Single microbead SELEX for efficient ssDNA aptamer generation against botulinum neurotoxin†‡

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An efficient and easy-to-execute single microbead SELEX approach is developed to generate high affinity ssDNA aptamers against botulinum neurotoxin.

Aptamers have recently been touted as potential antibody (Ab) replacements due to their high binding specificities and affinities.[1] Aptamers are single stranded (ss) nucleic acids that are able to fold into well-defined tertiary structures upon target binding. They are generated *via* the systematic evolution of ligands by exponential enrichment (SELEX) process,[2,3] which usually entails ~8 to 12 selection cycles. In recent years, capillary electrophoresis (CE) has been successfully used to generate high affinity aptamers in less than four cycles.[4,5] Since CE utilizes a very low amount of target, the ssDNA aptamers generated are observed to demonstrate high binding affinities. Several drawbacks of this approach, however, are that (i) only a very small volume of the interacting mixture is injected, and thus a large majority of the complex is not subjected to partitioning, and (ii) both binding conditions and CE running conditions for any given target have to be pre-determined, which is often time consuming.

We are interested in the generation of ssDNA aptamers toward the fabrication of aptasensors for biosecurity applications.[6,7] Herein we describe a novel and efficient approach to generate ssDNA aptamers through the use of only a single target-conjugated microbead (Fig. 1a). As opposed to traditional means exposing the ssDNA library to a column packed with thousands of target-derivatized microbeads, we exposed the library to only a single microbead. We hypothesized that by drastically reducing the amount of target molecules, only the tightest ssDNA binders are expected to remain bound to the exposed target after the incubation period (the weak binders will be displaced by the higher affinity binders). For this study, we have chosen two *Clostridium botulinum* neurotoxin (BoNT)-related targets, namely the BoNT-toxoid (aldehyde-inactivated toxin) and a short 19 amino acid (aa 1177–1195 of the heavy chain (Hc), Fig. 1b) BoNT Hc-peptide domain, as targets for ssDNA aptamer generation. The neurotoxins of the genus *Clostridium* are of great interest as one of its members, BoNT, is widely regarded as the "poison of all poisons".[8,9] BoNT is comprised of two primary domains, the light chain (Lc, 50 kDa) and the heavy chain (Hc, 100 kDa), that are connected *via* a single disulfide bond. Briefly, BoNT operates by first binding to the neuronal membrane receptors *via* its Hc domain. The Hc-peptide domain has been reported to be a putative ganglioside binding site (thus facilitating translocation)[10] and is also unique to BoNT as it is the antigenic site utilized to generate the anti-BoNT Ab.[11]

The BoNT/A Hc-peptide and toxoid were conjugated onto Ni-NTA agarose and amine-functionalized polystyrene TentaGel beads, respectively.‡ For each selection, a single functionalized microbead was incubated with 10 μL of DNA library (1 nmol) containing a 40 nucleotide (nt) randomized region in binding buffer (20 mM HEPES, pH 7.4; 150 mM NaCl; 5 mM KCl; 2 mM $MgCl_2$; 2 mM $CaCl_2$). After 2 h incubation with gentle agitation, the DNA library and microbead were transferred to a petri dish for subsequent washing steps conducted under a fluorescence stereomicroscope. The DNA library solution was carefully removed while the single microbead remained on the petri dish (if desired, the withdrawn unbound DNA library is applicable for re-selection of other targets in tandem). After extensive stringent washing in both binding buffer and water, the microbead was re-suspended in 5 μL of water and amplified by polymerase chain reaction (PCR) using fluorescent (forward) and biotinylated (reverse) primers. The resulting products were captured on streptavidin-coated beads, and the fluorescently labeled

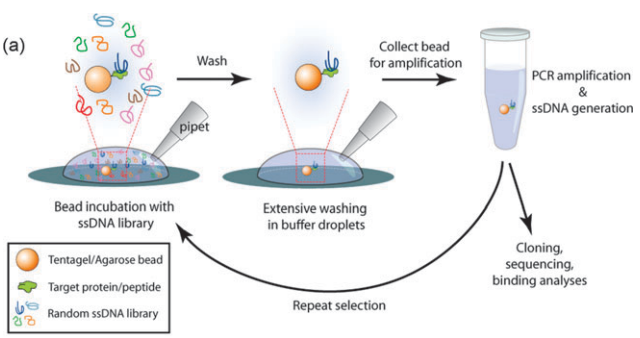

Fig. 1 (a) A schematic of the "single microbead" SELEX approach. (b) Sequences of the BoNT/A Hc-peptide target. The peptide target (underlined aa 11–19) is synthesized with a short glycine spacer and 6-His tag at its N-termini (aa 1–10) to facilitate efficient attachment onto Ni-NTA derivatized agarose. Not drawn to scale.

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† Electronic supplementary information (ESI) available: Fluorescence anisotropy binding and competition plots, list of all the sequenced aptamers and their respective predicted secondary structures, homology analysis plot. See DOI: 10.1039/b717936g

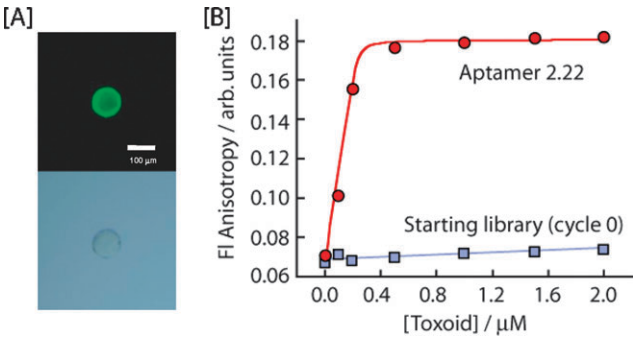

Fig. 2 Representative [A] fluorescence and [B] brightfield micrographs of BoNT/A Hc-peptide conjugated agarose microbead after incubation with Fl-ssDNA pool at cycle 2. (c) A representative fluorescence anisotropy plot of the starting ssDNA library and Apt 2.22 against BoNT toxoid.

ssDNA pool was eluted under alkaline conditions. Binding of the fluorescently labeled ssDNA pool to target-functionalized beads was readily monitored under the fluorescence stereomicroscope (Fig. 2a). Underivatized beads exhibited no fluorescence in the presence of the labeled ssDNA pool (data not shown), demonstrating that the selected pools are specifically binding to their intended targets.

A main advantage of this selection approach is the ability to monitor overall bulk binding dissociation constants (K_ds) for each enriched selection cycle *via* fluorescence anisotropy.[12,13] In both selections, minimal enhancement in the overall bulk K_ds was observed from selection cycles 1 to 2 (Table 1), thus both aptamer pools generated after round 2 were cloned and sequenced. After examining the cloned sequences for homology and secondary structure,[14] five unique Hc-peptide aptamer sequences and three unique toxoid aptamer sequences were selected for further affinity characterization (all the cloned and studied aptamer sequences are provided in the ESI†). We note that our obtained cloned sequences lack a single consensus family of binders. However, this is not uncommon in most SELEX approaches. We reasoned that since only a handful of aptamer clones were sequenced and studied for their affinities (*ca.* <0.001%), this could invariably lead to an incomplete survey of the entire pool of selected sequences and thus leading to the observation that a single consensus aptamer pool is absent. The binding measurements for each individual aptamer sequence were again performed using fluorescence anisotropy, and K_ds were determined using published procedures.[12] Table 1 summarizes the K_ds obtained for each aptamer against their respective targets. Toxoid aptamers exhibited K_ds between 3 and 51 nM, while Hc-peptide aptamers ranged from ~1–4 μM.

Table 1 A summary of the dissociation constants (K_ds) obtained for the aptamers (individual and bulk cycle pool) *via* fluorescence anisotropy measurements

Selection cycle	Bulk K_d/μM	Individual aptamer	Individual K_d/μM
(a) BoNT/A Hc-peptide			
0	n.b.[a]		
1	3.30		
2	1.46	2.5	4.20
		2.7	1.32
		2.12	1.25
		2.14	3.49
		2.20	1.09
(b) BoNT/A-Toxoid			
0	n.b.[a]		
1	0.068		
2	0.028	2.22	0.003
		2.26	0.019
		2.33	0.051

[a] n.b. = no binding observed.

Competition assays were performed to investigate if the generated aptamers were able to compete for binding against a commercially available anti-BoNT/A-toxoid monoclonal Ab.[11] Briefly, Fl-ssDNA was incubated with either the Hc-peptide or toxoid at a saturating concentration. If the Ab competes for the same binding site as the aptamers, the displacement of the Fl-ssDNA will effect a decrease in anisotropy. After titrating in increasing concentrations of Ab to the apt 2.20/Hc-peptide complex, there was a significant decrease in the observed anisotropy values (a complete list of all sequenced aptamers is in the ESI†). This indicates that the Fl-aptamer was displaced by the Ab, suggesting that the Hc-peptides are able to bind both Ab and our generated apt 2.20. We also observe the same binding trend for apts 2.5, 2.7, 2.12 and 2.14. Interestingly, we did not observe any decrease in anisotropy upon Ab addition to the aptamer–toxoid complex (apts 2.22, 2.26 and 2.33). This indicates that the Ab is not displacing the toxoid-bound aptamers, suggesting that the binding sites for both Ab and aptamers are distinct.

While Abs against the various serotypes of BoNT are commercially available, Abs generally suffer in structural robustness and affinity consistency (as they are being generated in different batches), hence limiting their effective use in biomolecular detection platforms. It is thus of interest to develop synthetic binding ligands against the various serotypes of BoNTs that are more structurally robust, readily synthesized, and cost-efficient to produce. Herein, we have demonstrated the application of an efficient and easy-to-execute "single microbead" SELEX approach to generate ssDNA aptamers against both BoNT/A Hc-peptide and toxoid. Even though agarose and Tentagel beads were used in this study, the technique should readily be applicable to other microbeads which are ~50 to 100 μm in diameter (the minimum size required for adequate handling and manipulation). Additionally, this selection approach does not require expensive equipment as only a single-wavelength fluorescent stereomicroscope is needed. Through this approach, we have demonstrated that low nM K_d ssDNA aptamers were generated against the toxoid target after only 2 cycles, whereas low μM K_d aptamers were selected against the Hc-peptide. Our binding data suggest that high-affinity aptamers can be generated in a single selection cycle, as there is only a minimal decrease in the overall bulk K_ds from cycles 1 to 2. Last, all of the tested anti-Hc-peptide ssDNA aptamers were able to competitively inhibit the interaction between the peptide and anti-BoNT Ab, which suggests that the ssDNA aptamer may potentially act as a therapeutic agent to prevent the docking of the BoNT-Hc to both ganglioside and binding protein on the synaptic vesicle surfaces.

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Notes and references

‡ *Materials*. Synthetic 29-mer BoNT/A peptide was ordered from EZBioLab (Westfield, IN), and was ascertained to be >95% pure by HPLC analysis. BoNT/A toxoid (toxin which is inactivated, usually *via* formaldehyde) was purchased from Metabiologics, Inc (Madison, WI). Anti-BoNT/A Antibody (Ab) was ordered from Abcam (Cambridge, MA). Ni-NTA derivatized agarose beads were purchased from Invitrogen (Carlsbad, CA) and polystyrene Tentagel-NH_2 beads (90 μm in diameter) from Rapp Polymere (Tubingen, Germany). The 40 randomized nt containing ssDNA library and both derivatized and underivatized primers were obtained from either Integrated DNA Technologies (Coralville, IA) or Biosearch Technologies Inc. (Novato, CA), and are of the following sequences: ssDNA Library: 5′-ATACCAGCTTATTCAATT-N_{40}–AGATAGTAAGTGCAATCT-3′; R-Primer: 5′-(Biotin)-AGATTGCACTTACTATCT-3′; F-Primer: 5′-(Fluorescein)-ATACCAGCTTATTCAATT-3′. *Conjugation of BoNT peptide and toxoid protein onto microbead*. The Hc-peptide, aa 1177 to 1195, 19-mer, incorporates both a short 4-mer glycine spacer and a 6-mer His tag on its N-terminus (total length 29-mer) to facilitate efficient attachment onto commercially available Ni-NTA derivatized agarose beads. According to both the manufacturer's specification (www.invitrogen.com) and our own calculation, a single Ni-NTA agarose bead is estimated to be able to conjugate to ~20–70 pmol of His-tagged target molecules. As the supplied agarose beads vary in their diameters (~60 to 140 μm), we only selected microbeads with a diameter of ~100 μm to ensure consistency. Briefly, after repeated washing of ~20–30 agarose microbeads in both water and binding buffer (20 mM HEPES, pH 7.4; 150 mM NaCl; 5 mM KCl; 2 mM $MgCl_2$; 2 mM $CaCl_2$), the microbeads were incubated with the Hc-peptide target at room temperature for 2 h with gentle agitation. During incubation, the NaCL concentration was adjusted to 1 M. The beads were then washed 2× with water and 2× with binding buffer *via* low-speed centrifugation, and the Hc-peptide conjugated microbeads were stored in binding buffer at 4 °C. For the BoNT/A toxoid, the protein is conjugated to Tentagel-NH2 microbeads, which were pre-swollen in PBS buffer overnight. Again according to the manufacturer's specification, the loading capacity of the Tentagel microbeads is 0.24 mmol g^{-1} and it is estimated that a single microbead has a loading capacity of ~80–100 pmol (www.rapp-polymere.com). For the conjugation process, the BoNT-A toxoid was first activated with ~3 eq of both 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), followed by incubation with ~5–10 Tentagel-NH2 beads for 2 h with gentle agitation. The beads were then quenched with ~3 eq. of ethanolamine with gentle agitation at room temperature for 5 min, and subsequently washed 2× with PBS and 2× with binding buffer *via* low-speed centrifugation. The toxoid conjugated beads were subsequently stored in binding buffer at 4 °C. *Single microbead SELEX selection*. In a 50 μL microcentrifuge tube, a single BoNT Hc-peptide or toxoid conjugated microbead was incubated with 10 μL of DNA library (1 nmol, suspended in binding buffer). It is estimated that with this concentration, the DNA library contains a diversity of ~10^{14} different sequences. The ssDNA library was heated at 95 °C for 7 min in a thermal cycler (Biorad, Hercules, CA) and gradually cooled to 20 °C over 30 min. After incubation for 120 min at room temperature with gentle agitation, the DNA library and bead suspension were carefully transferred to a petri dish and observed under a fluorescent stereomicroscope (Olympus, Model SZX12). It is noted that the input starting ssDNA library for selection cycle 1 was not fluorescently labeled, thus the microbead was monitored to ensure proper washing and handling processes. Using a hand-drawn glass micropipette, the DNA library solution was carefully withdrawn while the single microbead remained on the petri dish. The single microbead was then washed 7 times with binding buffer and 3 times with nuclease-free water (in our numerous prior observations, brief washes with a small volume of water do not adversely affect the interaction of aptamers with their intended targets). This was accomplished by carefully introducing 20 μL of the appropriate washing solution to the bead, gently blowing the water droplet by the glass micropipette to agitate the bead. Specifically, the bead was observed to circulate rapidly in the 20 μL wash droplet, which suggests that the bead was being efficiently washed. After the extensive washing process, the bead was re-suspended in 5 μL of distilled water. The whole suspension was then transferred to a fresh 100 μL microcentrifuge tube for amplification. The suspension was heated at 95 °C for 5 min and 1 μL of the suspension was quickly retrieved for pilot PCR to determine the number of PCR cycles needed for optimum product amplification. Both the forward and reverse primers used in the PCR amplification were both biotin and fluorescently (Fl)-labeled, respectively. Employment of these primers allowed efficient generation of the necessary "sense strand" Fl-labeled ssDNA after the amplification step. Briefly, the biotinylated-dsDNA was conjugated onto MyOne streptavidin magnetic beads (Invitrogen, Carlsbad, CA) and the fluorescent-ssDNA was eluted with 15 mM NaOH. After neutralization with 5% acetic acid (v/v), the Fl-ssDNA was desalted *via* BioRad's Micro Bio-spin 30 desalting columns (Hercules, CA). The Fl-ssDNA was then vacufuged to dryness and re-suspended in binding buffer, which was then analyzed *via* PAGE for its purity and UV spectroscopy for its concentration. The Fl-ssDNA was now ready for the second round of selection. Fl-ssDNA generated for individual aptamer and bulk rounds 1 and 2 binding studies were generated using the above approach. Prior to the second round of selection, 10 μL of the Fl-ssDNA library (~0.1 nmol, suspended in binding buffer) was again heated at 95 °C for 7 min in a thermal cycler and gradually cooled to 20 °C over 30 min. A single Hc-peptide or toxoid conjugated microbead was then added to the ssDNA library and incubated for 60 min at r.t. with gentle agitation. The same extensive washing steps were applied after incubation and the microbead was then observed under the fluorescent stereomicroscope. The fluorescent bead was then carefully transferred to a fresh microcentrifuge tube (ESI† Fig. S1) and the same pilot PCR and subsequent large scale amplification process, as described previously for selection cycle 1, was repeated. While the pilot PCR for selection cycle 2 employed both the biotin- and Fl-labeled primers (for both specificity and binding studies), the remaining ssDNA portion after round 2 selection was amplified *via* underivatized primers such that the resulting dsDNA generated could be efficiently ligated onto the Qiagen's (Valencia, CA) pDrive "U-overhang" vectors suitable for cloning.

1 S. D. Jayasena, *Clin. Chem.*, 1999, **45**, 1628.
2 C. Tuerk and L. Gold, *Science*, 1990, **249**, 505.
3 A. D. Ellington and J. W. Szostak, *Nature*, 1990, **346**, 818.
4 S. D. Mendonsa and M. T. Bowser, *J. Am. Chem. Soc.*, 2004, **126**, 20.
5 M. Berezovski, M. Musheev, A. Drabovich and S. N. Krylov, *J. Am. Chem. Soc.*, 2006, **128**, 1410.
6 N. O. Fischer, T. M. Tarasow and J. B. Tok, *Curr. Opin. Chem. Biol.*, 2007, **11**, 316.
7 N. O. Fischer, T. M. Tarasow and J. B. Tok, *Analyst*, 2007, **132**, 187.
8 G. Schiavo, M. Matteoli and C. Montecucco, *Physiol. Rev.*, 2000, **80**, 717.
9 L. L. Simpson, *Annu. Rev. Pharmacol. Toxicol.*, 2004, **44**, 167.
10 A. Rummel, S. Mahrhold, H. Bigalke and T. Binz, *Mol. Microbiol.*, 2004, **51**, 631.
11 www.abcam.com; product cat. # ab37344.
12 J. Cho and R. R. Rando, *Biochemistry*, 1999, **38**, 8548.
13 D. H. Ryu, A. Litovchick and R. R. Rando, *Biochemistry*, 2002, **41**, 10499.
14 M. Zuker, *Nucleic Acids Res.*, 2003, **31**, 3406.