Research Report

Use of Magnetic Beads in Selection and Detection of Biotoxin Aptamers by Electrochemiluminescence and Enzymatic Methods

BioTechniques 32:178-183 (January 2002)

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ABSTRACT

Systematic evolution of ligands by exponential enrichment (SELEX) was used to develop DNA ligands (aptamers) to cholera whole toxin and staphylococcal enterotoxin B (SEB). Affinity selection of aptamers was accomplished by conjugating the biotoxins to tosyl-activated magnetic beads. The use of magnetic beads reduces the volumes needed to perform aptamer selection, thus obviating alcohol precipitation and allowing direct PCR amplification from the bead surface. Following five rounds of SELEX, 5′-biotinylated aptamers were bound to streptavidin-coated magnetic beads and used for the detection of ruthenium trisbypyridine [$Ru(bpy)_3^{2+}$]-labeled cholera toxin and SEB by an electrochemiluminescence methodology. A comparison of control (double-stranded) aptamer binding was made with aptamers that were heat denatured at 96°C (single-stranded) and allowed to cool (conform) in the presence of biotoxin-conjugated magnetic beads. Results suggest that control aptamers performed equally well when compared to heat-denatured DNA aptamers in the cholera toxin electrochemiluminescence assay and a colorimetric microplate assay employing peroxidase-labeled cholera toxin and 5′-amino terminated aptamers conjugated to N-oxysuccinimide-activated microtiter wells. Interestingly, however, in the SEB electrochemiluminescence assay, double-stranded aptamers exceeded the performance of single-stranded aptamers. The detection limits of all aptamer assays were in the low nanogram to low picogram ranges.

INTRODUCTION

Systematic evolution of ligands by exponential enrichment (SELEX) is a relatively new and revolutionary method for generating very high-affinity receptors that are composed of nucleic acids instead of proteins (1–5,7–14). Thus, SELEX offers a completely in vitro combinatorial chemistry alternative to traditional protein-based antibody technology. SELEX is advantageous in terms of obviating animal hosts, reducing the production time, labor, and purification involved in generating specific binding receptors to a particular target agent or epitope. SELEX-generated receptors, whether composed of DNA or RNA, are referred to as "aptamers", from the Latin "aptus" meaning, "to fit" (10).

SELEX is typically performed by synthesizing a random oligonucleotide of 20 or more bases in length, which is flanked by known primer sequences (1, 2,10,12–15). Synthesis of the random region is achieved by mixing equimolar amounts of all four nucleotides at each locus in the sequence. Thus, the diversity of the random sequence is maximally 4^n, where n is the length of the sequence, minus the frequency of palindromes and symmetric sequences (10). In the present work, a random 20-mer sequence is employed, having a maximal diversity of roughly 10^{12} permutations. When the random SELEX library is exposed to an immobilized target analyte, sequences that bind without washing away are retained and amplified by PCR for subsequent rounds of SELEX consisting of alternating affinity selection and PCR amplification.

Since SELEX was first reported in 1990 (4,15), it has been used to generate high-affinity receptors to a broad array of targets. Aptamers are beginning to be exploited for detection (3) and potential therapeutic utility (7,10,12). Surprisingly, little work appears to have been done in the area of SELEX aptamer development to detect and quantitate biotoxins. Here, we present data on initial attempts to generate DNA aptamers to clinically and militarily important biotoxins (i.e., potential biowarfare agents) that may eventually function to detect and neutralize these toxins.

In the present work, we employ truncated versions of the template and PCR primers used by Tuerk and Gold (14) for SELEX. Truncated templates and primers were used because of the lack of sequence fidelity in synthesizing oligonucleotides longer than 50–60 bases, which could affect PCR amplification of selected oligonucleotides.

Use of magnetic beads for affinity separation of aptamers from an initial SELEX library affords several advantages such as the use of small sample volumes (50–100 µL), PCR amplification directly off of the magnetic bead surfaces, and the potential for automating SELEX by magnetically transferring magnetic beads to various reaction chambers. However, the use of magnetic beads in the SELEX process also requires some modifications to the standard methods employed in most laboratories performing SELEX. For example, the use of deionized water and heat to elute aptamers from target-conjugated magnetic bead surfaces followed by reconstitution of the ionic strength by the addition of an equal volume of 2× binding buffer (Figure 1).

Two methods of aptamer-based biotoxin detection are also compared in the present work: *(i)* an aptamer-magnetic bead toxin capture technique involving $Ru(bpy)_3^{2+}$-labeled toxins with

electrochemiluminescence detection (2,6,16,17) and *(ii)* a colorimetric enzyme-linked microplate assay similar to that developed by Drolet et al. (3). Magnetic beads were useful in both the affinity selection phase of SELEX and the electrochemiluminescence detection assays because magnetic beads offer the ability to work with small volumes of DNA solutions, hence obviating ethanol precipitation of aptamer DNA and enabling very sensitive electrochemiluminescence assays at the surface of the magnetized electrode in the ORIGEN® Electrochemiluminescence Analyzer (IGEN International, Gaithersburg, MD, USA) (6,16,17).

MATERIALS AND METHODS

Conjugation of Protein Toxins to Magnetic Beads

Cholera whole toxin and staphylococcal enterotoxin B (SEB) were obtained from Sigma (St. Louis, MO, USA) and were reconstituted in autoclaved deionized water. Toxins were added at a final concentration of 0.1 mg/mL to 1.3×10^8 tosyl-activated magnetic beads (M-280; Dynal, Lake Success, NY, USA) per mL of sterile 0.05 M sodium borate (pH 9.5). Magnetic beads were incubated with their respective toxins for 16 h at 37°C. Toxin-conjugated magnetic beads were washed in 1× binding buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5–7.6, 1 mM $MgCl_2$) (1,2, 4,5) with 0.1% Triton® X-100 and collected with a strong magnet. Toxin-magnetic beads were washed three more times in 1× binding buffer and stored at 4°C until needed.

Random DNA Libraries and PCR Amplification of Aptamers

SELEX DNA sets consisted of a single-stranded template with randomized (N) 20-mer midsection (5′-CCGGATCCGTTGATA-N_{20}-GTGGTGTTGGCTCCC-3′) and two primers (5′-GGGAGCCAACACCAC-3′ and 5′-CCGGATCCGTTGATA-3′) (14). One set of SELEX DNA possessed a 5′-amino-six carbon linker on the template and primers for conjugation to N-oxysuccinimide-activated microtiter wells in the colorimetric enzyme-linked detection assay. The other SELEX DNA set was identical, except that both the template and the primers were 5′-biotinylated to afford binding to streptavidin-coated magnetic beads in electrochemiluminescence assays. All oligonucleotides were obtained from Sigma-Genosys (Woodlands, TX, USA). All PCR reagents, except *Taq* DNA polymerase, were obtained from Applied Biosystems (Foster City, CA, USA). *Taq* DNA polymerase was obtained from Fisher Scientific (Pittsburgh, PA, USA).

PCR was carried out before exposure of the SELEX library to toxin-magnetic beads to empirically optimize annealing temperature. A 600-µL PCR master mixture consisting of 1 ng either capture or reporter SELEX DNA templates, 1 µM final concentration of appropriate primer (amino or biotinylated primers), 0.2 mM concentration of each deoxynucleotide, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5–7.6, 50 mM KCl, and 50 U *Taq* DNA polymerase in autoclaved, deionized water was aliquoted at 50 µL per PCR tube for empirical determination of annealing temperature using a RoboCycler® model 96 thermal cycler with a "hot top" assembly (Stratagene, La Jolla, CA, USA). PCR annealing temperature was studied between 36°C and 58°C (data not shown). Optimal PCR conditions were determined to be: initial denaturation at 96°C for 5 min, 40 cycles of 96°C for 1 min, 45°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min.

Magnetic Bead-Based SELEX

Figure 1 illustrates the general SELEX strategy used to select DNA aptamers to cholera and SEB toxin-conjugated magnetic beads. Initially, 200 µL (approximately 16 µg) of the original random capture or reporter DNA libraries in autoclaved deionized water were heated to 96°C to produce single-stranded libraries, which were immediately added to 200 µL toxin-magnetic beads (2.6×10^7) in autoclaved 2× binding buffer and allowed to cool to room temperature. The aptamer-toxin-magnetic beads were then washed three

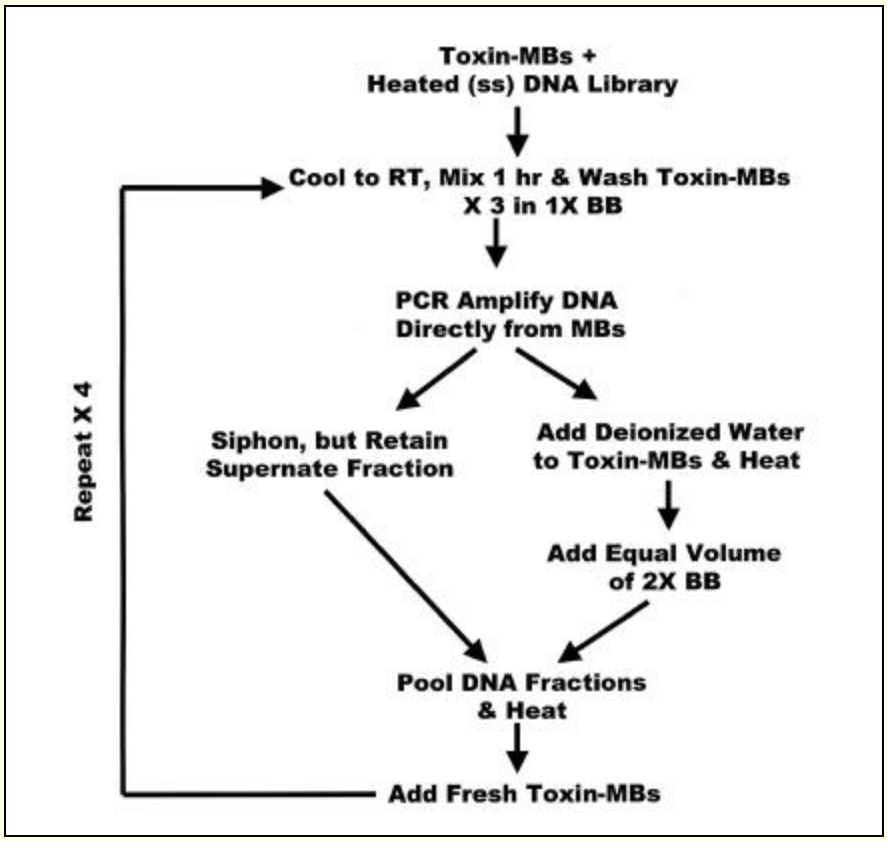

Figure 1. Generalized scheme used for magnetic bead-based SELEX aptamer development.

times in 1 mL 1× binding buffer by means of magnetic collection and careful siphoning. PCR was then carried out directly from the magnetic bead surfaces by the addition of 100 µL PCR master mixture containing either the capture (5′-amino) or reporter (5′-biotin) linkage systems (1,2). Following PCR, the supernate was collected while retaining the magnetic beads with a magnet. Autoclaved deionized water (100 µL) was added to the magnetic beads. The magnetic beads were resuspended and reheated to 96°C for 5 min to elute any residual magnetic bead-bound aptamers. Magnetic beads were recollected with a magnet, and the supernate containing aptamers in deionized water was separated from the magnetic beads and combined with 100 µL 2× binding buffer to reconstitute the ionic environment. The eluted fraction was recombined with the PCR supernate, reheated to 96°C, and added to fresh toxin-magnetic beads as before for the next round of SELEX. Five rounds of SELEX were accomplished. The presence of PCR product was checked after each round by the removal of 10 µL of the PCR product and electrophoresis in 2% agarose with ethidium bromide staining (data not shown and References 1 and 2).

Magnetic Bead-Electrochemiluminescence Detection Assay for Toxin Binding

Cholera and SEB toxins were labeled with N-hydroxysuccinimide-$Ru(bpy)_3^{2+}$ (IGEN International) in a 15:1 protein:N-hydroxysuccinimide-$Ru(bpy)_3^{2+}$ electrochemiluminescence label molar ratio as previously described (6,16). $Ru(bpy)_3^{2+}$-labeled toxins were purified on Sephadex® G25 columns (PD-10 columns; Amersham Biosciences, Piscataway, NJ, USA) in 1× binding buffer. Five microliters of cold (double-stranded) or 96°C heated (single-stranded) round 5 biotinylated reporter aptamers (A_{260} = 0.9) developed against cholera or SEB were added to 20 µL stock (200 µg) 5 µm diameter MPG® streptavidin-coated magnetic beads (CPG, Lincoln Park, NJ, USA) in 1 mL 1× binding buffer in 12 × 75 mm silanized glass tubes for 30 min at room temperature with vortex mixing at 85 rpm using an ORIGEN Electrochemiluminescence Analyzer. Magnetic beads were then magnetically collected and resuspended in various dilutions of $Ru(bpy)_3^{2+}$-labeled cholera or SEB toxin in 500 µL 0.2 M tripropylamine (2,6,15,16) buffer to yield the final concentrations reported in Figures 2 and 3. Electrochemiluminescence intensity measurements were obtained using the ORIGEN analyzer with a sample draw volume of 225 µL and an "assay gain" setting of 1000 (2,6,15,16).

Colorimetric Enzyme-Linked Oligonucleotide Assay for Cholera Binding

A colorimetric enzyme-linked oligonucleotide assay for aptamer binding similar to that reported by Drolet et al. (3) was devised for comparison of the cholera electrochemiluminescence detection assay to a more standard method. Peroxidase-labeled cholera whole toxin was obtained from Sigma and was dissolved in 1× binding buffer. Round 5 aptamers developed against cholera whole toxin were immobilized on DNA-Bind™ microplates (Corning Costar, Corning, NY, USA) using an N-oxysuccinimide linkage. Briefly, 100 µL round 5 anti-cholera toxin 5′-amino-aptamers (A_{260} = 0.9) were diluted 1:500 in PBS (Sigma) and added to each well of DNA-Bind™ plates. For both plates, cold (putative double-stranded) DNA was added to each well. DNA was allowed to bind to the plates for 2 h at 37°C. For the ssDNA plate, DNA was converted to its single-stranded form by placing the plate on the surface of a 98°C water bath for 5 min, followed by immediate decanting of the free DNA and three washes in 300 µL 1× binding buffer. All fluids were decanted from both plates, and peroxidase-labeled cholera toxin in 1×

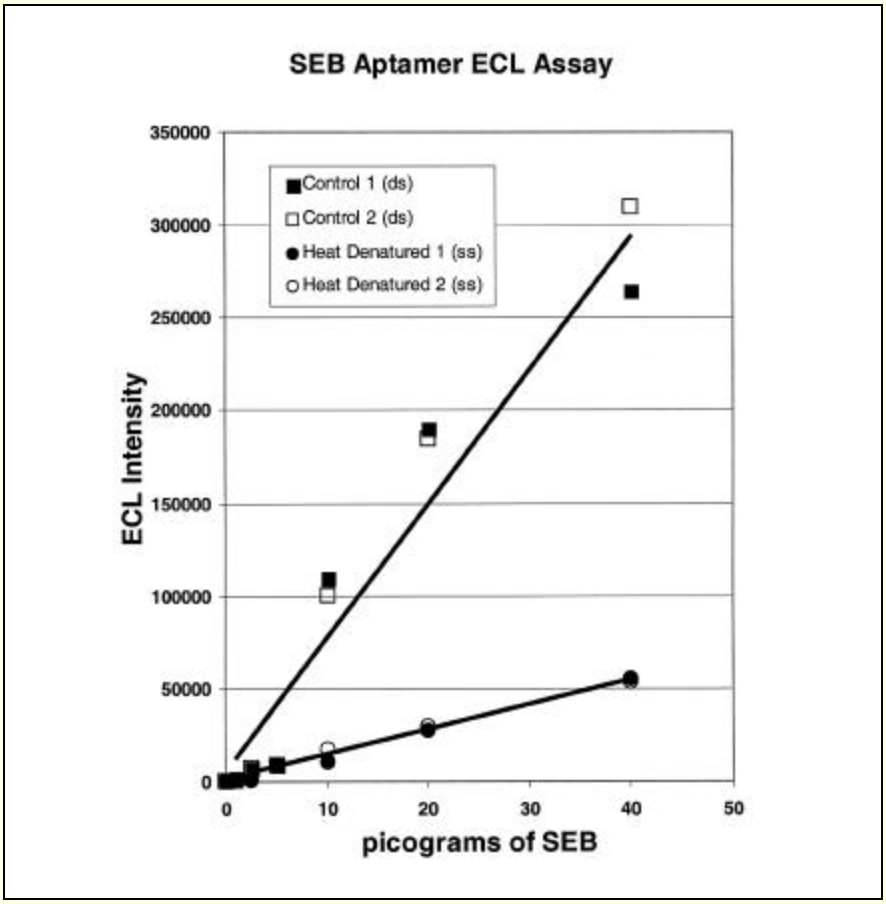

Figure 2. Magnetic bead-electrochemiluminescence assay results for SEB binding to single-stranded (96°C heated) versus control (double-stranded) round 5 DNA aptamers. Biotinylated reporter aptamers were immobilized on streptavidin magnetic beads and used to capture and detect $Ru(bpy)_3^{2+}$-labeled SEB.

binding buffer was added at the final concentrations indicated in Figure 4 for 1 h at room temperature. Wells were washed three times with 300 µL 1× binding buffer, and color was developed by the addition of 100 µL 2,2′-azino-di(3-ethyl-benzthiazoline-6-sulphonic acid) (ATBS) obtained as a one component mixture with H_2O_2 from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA) for 15 min at room temperature. The color development was halted by the addition of 100 µL 2% SDS in deionized water. Results were quantitated using a Model 550 automated microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at an absorbance of 405 nm.

RESULTS

Initial attempts at sandwich aptamer magnetic bead electrochemiluminescence (2) and plate assays for toxins resulted in poor sensitivity (data not shown). Rye and Nustad (13) encountered similar problems but overcame them by using a hybrid antibody-aptamer method and analyte capture on the surface of magnetic beads versus plates. In our case, a direct approach to capture and detection of either $Ru(bpy)_3^{2+}$-labeled or peroxidase-labeled biotoxins was adopted. This resulted in nanogram to picogram sensitivities, as depicted in Figures 2 through 4.

Clearly, the anti-SEB aptamers yielded a more sensitive electrochemiluminescence assay with a detection limit of less than 10 pg SEB, regardless of the "strandedness" of the aptamer (Figure 2), compared to a detection limit of less than 40 ng in the cholera electrochemiluminescence assay (Figure 3) and ≤ 10 ng in the colorimetric plate assay (Figure 4). Curiously, double-stranded control aptamers gave much greater electrochemiluminescence intensity in the SEB assay versus heat-denatured (single-stranded) aptamers (Figure 2). By contrast, both double- and single-stranded anti-cholera aptamers produced essentially the same binding curves in both the electrochemiluminescence (Figure 3) and colorimetric plate assays (Figure 4).

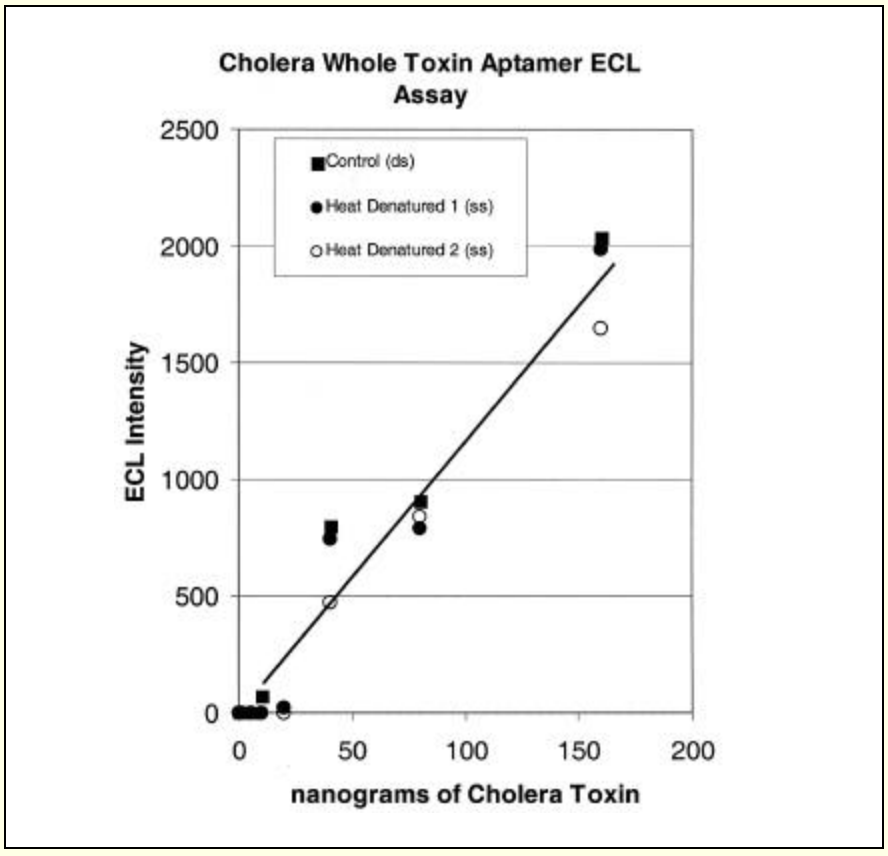

Figure 3. Magnetic bead-electrochemiluminescence assay results for cholera whole toxin binding to single-stranded (heat-denatured) versus double-stranded control round 5 DNA aptamers. Biotinylated reporter aptamers were immobilized on streptavidin magnetic beads and used to capture $Ru(bpy)_3^{2+}$-labeled cholera toxin.

DISCUSSION

Several interesting and somewhat surprising results were obtained in the current study, such as the disparity between the binding of single- versus double-stranded aptamers in the SEB electrochemiluminescence assay, but not in either of the cholera toxin assays. This observation underscores the necessity of empirically determining which type of nucleic acid (single-stranded versus potentially or partly ds-DNA or RNA) will work best in a given aptamer assay system. In addition, the lack of ability to develop a sandwich assay to either toxin suggests that some protein toxins may have limited "epitope" availability for SELEX aptamer development, especially when toxins are immobilized. Rye and Nustad (13) encountered similar difficulties in designing aptamer-antibody hybrid assays for thrombin by traditional schemes. However, with the use of magnetic beads and spacers for analyte capture, they were able to produce a sensitive assay for thrombin that employed an anti-thrombin aptamer.

Although we do not currently know the binding constants or nucleotide sequences of any of the anti-biotoxin aptamers reported here, the present work illustrates that individual researchers can easily develop their own aptamers for use in various types of assays. Such aptamers can be viewed as analogous to "polyclonal" antisera. Traditionally, researchers do not know the amino acid sequences of the antibodies they routinely use, but this fact certainly does not preclude the use of an antiserum. Similarly, the subsets of aptamers selected by five rounds of SELEX in the present work were sufficient to produce assays with nanogram to picogram toxin sensitivities.

The strandedness of the aptamers may affect their functionality, as witnessed in this work. Single-stranded

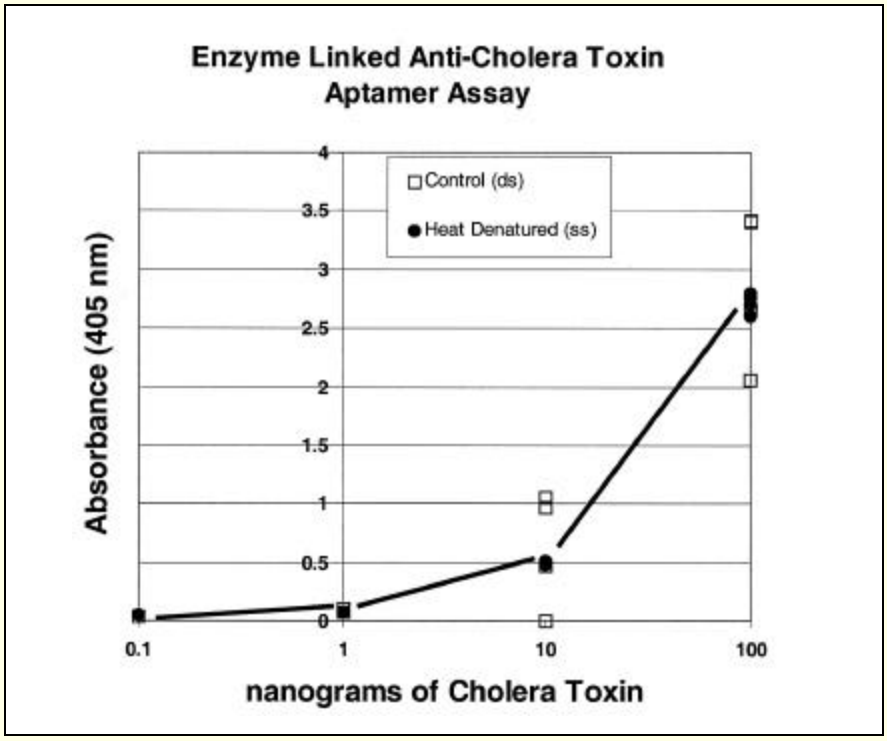

Figure 4. Colorimetric cholera toxin-aptamer microplate assay comparing single-stranded (heat-denatured) aptamers against double-stranded control aptamers. Peroxidase-labeled cholera toxin was captured by surface-immobilized aptamers and detected by the addition of ABTS.

aptamers probably tend to form binding pockets more readily than double-stranded aptamers. However, double-stranded aptamers may form pockets as well or may have single-stranded loop out regions that provide enhanced binding over their single-stranded counterparts. These anomalies underscore the need to empirically assess single- versus double-stranded aptamers.

In the present work, we chose to explore the use of magnetic beads for affinity selection because they afford the use of smaller volumes of DNA solutions, thus obviating ethanol precipitation of DNA from larger volumes. In addition, since magnetic beads can survive heat elution of DNA, they can be used directly in PCR amplification of aptamers. Magnetic beads can also be used directly in the detection of target binding by electrochemiluminescence or other means, which might be of value at the tail end of an automated SELEX system. Electrochemiluminescence was selected as one mode of detection partly because of our previous experience with highly sensitive immunomagnetic (6,17) and DNA-based (16) electrochemiluminescence assays for detection of biowarfare agents (6) and pathogens in foods (17). However, it is clear from Figure 4 that one can devise direct colorimetric enzyme-linked oligonucleotide microplate assays with comparable sensitivities.

ACKNOWLEDGMENTS

This work was funded by the U.S. Army, Edgewood Research, Development, and Engineering Center, Aberdeen Proving Ground, MD.

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Received 5 July 2001; accepted 30 August 2001.

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