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ARTICLE *in* ANALYTICAL CHEMISTRY · MAY 2011

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DNA Aptamers Binding to Multiple Prevalent M-Types of *Streptococcus pyogenes*

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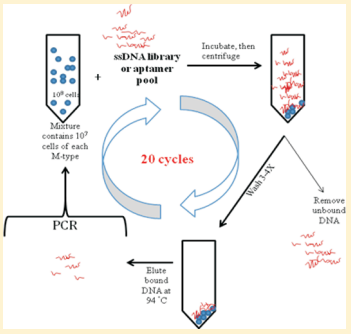
Supporting Information

ABSTRACT: This paper describes the selection of high affinity DNA aptamers binding to multiple M-types of the pathogenic species *Streptococcus pyogenes* (Group A *Streptococcus* or GAS). Unlike common aptamer selection techniques that use purified molecules of a monoclonal cell population as targets, this work has achieved the selection of aptamers against the various M-types of *S. pyogenes*. Cell mixtures containing equal numbers of the 10 most prevalent *S. pyogenes* M-types were incubated with 80-nucleotide DNA libraries, centrifuged, and washed to separate cell-bound from unbound DNA sequences. The DNA bound to the cells was amplified using the polymerase chain reaction, and the amplicons were tested for their binding to the target cells. The amplicons were also used as new DNA libraries for subsequent rounds of selection. Cloning, sequencing, and subsequent analysis of selected aptamers showed that they bind preferentially to GAS over other common and related bacteria. Resultant DNA aptamers showed strong and preferential binding to GAS, including the 10 most prevalent GAS M-types and another 10 minor M-types tested. Estimated K_d values were in the range of 4 to 86 nM. Two aptamers, 20A24P and 15A3P (with estimated binding dissociation constants of 9 and 10 nM, respectively), are particularly promising. These aptamers could potentially be used to improve the detection of GAS, a pathogen that is the causative agent of many infectious diseases, most notably strep throat.



Group A *Streptococcus* (GAS) is implicated in a variety of ailments, including streptococcal pharyngitis, necrotizing fasciitis, scarlet fever, streptococcal toxic shock syndrome (STSS), invasive systemic infections, and endocarditis.[1] Among the most common patient samples analyzed for GAS are throat and skin swabs and wound aspirate. Current point-of-testing methodology relies on either culture or antibody-based rapid antigen detection (RAD). The most common type of RAD is a 2-site sandwich immunoassay, to detect the Group A cell wall carbohydrate. Culture requires at minimum 6−8 h incubation, often overnight.[2] RAD is more rapid, taking only minutes but has poor sensitivity, and negative results require a confirmatory culture step.[3] The sensitivity of both culture and RAD depends also on the presence of a sufficient number of live cells in the inoculum. Thus, there is great need to develop alternative, improved methods for routine testing. Novel aptamer-based point-of-care tests could complement existing technologies.

Aptamers are short, synthetic nucleic acid molecules capable of binding to a target molecule with high affinity and selectivity.[4,5] They are created via a combinatorial chemistry process known as systematic evolution of ligands via exponential enrichment or SELEX.[4,5] Aptamers represent an ideal class of molecules for inclusion in laboratory diagnostic tests since they possess a variety of advantages over antibodies. These include ease of production and improved temperature stability. The affinity and selectivity of aptamers are comparable to or better than those of antibodies, with binding dissociation constants for aptamer−target complexes frequently in the micromolar to picomolar range.[5−11] The high affinity of aptamers for their targets translates into highly sensitive assays. In addition, aptamers can be easily modified and attached to surfaces with a variety of chemistries; aptamer coated surfaces can often be heated and reused. A critical limitation to wide application of aptamers is the shortage of identified aptamer sequences for molecular targets. There is no aptamer available for GAS.

We describe the selection of aptamers against a mixture of the 10 most prevalent GAS M-types in Canada: M1, M2, M3, M4, M6, M11, M12, M28, M77, and M89.[12] GAS is an ideal candidate for aptamer selection due to its well-characterized surface and protein-based serotyping system. The purpose of the use of whole live bacterial cells in SELEX is to negate the need for a priori purification of specific target molecules from the cell surface; similar methods have been employed previously on *L. acidophilus*, *E. coli*, *M. tuberculosis*, *S. aureus*, and *C. jejuni*.[13−16] However, these methods have focused on one cell type at a time, usually a monoclonal population. The present study is the first in which a mixture of bacterial types has been used with the aim of broadening aptamer selectivity. The advantage of this approach is that the resultant aptamer pool is expected to contain sequences that can recognize and detect a variety of *S. pyogenes* M-types.

Received: March 4, 2011
Accepted: April 8, 2011
Published: April 19, 2011

EXPERIMENTAL SECTION

Bacterial Strains and Culture Medium. All work with GAS was carried out in a Level 2 certified biosafety cabinet. Aseptic technique was practiced with all cultures. All bacterial waste was autoclaved at a minimum temperature of 121 °C for 45 min prior to disposal. *Streptococcus pyogenes* clinical isolates corresponding to M-types M1, M2, M3, M4, M5, M6, M11, M12, M28, M41, M49, M59, M75, M77, M82, M83, M89, M91, M92, and M114 were obtained from the National Centre for Streptococcus (Provincial Laboratory, University of Alberta, Edmonton, AB). *S. pyogenes* isolates were streaked out on 5% defibrinated sheep's blood agar (Teknova, Hollister, CA), and single colonies were cultured in Todd-Hewitt Broth (Oxoid, Nepean, ON). *Streptococcus bovis* and *Escherichia coli* were obtained from the American Type Culture Collection (ATCC) and were cultured in brain heart infusion (BHI) (Teknova) and Luria-Bertani (LB) media (BD Difco, Sparks, MD), respectively. *Streptococcus pneumoniae* (serotypes 4, 6B, 9 V, 14, 18C, 19F, 23F, 19A, 5, and 6A), *Enterococcus* sp. (*E. saccharolyticus*, *E. raffinosus*, *E. pseudoavium*, *E. mundtii*, *E. malodoratus*, *E. hirae*, *E. gallinarum*, *E. faecium*, *E. faecalis*, *E. durans*, *E. cecorum*, *E. casseliflavus*, *E. avium*) and Group B *Streptococcus* strains (975R547 IV, JM9 VIII, 7271 VII, 975R390 VI, 965R400 Ia, 975R384 V, 12351 IV, 965R155 Ia, 975R938 II, 975R27 Ib, 975R331 IV, 955R2028 IV, 975R591 III, 975R138 II, 975R570 Ib, 975R104 VII, 9842 VI, 975R594 III) were also obtained from the National Centre for Streptococcus. *S. pneumoniae* and *Enterococcus* sp. were on 5% defibrinated sheep's blood agar, and streaked out colonies were cultured in BHI broth; GBS isolates were cultured in TH broth. All bacteria were cultured overnight in aerobic conditions at 37 °C, and all liquid cultures were shaken at 200 rpm. *E. coli* DH5α-T1^R cells (Invitrogen, Carlsbad, CA) were used for all transformations.

DNA Library. An 80-nt oligonucleotide single-stranded DNA library consisting of a 40-nt randomized region flanked on both sides by 20-nt primer regions was used. The initial ssDNA library and the primers used to amplify it were obtained from Integrated DNA Technologies (Coralville, IA). DNA library or aptamer pools were rendered single stranded via heat denaturation at 94 °C for 10 min and subsequent cooling at 0 °C for 5 min.

PCR Amplification and Gel Electrophoresis. The primers used to amplify the ssDNA library and subsequent aptamer pools had the following sequences:

Forward: 5′-AGCAGCACAGAGGTCAGATG-3′

Reverse: 5′-TTCACGGTAGCACGCATAGG-3′

The PCR conditions for amplification of the DNA aptamer pools during SELEX were similar to those previously described.[13] After PCR, the reaction products were separated on 7.5% nondenaturing polyacrylamide gel electrophoresis (PAGE) in TBE buffer (Bio-Rad Protean III) at 60−120 V. The gels were stained with ethidium bromide and photographed under UV light. All PCR products were purified using Qiagen MinElute PCR Purification Kit (Qiagen Inc., Valencia, CA).

Aptamer Selection. SELEX was carried out using a procedure modified from Hamula et al.[13] Single colonies of 10 different *S. pyogenes* M-types (M1, M2, M3, M4, M6, M11, M12, M28, M77, and M89) were grown overnight in separate liquid cultures. The cells were then subcultured to a second set of tubes (with a 1:100 inoculum-to-media ratio) and were harvested upon reaching logarithmic phase (minimum OD_{600} of 0.3). Aliquots containing the same number of cells from each culture were combined only once growth was complete. Cell mixtures were centrifuged at 6000*g* and 4 °C for 10 min to remove media and wash supernatants. The varying SELEX incubation conditions and amounts of reagents per round are summarized in the Supporting Information Table S1. The ratio of DNA to cells was kept constant. SELEX was initiated with randomized ssDNA library (2 nmole initial round), and 100 pmole of aptamer pool was used as input in subsequent rounds. A total of 10^8 cells, containing an equal number of cells of each M-type (10^7), was used for each round of selection. An excess of tRNA and bovine serum albumin (BSA; Invitrogen) was added to the incubation buffer (20-fold molar excess of each in the initial round, up to a maximum 400-fold molar excess in round 20), and 0.05% w/v BSA was added to the wash buffer. The use of increasing amounts of BSA/tRNA increases the competition between the desired target (cells) and nontarget (BSA molecules) for aptamer molecules. The tRNA is present to compete with the aptamer sequences for target binding sites. A total of 20 rounds of SELEX was performed.

All washes and incubations were carried out in binding buffer (1× BB; 50 mM Tris−HCl (pH 7.4), 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$) at room temperature for 45 min. An initial incubation volume of 0.5 mL was used for round one, and this was decreased to 0.25 mL for subsequent rounds; four washes were carried out for each round of SELEX until round 8 at which the number of washes was decreased to three in order to increase the yield after amplification. To confirm the applicability of the multitarget cell-SELEX method, we also carried out a second SELEX set for 13 rounds, SELEX B. The detailed procedures for this set of SELEX are described in Supporting Information and Table S1.

Flow Cytometric Analysis of Aptamer Pool and Individual Aptamer Binding. A FACScan flow cytometer with PowerMac G4 workstation and CellQuest software (Flow Cytometry Facility, Faculty of Medicine and Dentistry, University of Alberta) was used to assess the binding of the aptamer pool and individual aptamer sequences to different types of cells (*S. pyogenes*, *Enterococcus* sp., *S. pneumoniae*, *S. agalactiae*, *E. coli* DH5a, *S. bovis*). The aptamer pools were fluorescently labeled via PCR amplification with 5′-FAM modified primers (IDT), whereas the individual aptamer sequences were purchased with the fluorescent label (5′-FAM) attached (IDT). Aptamer pools were heat denatured prior to incubation with bacterial cells. The binding assays were carried out by incubating 200 pmole of fluorescently labeled aptamer/aptamer pool with 10^8 cells for 45 min, as in the SELEX process, and then washing the cells once in binding buffer prior to resuspension in binding buffer for immediate flow cytometric analysis. In cases where mixtures of cells were used, an equal number of each cell type was combined to a total of 10^8 cells for screenings and 10^9 cells for obtaining binding curves. Forward scatter, side scatter, and fluorescence intensity (FL1-H) were measured, and gated fluorescence intensity above background (cells with no aptamers added) was quantified. The fluorescently labeled ssDNA library was used as a control for nonspecific binding in each experiment. Binding curves were run to estimate K_d values by varying aptamer concentrations (0−150 nM incubation) with a fixed number of cells (10^9). GraphPad Prism 5.0 software was used to obtain nonlinear regression curve fitting from which K_d values were estimated. All cultures used for flow cytometric screening were harvested in stationary phase in order to minimize differences in cell surface molecule expression.

Cloning, Sequencing, and Structural Analysis of Aptamers. The highest affinity aptamer pools measured via flow cytometry

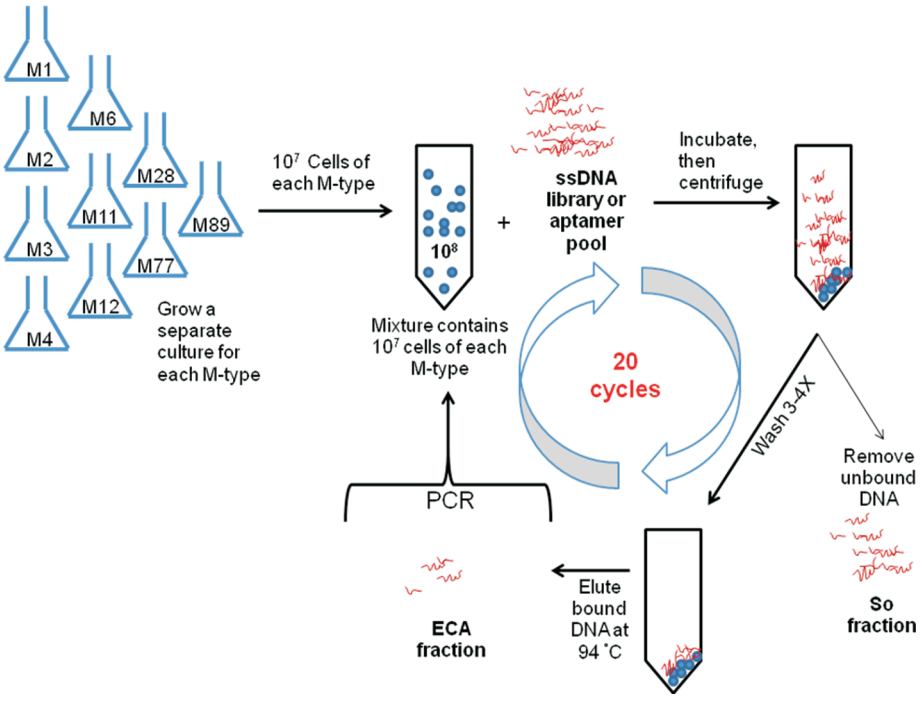


Figure 1. Schematic of bacterial cell SELEX against a mixture of the 10 most prevalent GAS M-types in Canada. A total of 10^8 cells were used for each round of selection, consisting of 10^7 cells of each M-type. Each M-type was cultured separately, and cells were combined immediately prior to selection. Other selection conditions are summarized in Table S1, Supporting Information.

were chosen for sequencing analysis: pools 15A, 13B, and 20A. Aptamer pools were cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen), transformed into *E. coli* DH5α-T1^R cells (Invitrogen), and colonies containing the vector were selected via overnight incubation at 37 °C on LB plates containing 50 μg/mL kanamycin. From each aptamer pool, 20 colonies were chosen for screening. The plasmid DNA was purified (Qiaex II gel extraction kit; Qiagen, Mississauga, ON) and analyzed for the presence of an 80 bp insert via digestion with 1 U of EcoR1 at 37 °C for 30 min, followed by 7.5% native PAGE. A total of 60 inserts were then sequenced (The Applied Genomics Centre, Department of Medical Genetics, University of Alberta), yielding of 57 useable sequences. The secondary structure of each sequence was predicted using Oligoanalyzer 3.0 (IDT), with input conditions of room temperature (21 °C) and 1 mM $MgCl_2$. The most likely sequence was chosen on the basis of the lowest predicted free energy of formation (ΔG; kcal/mol).

RESULTS

Aptamer Selection. The approach for generating aptamers is summarized in Figure 1. To select for aptamers that potentially recognize the common M-types of *S. pyogenes*, we chose a mixture containing an equal number of cells from each of the 10 most prevalent *S. pyogenes* M-types in Canada as the target. A randomized oligonucleotide library was incubated with the cell mixture, and cells were then separated from the supernatant via centrifugation. Fractions representing the incubation supernatant (So), washes (W), and heat-eluted cell-bound aptamers (ECA) were collected and amplified. As can be seen in Figure S1 (Supporting Information), the majority of the DNA is retained in the supernatant fraction. However, a substantial amount of DNA remains on the cells, as can be seen from the first three consecutive washes. DNA in the fourth wash was no longer detectable after PCR. The aptamers are then heat eluted from the cells at high temperature in low salt (ECA fraction). Even after heat elution, some sequences remain bound to the cells. The ECA fraction and the cells themselves were amplified for use as inputs in the next round of SELEX. A negative control consisting of cells without added DNA library was run concurrently through the entire procedure of incubation, washing, heat elution, and lysing of the cells. The negative control of these bacterial cells did not produce detectable PCR products of the expected aptamer size (80 nt).

The amplification products of the ECA fraction (Supporting Information Figure S3 lane 13) represent the DNA sequences bound to the cells. The observation of a single 80-bp band on the gel after each round of selection and PCR amplification of the ECA fractions suggests that the cells were able to bind to a pool of aptamer sequences. No DNA was detectable from the wash or ECA fractions of the negative control, which consisted of cells treated to the incubation, wash, and heat elution procedures without the addition of library or aptamer pool DNA (Supporting Information Figure S3 even-numbered lanes). The remaining gel photos for SELEX 2A to 20A (round 2 through round 20) are similar in that the So and W contained the unbound DNA and the ECA fractions showed the presence of bound DNA sequences.

Binding Affinity and Selectivity of Aptamer Pools Following Each Round of Selection. Flow cytometric analyses of incubation mixtures containing fluorescently labeled aptamer pools and the target cell mixture were carried out to assess the affinity of the aptamer pools for *S. pyogenes*. An increase in the number of fluorescent cells is due to the increased binding of the fluorescent aptamers to the target cells. Negative controls consisting of cells alone and target cells incubated with

fluorescently labeled randomized library were carried out concurrently. Controls carried out using fluorescent aptamer pools alone and buffer with BSA and tRNA alone did not yield any increase in gated fluorescence above background levels (data not shown).

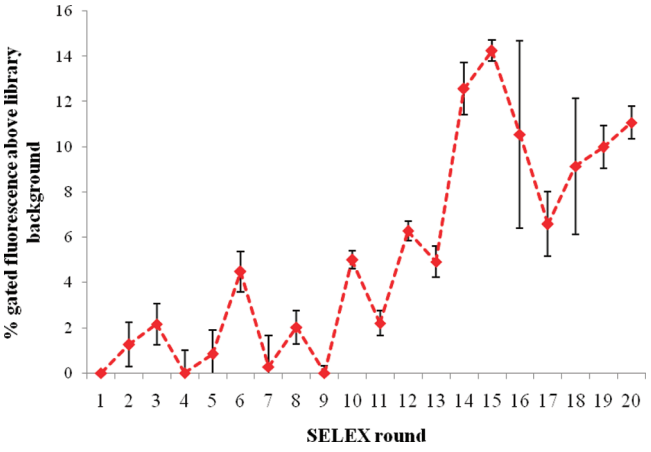


Figure 2. Average percent gated fluorescence intensity of *S. pyogenes* M-type mixture incubated with fluorescently labeled aptamer pools obtained from increasing SELEX rounds. A total of 10^8 cells, consisting of 10^7 cells of each of 10 separate M-types, were combined and pelleted at 6000*g* for 10 min. The medium was removed, and cells were washed twice in an equal volume of binding buffer prior to incubation with aptamers. Aptamer pools were fluorescently labeled with 5′-FAM and a total of 200 pmole of each aptamer pool was incubated with cells for 45 min at room temperature, under gentle rotation. Cells were then centrifuged at 6000*g* and 4 °C for 10 min, washed, and resuspended in binding buffer for flow cytometry analysis. A total of 10 000 events were counted for each analysis; triplicate analyses of triplicate incubations were carried out. A 5′-FAM labeled randomized library and cell control was run for each set of incubations, and average percent gated fluorescence of the cells bound to the library was subtracted from the values measured for each aptamer pool.

With increasing rounds of selection, the percent of cells with fluorescence above library background increased to a maximum average of 14% at round 15 for SELEX aptamer pools (Figure 2). Aptamer pool binding did not start to increase above background until round 10 (Figure 2); the percent of cells with fluorescence above library decreased from 14% at round 15 to 7% at round 17 before increasing again to 11% at round 20 (Figure 2).

Cloning and Sequence Analysis of Aptamer Pools. Aptamer pools from the 15th and 20th rounds of SELEX displayed the highest affinity for the target cells when screened via flow cytometry (Figure 2). The aptamer pools were cloned and sequenced; a total of 57 sequences were obtained. Analyses of all sequences both with and without primers revealed minimal sequence repetition with many sequences containing high GC content, indicative of secondary structure formation. Within the SELEX round 15 pool, sequences 15A2 and 15A15 are identical, as are sequences 15A8, 15A16, and 15A17 (Supporting Information Table S3). For the SELEX round 20 pool, 20A6, 20A15, and 20A17 are identical, as is 15A10 (Supporting Information Table S3). Sequences were chosen for further screening based not only on their repetitiveness but also on predicted secondary structures and free energies of formation.

Binding of Individual Aptamer Sequences to Target Cell Mixture. Fluorescently labeled aptamer sequences were incubated with the target mixture of ten *S. pyogenes* M-types used for selection and analyzed via flow cytometry. Aptamer sequences obtained after the 20th round of SELEX seem to have the highest affinity for the *S. pyogenes* M-type mixture. A total of nine sequences from that pool had greater than 50% gated fluorescence intensity above a randomized library control. These sequences are summarized in Table 1. Sequences 20A1, 20A1P (with primers), and 20A8 were the highest binders with gated fluorescence above background values of around 72%. The highest binding sequence in the 15A pool is 15A3P with a gated fluorescence above background of 48% (Figure 3).

Sequences 20A1, 20A8, and 20A9 form hairpins both in the absence and presence of the primer sequences. The affinity of these three aptamers for the *S. pyogenes* mixture changed minimally upon inclusion or exclusion of primers in the sequence; it remained at 72% gated fluorescence intensity above library for

Table 1. Tested Aptamer Sequences with (80 nt) and without (40 nt) Primers[a]

name	sequence
20A1	5′FAM/ CAGAACGCACCCGCACACCTCCATCACTCGCATGCACCCC-3′
20A1P	5′FAM/ TTC ACG GTA GCA CGC ATA GG CAGAACGCACCCGCACACCTCCATCACTCGCATGCACCCC CAT CTG ACC TCT GTG CTG CT-3′
20A8	5′FAM/ CCCCACGAATCGTTACTCTGGTCCTCTATTTCTCCTCCCC-3′
20A8P	5′FAM/ AGC AGC ACA GAG GTC AGA TG CCCCACGAATCGTTACTCTGGTCCTCTATTTCTC CTCCCC CCT ATG CGT GCT ACC GTG AA-3′
20A9	5′FAM/ CACACGCTGAAGAAACTGAGGTCGTAGGTTTTCTTCGGG-3′
20A9P	5′FAM/ AGC AGC ACA GAG GTC AGA TG CACACGCTGAAGAAACTGAGGTCGTAGGTTTTCTTCGGG CCT ATG CGT GCT ACC GTG AA-3′
20A12P	5′FAM/ TTC ACG GTA GCA CGC ATA GG GCCCGACACTCGTCCACCCGATACCTCTCATGTGTCCC CAT CTG ACC TCT GTG CTG CT-3′
20A14P	5′FAM/ AGC AGC ACA GAG GTC AGA TG GGCATGGGGAAGAGAAAGCGGGATAACTTCGTTACCGGGC CCT ATG CGT GCT ACC GTG AA-3′
20A24P	5′-FAM/ AGC AGC ACA GAG GTC AGA TG GGGGGAAGACACAGAGAAAGGCCGGGGTGAAGTGTAGAGG CCT ATG CGT GCT ACC GTG AA-3′
15A 3P	5′FAM/ TTC ACG GTA GCA CGC ATA GG GACAGCAAGCCCAAGCTGGGTGTGCAAGGTGAGGAGTGGG CAT CTG ACC TCT GTG CTG CT-3′

[a] Primer sequences are underlined.

dx.doi.org/10.1021/ac200575e |*Anal. Chem.* 2011, 83, 3640–3647

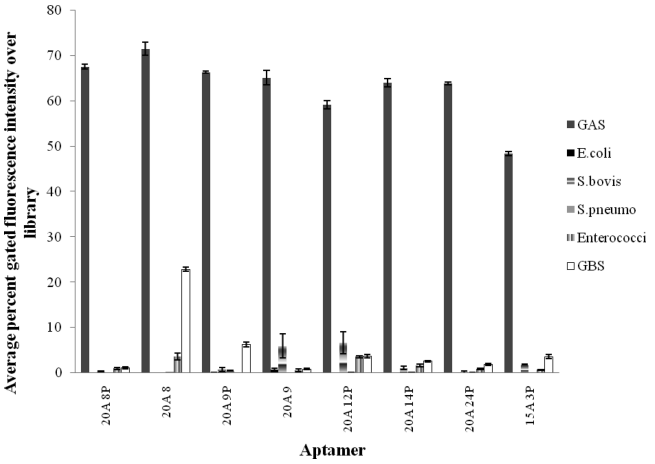


Figure 3. Sequences from SELEX 15A and 20A aptamer pools preferentially bind to the target GAS over other types of cells. All aptamers were fluorescently labeled with 5′-FAM. Incubations and centrifugation were carried out as in Figure 2. A ratio of 200 pmole of aptamer and 10^8 cells was used. Each species tested consisted of a mixture of separate strains, with each strain being cultured separately and combined immediately prior to selection. Cell types tested are summarized in Supporting Information Table S2, except for GAS. The GAS mixture analyzed was the target mixture of 10 separate M-types used during SELEX.

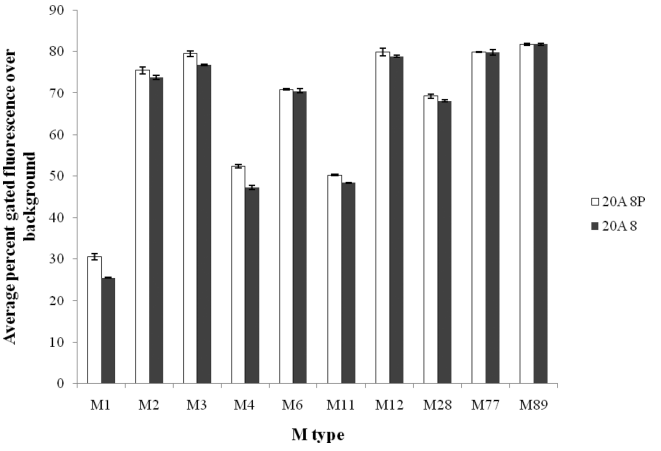


Figure 4. Binding of high affinity aptamer sequences to separate M-types. Analyses were carried out as in Figure 3, except that a total of 200 pmole of each aptamer sequence was incubated with 10^8 cells of each M-type separately, not as a mixture. 20A8P is an 80-nt full length sequence containing two 20-nt primers flanked on either end. 20A8 is a 40-nt sequence.

both 20A1 and 20A1P, 72% for 20A8 and 68% for 20A8P, and 65% for 20A9 and 66% for 20A9P (Figure 3). These results suggest that the variable region of the 40 nt sequences, not the 20 nt primers flanked at either end, is likely responsible for the affinity binding to the target.

Sequences with no or minimal affinity for the target cells tended to have minimal predicted secondary structures (data not shown). However, not all nonhairpin sequences have low affinity for the target cells. Sequence 20A24P forms a branched structure with high affinity for the target cell mixture (gated fluorescence intensity above library of 64% in Figure 3); removal of the primers negates this affinity (8% in Figure 3). The sequence 20A24 forms a hairpin.

Selectivity of High Affinity Aptamers for *S. pyogenes*. Fluorescently labeled aptamer sequences 20A8, 20A8P, 20A9, 20A9P, 15A3P, 20A24P, 20A12P, and 20A14P were tested against a variety of other bacteria including pathogens and commensal flora that could potentially interfere with a diagnostic test. Specifically, binding to other species of *Streptococcus* was tested using nonpathogenic *S. bovis*, and the pathogens Group B *Streptococcus* and *S. pneumoniae*. In addition, *Escherichia coli* DH5α was used to assess aptamer binding to a representative gram negative organism. Sequences were also screened against the human flora *Enterococcus* sp. For the *S. pneumoniae*, *S. agalactiae*, and *Enterococcus* isolates, mixtures were prepared containing an equal number of cells from multiple isolates, as were the *S. pyogenes* mixtures used for selection. A summary of the isolates for each mixture is presented in Supporting Information Table S2.

All selected aptamer sequences showed preferential binding to GAS over the other cells tested (Figure 3). The preferential binding confers the selectivity of these aptamers for GAS. The least selectivity was obtained with the aptamer 20A8, which seemed to have some affinity for the Group B *Streptococcus* (GBS) mixture (Figure 3). However, this affinity was low in comparison to the *S. pyogenes* target cell mixture. The percent gated fluorescence intensity above library background was 23% when 20A8 was incubated with GBS cells and

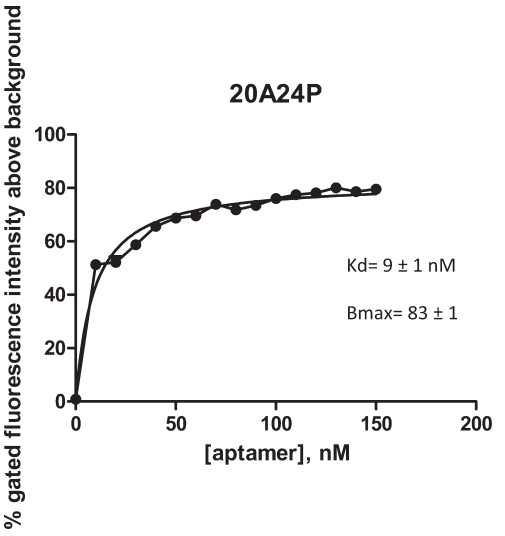
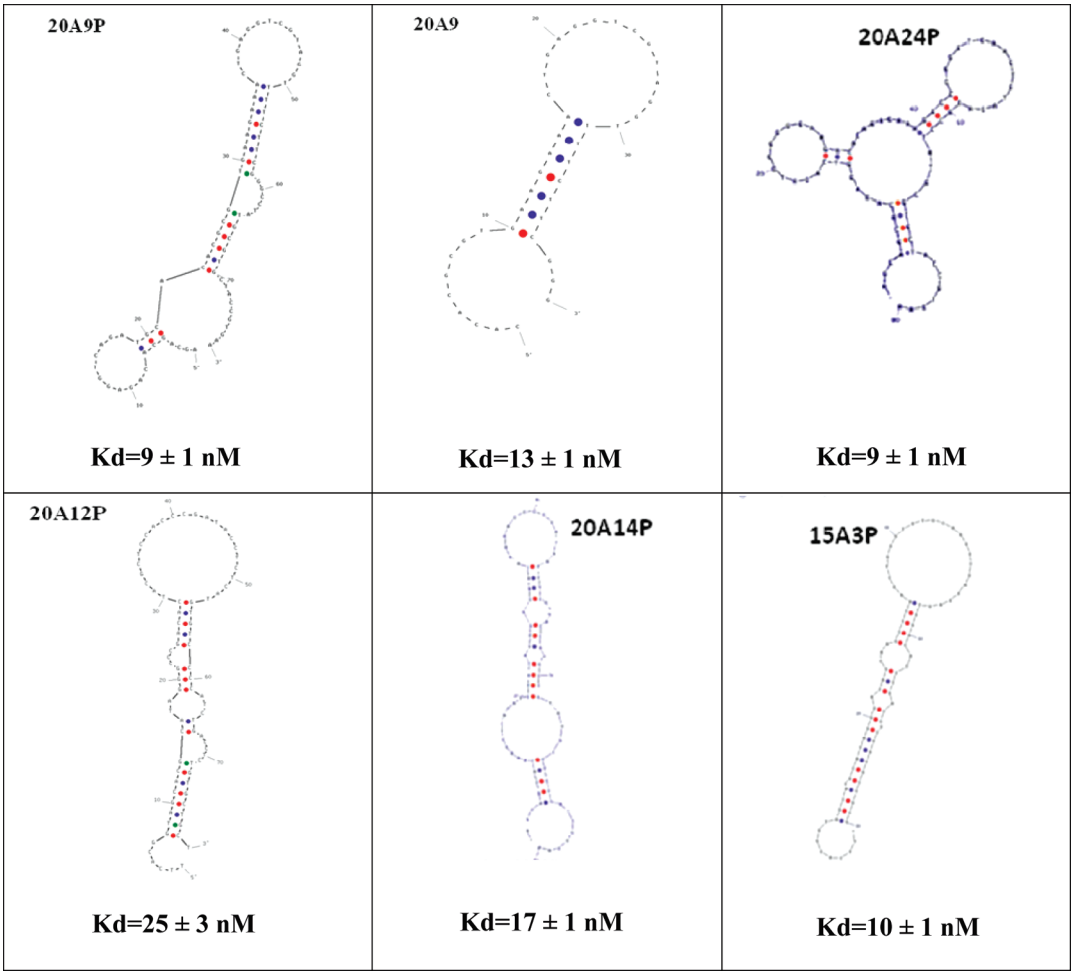


Figure 5. Binding saturation curve of aptamer 20A24P to the target mixture of *S. pyogenes* cells. A mixture containing a total of 10^9 cells, consisting of 10^8 cells of each of the 10 target M-types, was incubated with varying concentrations of 20A24P. Flow cytometry was carried out as done previously, except that cells were not washed after incubation, and library binding was not subtracted from the average gated fluorescence values. A nonlinear regression curve was fit to the data using GraphPad Prism 5.0. Similar experiments were run for all high affinity aptamer sequences, and the estimated K_d values are summarized in Table 2.

72% when incubated with the original *S. pyogenes* target cell mixture (Figure 3). Inclusion of the primer sequences (20A8P) seems to negate GBS binding, bringing the percent gated fluorescence intensity above library background down to 1%. The same sequence (20A8P) binding to the target GAS cells yielded 68% gated fluorescence above background (Figure 3). It can, thus, be concluded that the aptamer sequences tested are specific for *S. pyogenes*.

M-Type Selectivity of Aptamers. Fluorescently labeled individual aptamer sequences were also screened via flow cytometry against separate *S. pyogenes* M-types. As expected, these aptamers bind to all 10 individual M-types that were included in the selection

Table 2. Binding Dissociation Constants (K_d) and Predicted Secondary Structures of Aptamer Sequences That Have High Affinity and Selectivity for a Mixture of 10 Different *S. pyogenes* M-Types Used in SELEX



process. Aptamer sequences with high affinity to the 10 M-type mixture exhibited high binding to most of the M-types tested separately. For example, sequences 20A8 and 20A8P have very similar affinities for a given M-type, with greater than 50% gated fluorescence above background for all M-types except M1. M-types M2, M3, M6, M12, M77, and M89 have gated fluorescence above background greater than 70% (Figure 4). The high affinity sequences 20A24P and 15A3P showed a similar trend (data not shown). This trend of aptamer affinity for the target cell mixture mirroring aptamer affinity for individual M-types is also true for sequences with medium and low affinities (Figure S4a,b in Supporting Information). Also of note, the presence or absence of primers had little effect on GAS or M-type affinity of sequence 20A8/20A8P, indicating that the hypervariable region in the center of the aptamer sequence is responsible for binding (Figure 4).

The tested aptamer sequences also bound *S. pyogenes* M-types other than the common 10 used for SELEX. A cell mixture containing 10 M-types not used for selection was tested (Supporting Information Figure S5). The M-types included in the mixture were M5, M41, M49, M59, M75, M82, M83, M91, M92, and M114. Binding to this mixture was observed for all sequences tested, ranging from 23% for 20A8 to 1% for 20A9. Hence, it appears that the selection resulted in aptamer pools containing a mixture of sequences of similar high affinity for all target M-types and lower affinity to nontarget M-types.

Estimation of Binding Dissociation Constants (K_d) of High Affinity Aptamer Sequences. Figure 5 shows a typical binding saturation curve from flow cytometric analysis of the fluorescently labeled 20A24P aptamer and the mixture of 10 different *S. pyogenes* M-types used for selection (10^8 cells). Estimates from nonlinear regression curve fit showed a K_d value of 9 ± 1 nM and B_{max} of 83% gated fluorescence above background.

Binding of a fluorescently labeled randomized oligonucleotide library to the *S. pyogenes* cell mixture was also examined as a negative control. Average gated fluorescence intensity increased linearly with library concentration and was found to reach a maximum of 23% at a library concentration of 150 nM (data not shown). In contrast, all aptamer sequences tested exhibited saturation binding kinetics. The maximum percent gated fluorescence intensity leveled off before or at an aptamer concentration of 50 nM for all sequences tested.

Table 2 summarizes the estimated K_d and predicted secondary structures of the most selective aptamers for *S. pyogenes*. Sequences 20A24P, 20A9P, and 15A3P had the highest affinities and selectivities for the target cell mixture, as all had K_d values below or equal to 10 nM (9.1 ± 0.6 nM for 20A9P; 9.1 ± 0.8 nM

dx.doi.org/10.1021/ac200575e |*Anal. Chem.* 2011, 83, 3640–3647

for 20A24P; and 9.6 ± 0.3 nM for 15A3P). Sequences 20A8 and 20A9 had very high affinities for GAS (respective K_d values of 4 ± 1 nM and 9 ± 1 nM) but are less desirable than other sequences due to their high cross-reactivity with Group B *Streptococcus* and *Enterococcus* cells (Figure 3).

DISCUSSION

We selected aptamers against a mixture of the 10 most prevalent GAS M-types in Canada: M1, M2, M3, M4, M6, M11, M12, M28, M77, and M89.[12] Several resultant aptamer sequences were promising on the basis of the obtained affinity and specificity data. The sequence 20A24P had the highest affinity and selectivity for *S. pyogenes*, with an apparent K_d of 9 ± 1 nM and very low levels of binding to other *Streptococcus* and *Enterococcus* species. Another promising sequence was 15A3P, with high selectivity for *S. pyogenes* and an apparent K_d of 10 ± 1 nM. None of the sequences from these sets of SELEX were M-type specific. However, they are potentially useful in a point-of-care diagnostic test for *S. pyogenes*. Many of the high affinity sequences bound to GAS M-types other than those in the initial target mixture (Figure 4). The high affinity aptamers could also be used together to identify strains of GAS based on the binding pattern produced. Previous studies have used multiple cell-specific aptamers together as a panel for flow cytometric identification and typing of cancer cells, *Staphylococcus aureus*, and vaccinia-infected tissue culture cells.[15,18−20]

Binding of selected aptamer pools to the GAS mixture follows an upward trend with increasing rounds of selection, yet is highly variable. For example, aptamer pool binding rose to 14% in round 15 before plummeting to 6.6% in round 17 and finally increasing to 11% at round 20 (Figure 2). A key source of variability in aptamer pool binding is probably due to differences in expression of cell surface molecules between and within M-types. Differences in cell surface molecule expression and protein synthesis are greatest when cells are grown in logarithmic phase[17] which is when they were harvested for selection. Since the SELEX target is a mixture of 10 different M-types, it is much more complex than a monoclonal population. Small day-to-day variations in the surface of each M-type may be additive, resulting in high overall variability. This argument is supported by the results of our following experiments comparing binding of aptamer pools to cells from the same or different cultures. Parallel incubations using cells from two separate colonies showed substantial variability in their binding to aptamer pools (data not shown). This variability was minimal when duplicates from the same culture or duplicates of two separate stationary phase cultures were analyzed (data not shown). Hence, screening aptamer pools against stationary phase cultures results in decreased variability of aptamer pool binding and is the procedure used for all remaining binding experiments.

Our choice of a SELEX target mixture of different M-types decreases the M-type selectivity of the resultant aptamers. This observation is in accordance with previous studies wherein aptamer selectivity is broadened by alternating targets.[21] Species cross-reactivity can be obtained in this manner.[21] Until our study, whole cell SELEX had not previously been carried out against a mixture of cell types with the aim of broadening aptamer selectivity. With the success of obtaining aptamers that bind to a group of cells, further research could incorporate a negative selection step (counter selection) into our SELEX procedures to improve selectivity. Previous work has shown that aptamer selectivity and specificity for a cell type could be refined by carrying out one or more counter selection steps against a nontarget cell to remove unwanted sequences.[22]

Current point-of-care testing methodology for Group A *Streptococcus* (GAS) relies on antibody-based rapid antigen detection (RAD), a 2-site sandwich immunoassay, to detect the Group A cell wall carbohydrate.[3] RAD has poor sensitivity and frequent false negative results, which account for 70−80% of the results obtained, requiring a confirmatory culture step.[3] The aptamers generated here for GAS could be used to improve on these assays.

Aptamers have been proven to be useful in detection of bacteria in such assay formats as enzyme-linked oligonucleotide assays, flow cytometry, chemiluminescent sandwich aptasensors, aptamer-quantum dot fluorescence assays, and fluorescence resonance energy transfer-based assays[13,14,23−27]. With the sequences reported in this paper, aptamers can be chemically modified and conjugated to highly sensitive detection probes (for example fluorescent and enzymatic). The flexibility of aptamer reagents enables the development of novel point-of-care diagnostic assays for GAS.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

We thank the Natural Sciences and Engineering Research Council of Canada (NSERC), Alberta Health and Wellness, the Alberta Water Research Institute, and the Canadian Water Network for funding this project and NSERC and the Alberta Heritage Foundation for Medical Research for providing a studentship to C.L.A.H. We also thank Drs. Christopher Cairo, Jeff Fuller, and Gregory Tyrrell of the University of Alberta for their valuable discussions and suggestions.

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