGAAGAGCA CACGTCTGAACTCCAGTCACTTAGGCATC**AGATAGTAAGTGCAATCT**
Ran.2	Randomized SELEX	**ATACCAGCTTATTCAATT**GTATACGGAGTGGATATCGATCTGTAACGTGA GTGAGATAATGTGATGCATAGTCGTGGAG**AGATAGTAAGTGCAATCT**
Red.1	Redirected SELEX	**ATACCAGCTTATTCAATT**AGATAGTAAGTGCAATCTAGATCGGAAGAGC ACACGTCTGAACTCCAGTCACTTAGGCATC**AGATAGTAAGTGCAATCT**
Red.2	Redirected SELEX	**ATACCAGCTTATTCAATT**GGCCGTGTAGATAGTAAGTGCAATCTGATCGG AAGAGCACACGTCTGAACTCCAGTCACCG**AGATAGTAAGTGCAATCT**
Hcy10	Homocysteine SELEX[27]	**ATACCAGCTTATTCAATT**GTGGAAAGCCGAATGTGATTAGGGACCAGTG GAGAAGTAGTACGGACTGACCTCGCGTGTA**AGATAGTAAGTGCAATCT**

[a]The forward and reverse primer binding sites are indicated in green and blue, respectively, for the original SELEX and in mauve and yellow for the randomized and redirected SELEX experiments and Hcy10, respectively. Conserved motifs are shown in red and underlined.

both sets of primers were examined using the Multiple Primer Analyzer tool (Thermo Fisher Scientific) and were not expected to form primer dimers; therefore, this result was unexpected. No change in the DNA library size was observed using AptaCluster to indicate mispriming. These reverse primer-binding sites appeared in many of the most promising aptamer candidates, indicating they may have been incorporated into secondary structures that participated in target binding.

Appreciable sequence homology was observed among the aptamer candidates. Sequences Ran.1 and Red.1 were identical except for nucleotide 37. Because these sequences were obtained from two different selection experiments, this sequence was a good candidate for further characterization. Sequence Red.2 was also very similar to Ran.1 and Red.1. In all three sequences, there was a conserved motif of 37 nucleotides (5′-GGAAGAGCACACGTCTGAACTCCAGTCACT-TAGGCATC-3′), shown in red in Table 2. Interestingly, a 13-nucleotide portion of this motif (5′-GGAAGAGCACACG-3′) was found in sequence Ser.1, which was obtained from the original pool SELEX. Because this original selection had no bias and still produced a conserved motif observed in candidates from each SELEX experiment, this motif could be important for L-serine binding. Aptamers that had been previously selected for amino acid targets (shown in Table 1) were manually examined for the presence of this motif. The 7-nucleotide sequence 5′-GGAAGAG-3′, which is a portion of this long motif, was identified in the middle section of a L-arginine-binding aptamer selected by Geiger et al.[14] This suggests that this 5′-GGAAGAG-3′ motif could have affinity for the amino acid structure. A shorter motif 5′-GATC-3′ was present in all six L-serine aptamer candidates and not in the L-homocysteine-binding aptamer, Hcy10, which could indicate the involvement of the motif in L-serine binding.

Evaluating Aptamer Affinity. The binding affinity of an aptamer to its target is described by its dissociation constant (K_d). The apparent K_d values of the aptamer candidates were determined for L-serine both immobilized to Sepharose beads and in solution using MST. A range of aptamer concentrations (from 50 nM to 2 μM) was mixed with equal volumes of L-serine-conjugated Sepharose beads, and binding DNA was eluted and quantified. Tris contains three alkyl hydroxyl groups and was used during SELEX to provide additional selective pressure. To avoid interference from Tris, PBS buffer was used for affinity experiments, and aptamer affinity for Tris was measured separately. To examine the selectivity of these aptamer candidates, Tris-conjugated Sepharose beads and L-glycine-conjugated Sepharose beads were incubated with a range of aptamer candidate concentrations, and their apparent K_d values are also displayed in Table 3.

All aptamer candidates showed binding to immobilized L-serine in the low micromolar range, except for Ser.2 and Ran.1, which showed binding to L-serine but had an apparent K_d outside the range of concentrations tested (Table 3). This low micromolar affinity was comparable to other amino-acid-binding aptamers shown in Table 1. Given that the goal of the aptamer is to detect spikes in the serine concentration as a result of root exudation, the low micromolar affinity could allow for discrimination from basal serine levels in soil. Red.1 showed the most affinity for immobilized L-serine, with an apparent K_d of 2.8 ± 2.0 μM. The aptamer candidates showed binding to Tris ranging from 0.85 to 4.0 μM, except Ran.1 and Ran.2, which showed weak binding outside the range of concentrations tested. Tris was ubiquitous throughout all three

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Table 3. Apparent K_d Values for Aptamer Candidates Determined Using Sepharose Beads Conjugated to L-Serine, Tris, or L-Glycine as Target Molecules[a]

	apparent K_d (μM)		
aptamer	L-serine	Tris	L-glycine
Ser.1	3.0 ± 2.7	0.85 ± 0.68	2.3 ± 0.13
Ser.2	inconclusive[b]	2.0 ± 1.0	4.7 ± 5.9
Ran.1	inconclusive	inconclusive	inconclusive
Ran.2	2.9 ± 3.2	inconclusive	no binding[c]
Red.1	2.8 ± 2.0	4.0 ± 2.7	2.6 ± 1.3
Red.2	3.5 ± 2.9	0.72 ± 0.057	inconclusive

[a]SigmaPlot simple ligand binding was used to calculate K_d, and the error value represents the standard deviation between trials (N = 3). [b]Inconclusive refers to a possible K_d value outside the concentrations tested/non-specific binding to Sepharose. [c]No binding refers to no trend seen.

SELEX experiments, because it was used as the buffering agent during selections as well as a blocking agent for any activated esters that remained after L-serine conjugation to Sepharose beads. Counter selections against Tris were performed prior to every SELEX round; however, affinity for Tris remained. This could be due to the structural similarity between Tris and L-serine, with Tris offering three alkyl hydroxyl groups as binding sites. L-Glycine was chosen as a structural control, differing from L-serine by only one alkyl hydroxyl group. Ser.1, Ser.2, and Red.1 showed affinity for L-glycine with apparent K_d values ranging from 2.3 to 4.7 μM, while Ran.1 and Red.2 showed weak binding outside the range of concentrations tested (Table 3). Ran.2 was the most selective of all of the aptamer candidates, showing no binding to L-glycine and the weakest binding to Tris of all of the aptamer candidates (Table 3). Red.1 showed the highest affinity to L-serine-conjugated beads, which was a result of target binding rather than non-specific binding when compared to a random aptamer of similar length (see Figure S2 of the Supporting Information).

The L-serine-conjugated Sepharose bead assay was used to screen aptamer candidates, but any binding interference from the beads could not be ruled out with L-serine being immobilized. To determine the apparent binding affinities of these L-serine-binding aptamer candidates in solution, MST was used. The aptamer candidates were labeled with a fluorophore, mixed with varying L-serine concentrations, and examined using MST. Aptamer candidates Ser.1, Ran.2, and Red.1 were all able to bind L-serine in solution, with apparent K_d values of 14.9 ± 5.9, 17.2 ± 7.3, and 7.9 ± 3.6 μM, respectively, shown in Figure 5. These three candidates also bound immobilized L-serine most strongly (Table 3). The ability of these aptamers to bind immobilized L-serine as well as L-serine in solution showed that the binding interaction to L-serine was not dependent upon or hindered by the solid support. Ser.2, Ran.1, and Red.2 were unable to bind L-serine in solution (Figure S3 of the Supporting Information), indicating that any affinity seen in the bead assay could be attributed to binding to the solid support. Aptamer candidate Red.1 showed the highest affinity for L-serine in both the bead assay and MST and was used for further testing.

Minimer Design and Characterization. Before selected aptamers were moved into their intended applications, it can be useful to find the minimal aptamer, termed minimer. A full-length aptamer contains a primer-binding region at each end of the DNA sequence used during SELEX for DNA amplification steps. By elimination of bases from the sequence that are not important for target binding, the cost of the aptamer can be reduced and, in some cases, the affinity of the aptamer can be improved.[29,30] Figure 6A shows the structural prediction of the Red.1 aptamer used to design minimers, because Red.1 had the highest affinity for L-serine. The random region minimer was generated by removing the 18-base primer-binding regions from the 5′ and 3′ ends of the DNA sequence, which is a common method for designing a minimer.[35] The structural prediction in Figure 6A shows that these primer-binding regions may be part of secondary structures that could be important for target binding. Minimers were designed to include the two stem loops on the 5′ end (5′ loop) and the larger stem loop structure on the 3′ end (3′ loop), seen in Figure 6A. Because the 3′ primer-binding region was involved in a stem structure but not part of the loop, a minimer was designed with this region removed (minus 3′ primer). The sequences of the four minimers designed from Red.1 are displayed in Figure 6B.

The K_d values for the minimer sequences derived from the Red.1 aptamer binding to L-serine were determined in solution using MST and are shown in Table 4. The 5′ loop minimer and the random region minimer showed apparent K_d values of 9.6 ± 4.4 and 5.2 ± 2.8 μM, respectively. The 3′ loop minimer and the minus 3′ primer minimer were unable to bind L-serine

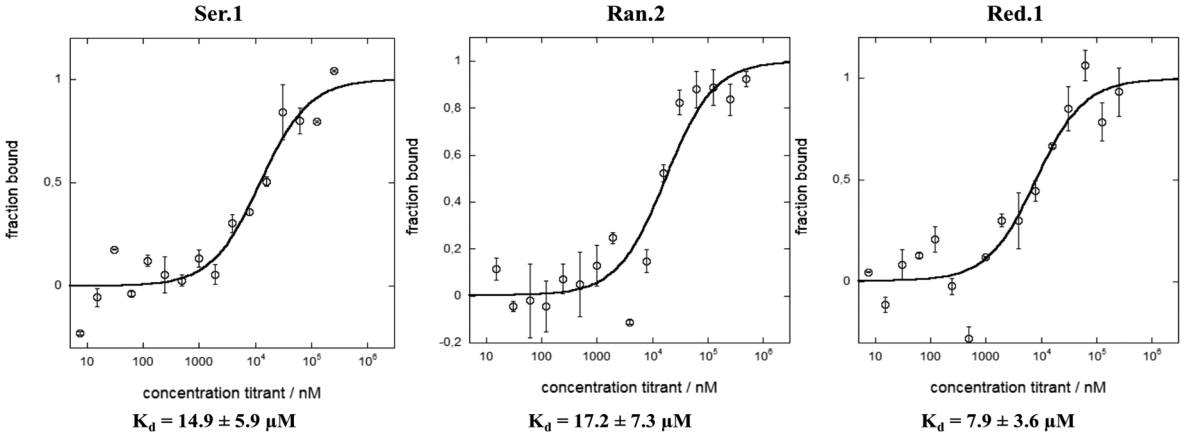

Figure 5. Aptamer candidates Ser.1, Ran.2, and Red.1 binding a range of L-serine concentrations in solution, measured by MST performed by 2bind. Fluorescently modified DNA (20 nM) was examined after incubation with a range of L-serine concentrations (from 7.6 nM to 250 μM).

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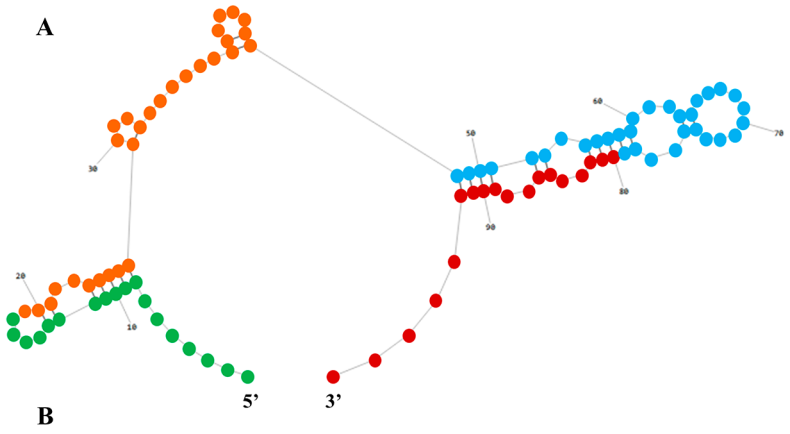

Red.1 Minimer	DNA Sequence (5'-3')
5'Loop	ATACCAGCTTATTCAATTAGATAGTAAGTGCAATCTAGATCGGAAGA
3'Loop	GCACACGTCTGAACTCCAGTCACTTAGGCATCAGATAGTAAGTGCAATCT
Random Region	AGATAGTAAGTGCAATCTAGATCGGAAGAGCACACGTCTGAACTCCAGTC ACTTAGGCATC
Minus 3' Primer	ATACCAGCTTATTCAATTAGATAGTAAGTGCAATCTAGATCGGAAGAGCAC ACGTCTGAACTCCAGTCACTTAGGCATC

Figure 6. Minimers were designed from the full-length Red.1 aptamer. (A) Red.1 structure determined by RNAstructure software,[36] where the colors of each segment describe the design of minimer sequences. The green and red portions of this sequence represent the 5′ and 3′ primer-binding regions, respectively. (B) Each minimer sequence designed from the Red.1 aptamer sequence, where the colors of the sequences reflect their position in the Red.1 aptamer sequence.

Table 4. Dissociation Constants of Red.1 and Its Minimer Sequences Determined by MST

aptamer candidate	K_d (μM)
Red.1	7.9 ± 3.6
5′ loop	9.6 ± 4.4
3′ loop	no binding
random region	5.2 ± 2.8
minus 3′ primer	no binding

in solution. Because Red.1 and its random region minimer were able to bind L-serine with the highest affinities, RNAstructure software was used to predict common secondary structures that could be present in these sequences and could be implicated in L-serine binding, shown in Figure 7. When these predicted secondary structures were examined for the presence of conserved sequence motifs, three motifs were found to participate in the stem−loop structure toward the 5′ end: the reverse primer-binding region (5′-AGATAGTAAG-TGCAATCT-3′) that had been inserted into Red.1, Red.2, and Ran.1, the 13-base conserved motif (5′-GGAAGAGCAC-ACG-3′) present in Ser.1, Ran.1, Red.1, and Red.2, and the GATC motif found in all aptamer candidates. Many aptamers have been found to interact with their targets through these

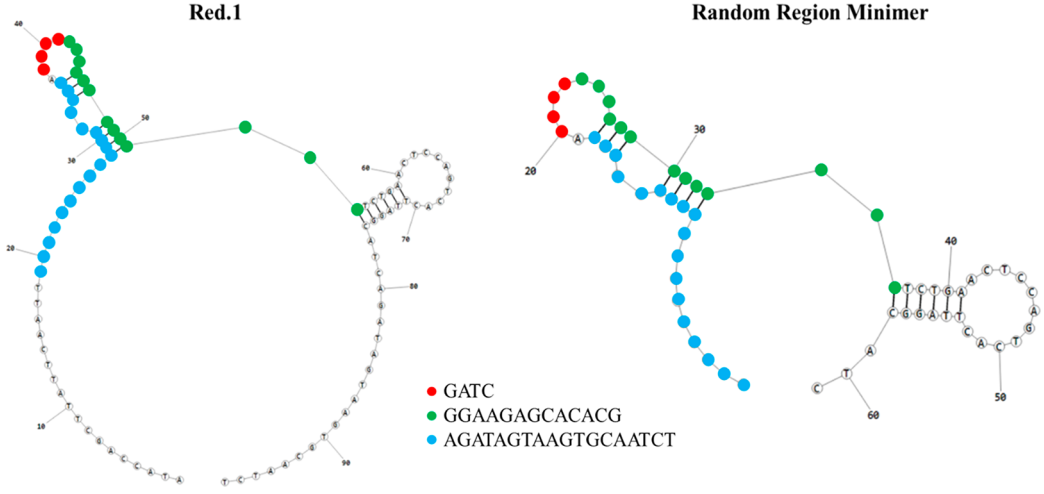

Figure 7. Conserved sequence motifs found in the shared predicted secondary structures of Red.1 and its random region minimer. Structure prediction was performed using RNAstructure software,[36] and conserved sequence regions are indicated by colored circles.

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loops and bulges, such as aptamers for dopamine, protein A, and ATP for example.[37−39] The presence of these conserved sequence motifs within the predicted stem−loop structure suggests that this structure could play an important role in binding L-serine. This stem−loop structure could be further studied using mutational analysis to determine its involvement in L-serine binding. It could be possible to shorten the minimer even further by eliminating non-conserved bases on the 3′ end.

Spiegelmer Binding. Incorporating aptamers into sensor and therapeutic applications often requires modifications to the DNA to improve its biological and chemical stability. Traditional DNA aptamers are composed of D-deoxyribonucleotides, which can be easily degraded by naturally occurring nucleases. Spiegelmers are mirror-image aptamers composed of L-deoxyribonucleotides, which afford Spiegelmers a resistance to nucleases that is orders of magnitude higher than traditional aptamers, because nucleases are stereoselective.[40] Serine contains a chiral carbon, generating L/D enantiomers. The selection experiments were completed using L-serine as the target, with no counter selection against its enantiomer D-serine. Synthesizing the random region minimer as a Spiegelmer allowed us to investigate the stereoselectivity of the binding pocket. MST was used to evaluate the binding affinities of the random region minimer and its corresponding Spiegelmer to L- and D-serine as well as to similar molecules Tris and L-glycine, and the K_d values are displayed in Table 5.

Table 5. Apparent K_d Values for the Random Region Minimer and Spiegelmer Binding L- and D-Serine, L-Glycine, and Tris Obtained by MST

	apparent K_d (μM)			
minimer	L-serine	D-serine	L-glycine	Tris
random region minimer	5.2 ± 2.7	13.3 ± 3.4	19.8 ± 4.9	0.2 ± 0.06
Spiegelmer	2.0 ± 0.8	3.7 ± 1.5	13.7 ± 8.6	5.2 ± 1.9

The random region minimer bound the target L-serine with an apparent K_d of 5.2 ± 2.7 μM and bound D-serine more weakly with an apparent K_d of 13.3 ± 3.4 μM. The Spiegelmer bound the target L-serine most strongly with an apparent K_d of 2.0 ± 0.8 μM while binding D-serine only slightly more weakly with an apparent K_d of 3.7 ± 1.5 μM. Because the Spiegelmer was able to bind strongly to both serine enantiomers, it is likely that the chiral carbon of serine was not part of the binding site or that the binding pocket was flexible enough to allow either serine enantiomer to bind. During the selections, the amino group of serine was used to immobilize L-serine to the Sepharose solid support and was therefore unlikely involved in the binding interaction. The remaining functional group was the alkyl hydroxyl side chain of serine, which was most likely the binding site for the random region minimer and Spiegelmer. The minimer and Spiegelmer had less affinity for L-glycine, with apparent K_d values of 19.8 ± 4.9 and 13.7 ± 8.6 μM, respectively, which could be attributed to the lack of an alkyl-hydroxyl group of L-glycine. Both the minimer and Spiegelmer were able to tightly bind Tris, with apparent K_d values of 0.23 ± 0.07 and 5.2 ± 1.9 μM, respectively. Tris offers three alkyl hydroxyl groups as binding sites, which could explain the affinity of the minimer and Spiegelmer. Of all of the aptamer candidates and minimers tested, the Spiegelmer showed the highest affinity for the target L-serine, with an apparent K_d of 2.0 ± 0.8 μM.

Spiegelmer Nuclease Resistance. Because aptamers are composed of DNA, their application is challenged by rapid nuclease degradation. Unmodified DNA aptamers in serum were shown to be degraded by nucleases in minutes.[41] Nucleases have to be considered when designing an aptamer for use in agricultural applications, because extracellular nucleases become released by soil bacteria.[42−44] The L-serine-binding Spiegelmer could provide nuclease resistance without negatively affecting target binding. To examine the stability of the L-serine-binding Spiegelmer compared to the random region minimer, each one was exposed to the nuclease DNase I at 37 °C for a range of time points. DNase I is an endonuclease, which non-specifically cleaves DNA, resulting in smaller oligonucleotide fragments. The results of this nuclease incubation with the random region minimer and the corresponding Spiegelmer are displayed in Figure 8.

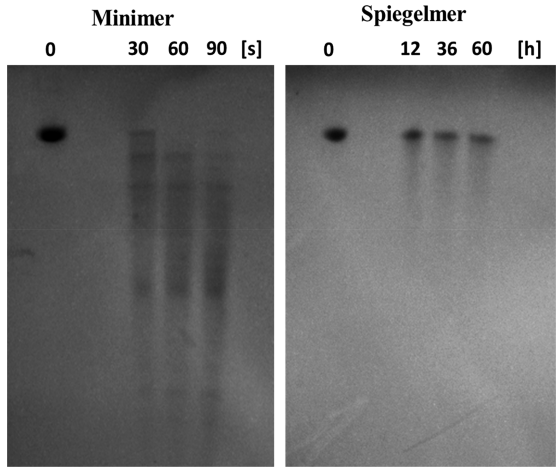

Figure 8. Stability of the L-serine-binding minimer (D-DNA) and Spiegelmer (L-DNA) in the presence of DNase I at 37 °C. After the time period indicated (time scale for the minimer is in seconds, while the time scale for the Spiegelmer is in hours), EDTA was added and DNA was heated to 90 °C to inactivate the enzyme and was stored at −20 °C until 19% denaturing PAGE could be performed.

For the random region minimer, which was composed of the natural D-DNA enantiomer found in nature, nuclease degradation proceeded quickly. After 30 s of DNase I exposure, the full-length minimer could still be observed but several degradation products were also visible. After only 1 min of DNase I exposure, no full length minimer could be observed. The Spiegelmer, composed of L-DNA, was exposed to DNase I at 37 °C for 60 h, and even after this extended exposure, full-length Spiegelmer was observed (Figure 8). The fainter bands observed over time suggested a low level of Spiegelmer degradation; however, the full-length Spiegelmer was still predominant. Naturally occurring nucleases show stereospecificity toward the degradation of D-DNA, allowing the L-DNA of Spiegelmers to escape degradation.[34] Using the L-serine binding Spiegelmer for further applications would maintain the binding affinity while also providing the nuclease resistance needed for incorporation into agricultural biosensing applications. Several selection strategies were explored in parallel for the discovery of a L-serine binding aptamer. Analysis of each SELEX experiment showed that using an original library of DNA sequences as the starting pool provided the most sequence diversity as well as the biggest change in

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enrichment over the selection. Using a randomized aptamer library showed the biggest change in sequence diversity. Using the final rounds from a previous L-homocysteine selection as the starting library provided the least diversity in the starting pool but resulted in the highest level of enrichment. Considerable sequence homology was found among aptamer candidates from each SELEX experiment, which provided possible L-serine binding motifs for further study. Each SELEX strategy, combined with HTS, allowed for the identification of L-serine-binding aptamer candidates enriched in soil solution, which can now be further characterized for use in agricultural biosensing applications.

Each of the three SELEX experiments performed for L-serine generated one aptamer candidate that bound only to immobilized L-serine and one candidate that bound both immobilized and free L-serine. This finding suggested that all three SELEX strategies employed herein could be useful, because L-serine-binding aptamers with micromolar affinity were generated from an original library, from a redirected L-homocysteine selection, and from a randomized L-homocysteine aptamer library. The aptamer Red.1, from the redirected L-homocysteine SELEX library, showed the most affinity for L-serine in both the Sepharose bead test and MST, with apparent K_d values of 2.8 ± 2.0 and 7.9 ± 3.6 μM, respectively. The ability to reuse libraries after several rounds of SELEX for similar molecules could save researchers time as well as produce high-quality aptamers at a lower cost. The Ran.2 aptamer from the randomized aptamer library may provide the most selectivity, because it did not appear to bind to Tris strongly or to L-glycine in the Sepharose tests. These results underscore the importance of performing negative selections in SELEX for small molecules, because both the randomized SELEX pool and the redirected SELEX pool had undergone several negative selections against Sepharose beads before beginning the L-serine SELEX. These extra negative selections could have contributed to the selection of aptamers with higher affinity and selectivity.

Removing the primer-binding regions from Red.1 improved the binding affinity to 5.2 ± 2.7 μM, and one stem−loop structure containing three conserved sequence motifs was predicted to be important for L-serine binding. A serine-binding Spiegelmer was generated, which suggested that the alkyl hydroxyl side chain of serine was important for aptamer binding. The Spiegelmer had the highest affinity for L-serine, with an apparent K_d of 2.0 ± 0.8 μM. This Spiegelmer was also shown to resist nuclease degradation for at least 60 h. This Spiegelmer has the affinity for L-serine and stability from nucleases, which could allow it to be incorporated into aptamer-based materials for agricultural applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c06796.

Binding isotherm of Hcy10 to L-serine (Figure S1), count and enrichment scores of aptamer candidates from HTS and AptaCluster analysis (Table S1), non-specific binding control to L-serine Sepharose (Figure S2), MST results for Ser.1, Ran.1, and Red.2 (Figure S3), detailed SELEX conditions (Table S2), and HTS preparation (Table S3) (PDF)

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Notes

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