



DNA aptamers bind specifically and selectively to (1 → 3)-β-D-glucans

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ARTICLE INFO

Article history:

Received 16 November 2008

Available online 4 December 2008

Keywords:

Aptamer
(1 → 3)-β-D-Glucans
SELEX
Curdlan
Biosensor
Bioaerosol
Aerosol exposure
Asthma

ABSTRACT

(1 → 3)-β-D-Glucans are structural cell wall components of fungi, plants, and some bacteria and have been linked with human respiratory symptoms following aerosol exposure. A clear interpretation of the health impact of (1 → 3)-β-D-glucans is limited by the high cost and uncertainties associated with current glucan quantitation methods. The objective of this research is to develop DNA aptamers for the measurement of (1 → 3)-β-D-glucans. Aptamers are synthetic DNA functional binding molecules that fold into unique conformations, allowing them to bind specifically to their target. Through the *in vitro* selection process SELEX, we have produced aptamers that are able to bind with sub-micromolar affinity to curdlan, a linear unbranched form of (1 → 3)-β-D-glucans. These aptamers display high selectivity to curdlan and do not bind to non-(1 → 3)-β-D-polysaccharides, suggesting specificity for the β-(1 → 3)-glycosidic linkage. The aptamers produced here will enable the production of more cost-effective, less ambiguous assays for the environmental measurement of (1 → 3)-β-D-glucans.

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(1 → 3)-β-D-Glucans are water-insoluble structural cell wall components of most fungi, some bacteria, and plants [1]. Glucans comprise up to 60% of the dry weight of the cell wall of fungi, of which the primary constituent is (1 → 3)-β-D-glucans. The overall structure of (1 → 3)-β-D-glucans includes glucose polymers with variable degrees of branching. These polymers can exist as triple helix, single helix, or random coils [2]. Due to a wide variety in the degree of branching and polymerization, molecular weights of (1 → 3)-β-D-glucans range from 10³ to 10⁶ Da [3,4].

These compounds are considered potent respiratory hazards. *In vivo* toxicology studies using animal models have shown that intravenous administration of (1 → 3)-β-D-glucans resulted in the development of pulmonary diseases, enhanced endotoxin sensitivity, increased susceptibility to Gram-negative infections, and the onset of microembolism [4]. Epidemiological studies designed to link adverse health effects with environmental exposure to (1 → 3)-β-D-glucans have noted symptoms including airway inflammation, upper airway irritations and fatigue/tiredness, decreased lung function, and decreased airway responsiveness [5]. Studies based on subgroup analyses have suggested a higher susceptibility to these glucan-related health effects among atopics and/or subjects with pre-existing symptoms [6,7]. However, other epidemiological studies have not been able to verify the abovementioned associations between glucan exposure and health effects [5].

Chief among the problems in the clear interpretation of potential health effects from (1 → 3)-β-D-glucan exposure are the difficulties and uncertainties in the methods available to quantify environmental glucan levels [5]. The two widely used methods for (1 → 3)-β-D-glucan analysis are the glucan-specific limulus amoebocyte lysate (LAL) assay (Associates of Cape Cod, Falmouth, MA) [8], which is based on glucan-activation of factor G, a protease zymogen of the horseshoe crab (*Limulus polyphemus*); and a recently developed enzyme-linked immunosorbent assay (ELISA) using either rabbit or mouse-derived (1 → 3)-β-D-glucan antibodies [9,10]. While the LAL assays are capable of great sensitivity (with a limit of detection of 1–10 pg/ml), large differences in the (1 → 3)-β-D-glucan content as measured by the LAL assay might result if different extraction protocols are employed [5]. In addition, there is a risk of incurring false positive results with a modified version of the LAL assay [8] if endotoxins are present in environmental samples; endotoxins are toxic, natural compounds found inside bacteria which could also activate protease zymogen factors that are necessary for LAL glucan measurement. Antibodies specific for (1 → 3)-β-D-glucans have been developed in response to these LAL assay inconsistencies. However, *in vivo* production required for antibodies is complex and requires the use of an animal, thus resulting in high costs for antibody-based assays. ELISA methods for glucan detection are less prone to inhibitory effects than the enzymatic reaction-dependent LAL assays but have detection limit concentrations greater than 80 times that of the LAL method.

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In this study, we report the first development of DNA aptamers that are able to bind to (1 → 3)-β-D-glucans with high affinity and selectivity. Aptamers are synthetic, single-stranded DNA or RNA molecules that fold into unique three-dimensional structures [11]. The unique conformations that aptamers fold into allow them to bind specifically to other target molecules. The (1 → 3)-β-D-glucan-specific aptamers developed in this study were isolated from a combinatorial library consisting of DNA molecules having a 60 nt-long randomized region, using the *in vitro* selection process known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) [12]. The results presented in this report provide the first-step in the development of an affordable, sensitive and high-throughput assay for the detection of (1 → 3)-β-D-glucans in environmental samples, offering a possible viable alternative to current methods of glucan quantitation.

Materials and methods

Immobilization of curdlan onto streptavidin columns. Common to all fungal glucans, irrespective of the degree of branching, is the primary backbone structure consisting of (1 → 3)-β-D-linked glucose units. As this is the structure for curdlan, it was chosen as the (1 → 3)-β-D-glucan upon which the aptamer development work was based. Curdlan has an average molecular weight ranging from 5.3×10^4 to 2.0×10^6 Da [13]. This expected molecular weight lies within the same range as that of most fungal glucans [3]. Anhydrous biotinylated curdlan (CarboMer, San Diego, CA) was solubilized by alkaline treatment followed by neutralization. Briefly, 1.5 ml of 3 N NaOH was added to 50 mg of curdlan powder and stirred overnight. Subsequently, 10 ml of 0.2 M K_2HPO_4 was added, and the pH was dropped to 7 through the addition of 0.5 ml of 5 N HCl. The solubilized biotin–curdlan was immobilized onto a streptavidin column (Hydros, Inc., Bourne, MA), each containing 20 μg of streptavidin covalently coupled to a porous plastic polymer matrix, by incubating 50 μl of the solution in the column at room temperature for 1 h. Unbound biotinylated curdlan was removed through two washing steps using a wash solution comprising 0.15 M NaCl, 0.001 M $MgCl_2$, and 0.01% SDS.

In vitro selection of aptamers. A starting aptamer library consisting of 95-mer oligonucleotides with central 60-base long randomized sequences was synthesized (Sigma-Genosys, St. Louis, MO). The sequence of each aptamer is 5'-CGACTGACGCCTCCAAGC-N₆₀-GCATGCATCGCTACGTG, where N represents a randomized nucleotide of either A, G, C, or T. Primer 1 (5'-CGACTGACGCCTCCAAGC) and primer 2 (5'-CACGTAGCGATGCATGC) were used for PCR amplification of the DNA library. The aptamer library was initially purified by electrophoresis on a 6% non-denaturing polyacrylamide gel. The bands were visualized by UV-shadowing, excised, the DNA recovered by ethanol precipitation, and resuspended in 25 μl of DNase-free water. Prior to *in vitro* selection of curdlan-binding aptamers, the library was subjected to PCR amplification for 20 cycles in a 50 μl mixture containing 0.4 pmol DNA template, 15 pmol each of primer 1 and 2, 0.2 mM each dNTPs, 1.5 mM $MgCl_2$, and 2.5 U Taq DNA polymerase. To generate single-stranded aptamers, the resulting amplicons were then subjected to eight cycles of asymmetric PCR containing 15 pmol of primer 1. As a control, the amplified aptamer pool was then incubated (~1 h) with an unmodified streptavidin column to remove DNA molecules that bind non-specifically to streptavidin. To start the selection process, the flow-through fraction from this control incubation was subjected to PCR followed by asymmetric PCR under the same conditions as mentioned above, and subsequently transferred to a curdlan-modified affinity column and incubated for 5 min. Following the incubation period, the column was washed four times with 50 μl of wash solution to remove unbound DNA molecules. Bound aptamers were eluted by incubating 50 μl of a solution comprising

7 M urea, 0.01 M EDTA and 0.001% SDS for 1 h, followed by flushing and collecting into a 1.5 ml microcentrifuge tube. The eluted aptamers were then amplified by PCR for 20 cycles, followed by eight cycles of asymmetric PCR. This new aptamer pool was then applied to a fresh curdlan-modified affinity column for the next round of selection. A total of 10 selection cycles was completed for the *in vitro* selection process.

Cloning and sequencing of aptamers. After *in vitro* selection, aptamers were cloned using a TOPO TA cloning kit with the pCR[®]4-TOPO[®] vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Colonies were subsequently picked and grown overnight in a culture of 5 ml LB medium under vigorous shaking, and plasmid DNA from these colonies were harvested with a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). Cloned inserts from the plasmid DNAs were sequenced at the DNA Analysis Facility at Yale University using an Applied Biosystems 3730 DNA analyzer (Foster City, CA). Sequence analysis and alignments were performed using the ClustalX program. The *mfold* tool [14] was used to estimate the secondary structures of sequenced aptamers.

Determination of dissociation constants (K_D) of individual aptamers. Radiolabeled aptamers were prepared by incubating approximately 500 pmol of a single aptamer clone in a 50 μl reaction containing $1 \times T4$ polynucleotide kinase reaction buffer, 2.5 μCi [γ -³²P]ATP (10 Ci/mmol) (Perkin-Elmer, Waltham, MA) and 100 U of T4 polynucleotide kinase (New England Biolabs, Ipswich, MA), at 37 °C for 30 min. Unincorporated nucleotides were removed by using G-25 sephadex columns (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

Saturation binding assays for the determination of dissociation constants of selected aptamer clones to their target curdlan were carried out in the same procedure as outlined earlier for the binding and elution stages of the selection process, but by incubating increasing concentrations of radiolabeled aptamer (between 0.01 and 1–2 μM) with a curdlan-modified affinity column. The amount of aptamers that were bound to the column for each input aptamer concentration was quantified by liquid scintillation counting in a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin-Elmer, Waltham, MA), after elution from the column. Saturation curves were plotted based on these data and the dissociation constants K_D computed by non-linear regression analysis.

Specificity testing of individual aptamers by affinity elution assays. Selected aptamer clones were tested for their ability to bind to different types of (1 → 3)-β-D-glucans (laminarin, barley glucan, and free non-immobilized curdlan) and non-target polysaccharide competitors that lack the β-(1 → 3)-glycosidic linkage (dextran, mannan, and endotoxin). The polysaccharides used in the specificity study are described in Table 1, and were obtained from Sigma-Aldrich (St. Louis, MO), with the exception of curdlan, which was obtained from CarboMer (San Diego, CA). Upon receipt, the polysaccharides were solubilized according to suppliers' information. The final concentrations of the respective polysaccharide solutions were: 2.3×10^{-3} M laminarin; 4.4×10^{-5} M barley glucan; 1.01×10^{-5} M free curdlan; 10×10^{-3} M dextran; 8×10^{-4} M mannan; and 4000 EU/ml endotoxin.

Specificity testing was performed through the use of affinity elution assays. These assays were carried out by incubating 5000 pmol of fluorescein-labeled selected aptamer clones with a curdlan-modified affinity column for 1 h. Following a series of four washing steps to remove unbound aptamers, bound aptamers to the curdlan column were incubated for 1 h with 50 μl of an affinity wash solution containing the glucan target or non-target competitor at their respective solubilities, and subsequently flushed into a 1.5 ml microcentrifuge tube. Remaining aptamers not eluted by the polysaccharide affinity elution wash were removed by incubation for 1 h with 50 μl of urea wash, flushed, and collected into a 1.5 ml microcentrifuge tube. The amounts of aptamers in both

Table 1
Polysaccharides used in specificity testing for this study.

Polysaccharide	Source	Linkage ^a	Molecular weight
<i>Non-(1 → 3)-β-D-glucan</i>			
Dextran	<i>Leuconostoc mesenteroides</i>	(1 → 6)(1 → 3)(1 → 4)(1 → 2)-α-D-Glucan (B)	10,000
Endotoxin	<i>Escherichia coli</i>	(1 → 2)-β-D-Oligoglucosides	8000
Mannan	<i>Saccharomyces cerevisiae</i>	(1 → 2)(1 → 3)(1 → 6)-α-D-Glucan (B)	48,000
<i>(1 → 3)-β-D-glucan</i>			
Barley glucan	Barley plant	(1 → 3)(1 → 4)-β-D-Glucan (L)	216,000
Curdlan	<i>Alcaligenes faecalis</i>	(1 → 3)-β-D-Glucan (L)	136,000
Laminarin	<i>Laminaria digitata</i>	(1 → 6)(1 → 3)-β-D-Glucan (LSB)	8600

^a L, linear; B, branched; LSB, linear with side branches.

the affinity elution wash and urea wash were quantified by relative fluorescence measurement using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

Results

In vitro selection and sequence analysis of (1 → 3)-β-D-glucans-binding aptamers

Ten rounds of *in vitro* selection were used to isolate aptamers that show favorable binding properties to curdlan. Forty clones derived from aptamers remaining in the final round were selected for sequencing. Sequence alignment using the ClustalX software program revealed that, of the 32 readable round #10 clone sequences, sequence similarities existed in clones seq20 and seq24, seq6 and seq18, and seq16, seq27 and seq30, (Supplementary

Table S1), differing by only 1–3 bases within each respective group. All other sequences were observed to be distinct. There was no single consensus sequence that is conserved among the entire population of sequenced clones. Similarly, secondary structural prediction using *mfold* did not reveal any global structural similarities among the 32 selected aptamers. All of the clone sequences showed a tendency towards C-richness, T-richness, or both. Aptamers that are contained within homologous groups are likely to have been strongly preferentially chosen during the selection process. Hence, two aptamers displaying such favorable properties, seq6 and seq30, were selected for further characterizations of binding affinity and specificity. Fig. 1 shows the *mfold*-structural analysis of the two chosen aptamers.

Dissociation constants (K_D) of selected aptamers

The dissociation constants of the two selected aptamers, seq6 and seq30, were determined by carrying out saturation binding experiments in which the radiolabeled aptamer clone was incubated with a fixed amount of target curdlan (~200 pmol) in an affinity column. Saturation curves plotting the amount of bound aptamer clones measured against the corresponding input aptamer concentration used are presented in Fig. 2. Using non-linear regression analysis, the aptamer seq6 was found to have a K_D value of $0.303 \pm 0.067 \mu\text{M}$ (standard error) while seq30 was found to have a K_D value of $0.326 \pm 0.089 \mu\text{M}$.

Determination of aptamer specificity by affinity elution tests

In free solution, aptamers seq6 and seq30 were tested for their ability to bind (1 → 3)-β-D-glucans (laminarin, barley glucan, and

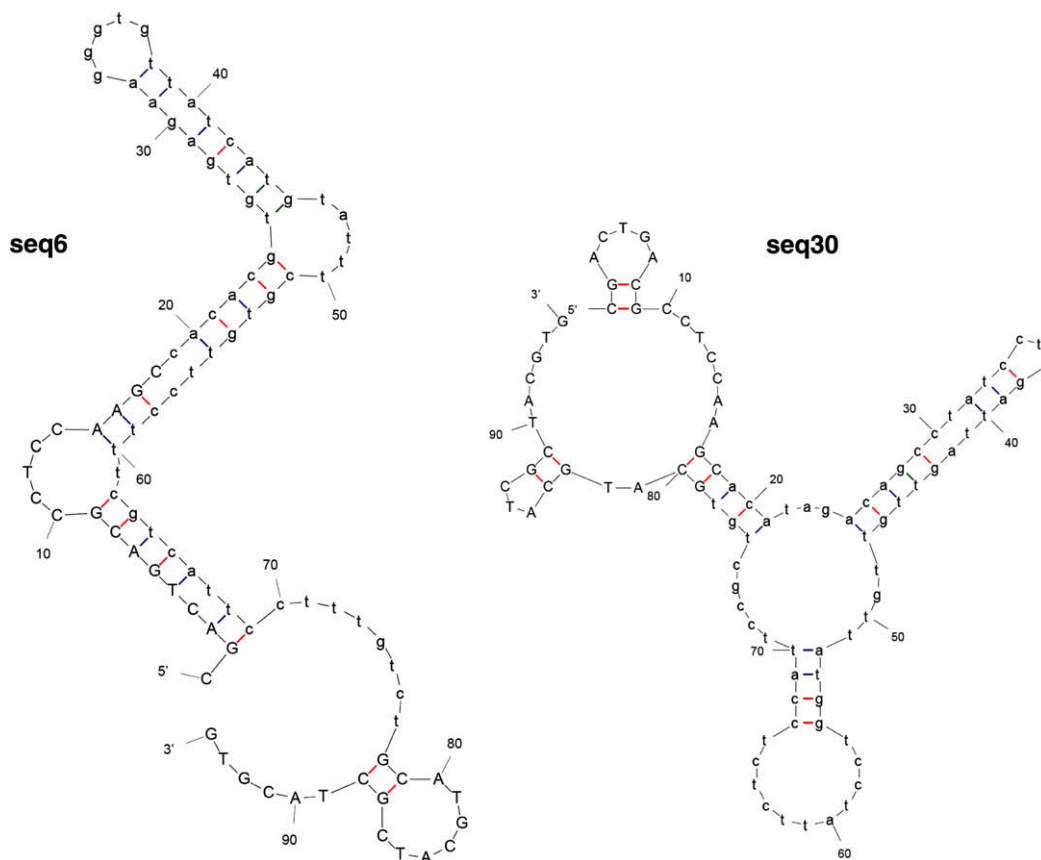


Fig. 1. Secondary structural predictions of round #10 aptamers, seq6 and seq30. The primer regions are in upper-case letters. The central non-primer nucleotide sequences of these two aptamers were found to be similar to other aptamer clones in the final selection round (seq6 with seq18; seq30 with seq16 and seq27).

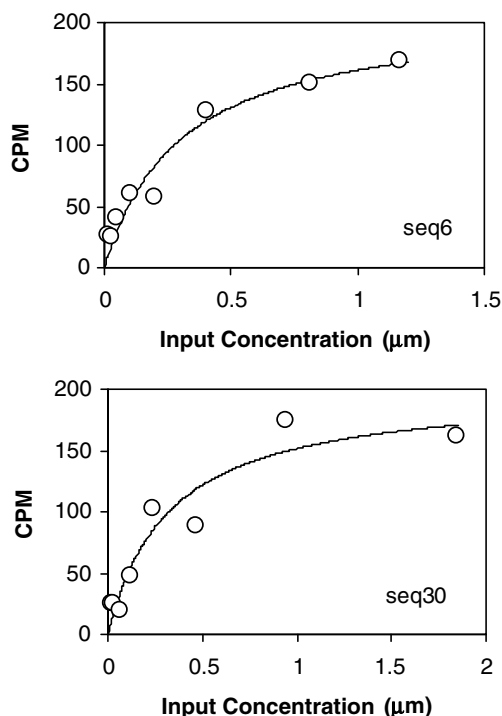


Fig. 2. Saturation curves of aptamer clones seq6 and seq30. A concentration series of each radiolabeled clone, ranging from 0.01 to 1–2 μM , was incubated with a fixed amount (~ 200 pmol) of target in a curdlan affinity column. (K_D) of the respective aptamer clones were computed by non-linear regression analysis.

curdlan) and non-(1 \rightarrow 3)- β -D-glucans (dextran, mannan, and endotoxin). The selection of polysaccharides used in this study was based upon corresponding work carried out in testing the specificity of the glucan-specific ELISA and LAL assays [8–10]. It was found that, of the polysaccharides tested, both seq6 and seq30 display significantly higher affinity for (1 \rightarrow 3)- β -D-glucans than non-(1 \rightarrow 3)- β -D-glucans ($p < 0.001$, t test), thus suggesting a greater specificity for (1 \rightarrow 3)- β -D-glucan targets (Fig. 3). Endotoxin, comprising of (1 \rightarrow 2)- β -D-oligoglucosides, only eluted 17.8% of bound seq6 aptamers and 10.8% of seq30 aptamers. For dextran, a branched (1 \rightarrow 6)(1 \rightarrow 3)(1 \rightarrow 4)(1 \rightarrow 2)- α -D-glucan, only 7.4% and 6.9% of curdlan-bound seq6 and seq30 aptamers, respectively, were eluted by the affinity wash. No detectable

amounts of either aptamer bound on the curdlan column were eluted by a solution of mannan, a branched (1 \rightarrow 2)(1 \rightarrow 3)(1 \rightarrow 6)- α -D-glucan. On the other hand, both seq6 and seq30 could bind to free non-immobilized curdlan in solution: the curdlan solution eluted 86.5% of bound seq6 and 89.5% of bound seq30. The two aptamers were also found to be able to bind to other polysaccharides having the β -(1 \rightarrow 3)-glycosidic linkage: for barley glucan, a linear (1 \rightarrow 3)(1 \rightarrow 4)- β -D-glucan, 64.8% of bound seq6 and 67.1% of bound seq30 were eluted by the affinity wash; for laminarin, a (1 \rightarrow 3)- β -D-glucan with side branches of glucose monomers connected to the primary backbone via β -(1 \rightarrow 6)-glycosidic linkages, the affinity wash eluted 76.3% of bound seq6 and 81.4% of bound seq30.

Discussion

In this report, we describe the development of aptamers that could bind with high affinity and specificity to the biotoxin, (1 \rightarrow 3)- β -D-glucans. These represent the first aptamers developed for common indoor contaminants. The use of these aptamers in sensors or detection assays should lead to increased accuracy and decreased expense in exposure monitoring and epidemiological investigations for environmental respiratory diseases.

The lack of global consensus in the aptamer clone sequences and absence of a common predominant predicted structural motif suggests that a diversity of DNA structures are able to bind to the (1 \rightarrow 3)- β -D-glucan target under the selection conditions used. The results also suggest an association between sequences that are rich in either cytidine or thymidine, or both, and an affinity for (1 \rightarrow 3)- β -D-glucans.

Two aptamers, seq6 and seq30, were selected for further characterizations of binding properties, and were found to display good binding affinity for their polysaccharide target, with sub-micromolar dissociation constants, around 0.3 μM each, to (1 \rightarrow 3)- β -D-glucans. In prior work on aptamers targeting polysaccharides, Yang et al. [15] found cellobiose-targeting aptamers with K_D values ranging from 10^{-7} to 10^{-5} M, while Masud et al. [16] developed an aptamer for sialyllactose with a dissociation constant of 4.9 μM . Similar interactions between lectins and carbohydrates were observed to have affinities ranging from 10^{-5} to 10^{-3} M [17], while those for anticarbohydrate antibodies were found to be between 10^{-5} and 10^{-2} M [18,19]. It has been postulated that aptamers to targets containing sugar groups, which would include (1 \rightarrow 3)- β -D-glucans, are not likely to display the nanomolar or

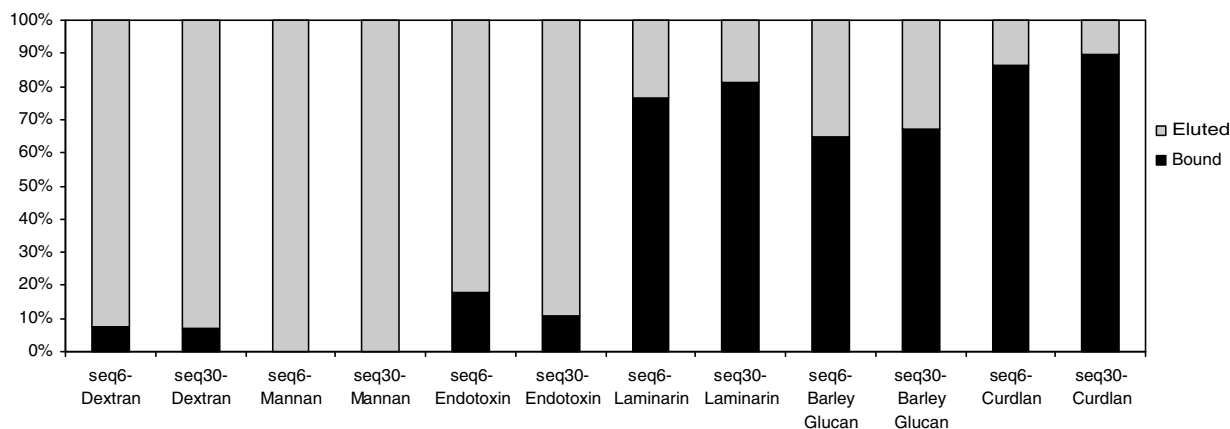


Fig. 3. Specificity of selected aptamer clones, seq6 and seq30, for (1 \rightarrow 3)- β -D-glucans (laminarin, barley glucan, and curdlan) and non-(1 \rightarrow 3)- β -D-glucans (dextran, mannan, and endotoxin). Affinity elution washes using the respective polysaccharide solutions, followed by urea washes, were carried out in turn to remove fluorescein-labeled aptamers bound on a curdlan-modified affinity column. Low percentages of aptamers eluted by the affinity washes correspond to a low affinity between the aptamer and the corresponding polysaccharide, and vice versa. Triplicate runs of experiments yielded standard errors ranging from 1% to 9%.

even picomolar affinities that might otherwise be achievable with targets of other nature [20], since sugar groups lack both positively charged motifs and aromatic ring structures with which to facilitate stable non-covalent interactions with nucleic acids like aptamers [15].

Both selected aptamers also exhibit high specificity for (1 → 3)-β-D-glucans, versus polysaccharides lacking the β-(1 → 3)-glycosidic linkage. The markedly different amounts of both seq6 and seq30 that were eluted across the (1 → 3)-β-D-glucan and non-(1 → 3)-β-D-glucan polysaccharide affinity washes suggest that the aptamers do not bind specifically to any of the moieties on the common individual glucose monomer unit of the various polysaccharides, such as hydrophobic sites, hydroxyl groups or pyranose ring oxygens. The limited binding behaviors shown towards non-(1 → 3)-β-D-glucan polymers such as endotoxin (<18% eluted), dextran (<7.5% eluted) and mannan (0% eluted) are indications that the aptamers have low affinity for the non-β-(1 → 3)-glycosidic bonds linking the glucose monomers together in these polysaccharides. In contrast, the significantly higher elution amounts ($p < 0.001$, t test) for the aptamers towards (1 → 3)-β-D-glucans like barley glucan (>65% eluted), laminarin (>75% eluted), and curdlan (>86% eluted), coupled with the earlier results for non-(1 → 3)-β-D-glucans, suggest the aptamers exhibit specific affinity for the characteristic β-(1 → 3)-glycosidic linkages in the primary backbone of (1 → 3)-β-D-glucans. This is desirable since the β-(1 → 3)-glycosidic linkages are the defining and common traits among the wide variety of environmental (1 → 3)-β-D-glucans. For this reason, curdlan, a (1 → 3)-β-D-glucan consisting exclusively of glucose monomers linked by β-(1 → 3)-glycosidic linkages in a primary backbone without any side branches, was chosen as the target for aptamer selection. The slightly lower elution amounts shown by laminarin and barley glucan in comparison with curdlan may be due to the fact that the β-(1 → 6)-glycosidic branching in laminarin, and the mix of β-(1 → 4)-glycosidic linkages in the primary backbone of barley glucan, could have impeded aptamer binding to the target β-(1 → 3)-glycosidic bonds, either through steric hindrance, or by limiting the number of available binding sites. The results of the specificity study suggest, therefore, that the aptamers developed in the study are highly specific for (1 → 3)-β-D-glucans over other similarly structured but non-target polysaccharides. In addition, the aptamers were also shown to be able to bind (1 → 3)-β-D-glucans over a wide coverage of molecular weights, ranging 10^3 Da (laminarin, 8600 Da) to 10^6 Da (barley glucan, 216,000 Da).

Finally, the development of aptamers that display good binding affinity and specificity to (1 → 3)-β-D-glucans suggests that aptamers have strong potential to be a viable alternative for assaying (1 → 3)-β-D-glucans in environmental samples. Since aptamers can be economically produced using chemical synthesis or by PCR, manufacturing and assay costs of aptamers are expected to be significantly lower than those which might be incurred with the LAL or ELISAs. It is estimated that production costs of aptamers are about 10–50 times less than antibodies. In addition, the results from this study suggest that aptamers, being highly specific for (1 → 3)-β-D-glucans over endotoxins, would likely be less prone to false positive results in endotoxin-tainted samples, an aspect to which the modified LAL assay [8] is reportedly susceptible [5]. Aptamers can also readily be labeled with reporter and quencher moieties to form molecular beacons, and functional groups for immobilization on microarrays, facilitating the construction of a biosensor. Contemporary sensor platforms have demonstrated detection levels on the order of 10 pg/ml with sub-micromolar

affinity aptamers [21], suggesting that aptamer-based assays can achieve detection limits competitive with the sensitive LAL assay (1–10 pg/ml). The development of these sensitive and specific aptamers is the first step in developing a robust, cost-effective assay that will close the gap between environmental (1 → 3)-β-D-glucan exposure and clinical outcomes.

Acknowledgment

This work was supported by NIH Grant RES015312A.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2008.11.087](https://doi.org/10.1016/j.bbrc.2008.11.087).

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