

# Llama-derived single-domain antibodies for the detection of botulinum A neurotoxin

Marla D. Swain · George P. Anderson · Dan Zabetakis · Rachael D. Bernstein ·  
Jinny L. Liu · Laura J. Sherwood · Andrew Hayhurst · Ellen R. Goldman

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**Abstract** Single-domain antibodies (sdAb) specific for botulinum neurotoxin serotype A (BoNT A) were selected from an immune llama phage display library derived from a llama that was immunized with BoNT A toxoid. The constructed phage library was panned using two methods: panning on plates coated with BoNT A toxoid (BoNT A Td) and BoNT A complex toxoid (BoNT Ac Td) and panning on microspheres coupled to BoNT A Td and BoNT A toxin (BoNT A Tx). Both panning methods selected for binders that had identical sequences, suggesting that panning on toxoided material may be as effective as panning on bead-immobilized toxin for isolating specific binders. All of the isolated binders tested were observed to recognize bead-immobilized BoNT A Tx in direct binding assays, and showed very little cross-reactivity towards other BoNT serotypes and unrelated protein. Sandwich assays that

incorporated selected sdAb as capture and tracer elements demonstrated that all of the sdAb were able to recognize soluble (“live”) BoNT A Tx and BoNT Ac Tx with virtually no cross-reactivity with other BoNT serotypes. The isolated sdAb did not exhibit the high degree of thermal stability often associated with these reagents; after the first heating cycle most of the binding activity was lost, but the portion of the protein that did refold and recover antigen-binding activity showed only minimal loss on subsequent heating and cooling cycles. The binding kinetics of selected binders, assessed by both an equilibrium fluid array assay as well as surface plasmon resonance (SPR) using toxoided material, gave dissociation constants ( $K_D$ ) in the range  $2.2 \times 10^{-11}$  to  $1.6 \times 10^{-10}$  M. These high-affinity binders may prove beneficial to the development of recombinant reagents for the rapid detection of BoNT A, particularly in field screening and monitoring applications.

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M. D. Swain · G. P. Anderson · D. Zabetakis · J. L. Liu ·  
E. R. Goldman (✉)  
Center for Bio/Molecular Science and Engineering,  
Naval Research Laboratory,  
4555 Overlook Ave. SW,  
Washington, DC 20375, USA  
e-mail: ellen.goldman@nrl.navy.mil

R. D. Bernstein  
Nova Research Inc.,  
1900 Elkin Street, Suite 230,  
Alexandria, VA 22308, USA

L. J. Sherwood · A. Hayhurst  
Department of Virology and Immunology,  
Southwest Foundation for Biomedical Research,  
7620 NW Loop 410,  
San Antonio, TX 78227, USA

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## Introduction

Botulinum neurotoxin, a protein produced by *Clostridium botulinum* bacterium, is known to be the most toxic substance in existence [1, 2]. It is considered to be 15,000 times and 100,000 times more toxic by weight than the nerve gases VX and sarin, respectively, both of which are considered to be weapons of mass destruction. Due to botulinum neurotoxin's extreme toxicity and ease of production, its potential use as a bioterror agent is a legitimate threat, and has led to the toxin's designation as a Category A risk agent by the CDC. There are seven known structurally similar BoNT serotypes (A–G) which pose a

potential threat to both military and civilian populations. However, exposure to BoNT serotype A (BoNT A) has been responsible for most foodborne outbreaks, and has been known to cause more acute symptoms that lead to higher mortality [3, 4]. Currently, laboratory diagnosis of BoNT in clinical specimens and food is performed using the mouse bioassay [5]. Though this bioassay method can detect amounts of BoNT as small as 0.03 ng, it is labor intensive and results are often not available for several days [6]. Thus, there is a need to develop a more rapid, sensitive assay that allows for the early detection of natural or intentional BoNT exposure.

Several methodologies have been employed in efforts to improve assays that are used to detect botulinum toxins, such as ELISA, microsphere arrays, evanescent wave fiber-optics, and electrochemiluminescence [7–12]. Most of these immunoassay formats utilized rabbit or mouse polyclonal or monoclonal IgG as the recognition element. However, both sensitive and specific immunoassays that use conventional antibodies often lack stability and succumb to chemical or heat exposure [13]. The use of various IgG derivatives (e.g., Fab, Fab'2 and scFv fragments) has also been proven to be problematic due to decreased stability compared to the parent molecule and limited solubility [14]. More recently developed single-domain antibodies (sdAb), derived from members of the *Camelidae* family, may be poised to replace conventional antibodies in immunoassay formats because of their observed stability, solubility and ability to refold after denaturation [15–17].

SdAb are recombinantly expressed variable regions from camelid heavy-chain antibodies. Unlike antigen-binding regions of conventional IgG that are derived from the pairing of light-chain and heavy-chain variable domains, heavy-chain antibodies bind antigen through unpaired variable heavy domains [16, 18]. As a result, the sdAb are smaller and often observed to be more stable than recombinantly expressed conventional antibody variable domains (scFv) [15, 16, 19]. SdAb that have affinities and specificities for a variety of targets, including proteins, small molecules, bacteria, and viruses, have been described [20, 21]. Previously, we have reported on the selection of sdAb against the biothreat agent ricin [22]. In addition, we have shown that sdAb derived from llamas immunized with a cocktail of botulinum neurotoxin-complex toxoids specifically bind to toxoided and untoxoided BoNT A complex in buffer and milk; however, those sdAb were all found to bind to one of the neurotoxin-associated proteins (NAPs), hemagglutinin 33 (Hn-33) [23]. Recently, Conway et al. described the isolation and characterization of sdAb against the seven serotypes of BoNT [24]. A llama was immunized simultaneously with all seven toxin toxoids and binders selected in solution towards biotinylated toxin. Other newly published reports have described the isolation of sdAb derived from naïve libraries that inhibit the catalytic activity of BoNT

A Tx [25, 26]. In this paper, we report the development of a phage-display library prepared from a llama that had been immunized with only BoNT A Td, and on the use of bead-bound and plate-immobilized targets for selection. Binder specificity and affinity were evaluated by Luminex direct binding and sandwich assay and by surface plasmon resonance (SPR). We more thoroughly examined the thermal stability of selected BoNT A binders by examining direct binding to bead-immobilized toxin after thermal cycling.

## Materials and methods

**Reagents** BoNT A toxoid (BoNT A Td)- and BoNT A toxin (BoNT Tx)-conjugated microspheres were purchased from Metabio, Inc. (Madison, WI, USA). BoNT A complex toxoid (BoNT Ac Td) was obtained from the U.S. Department of Defense Critical Reagents Program (CRP). Rabbit anti-BoNT A/B and monoclonal anti-BoNT A were from the CRP; monoclonal anti-BoNT B was from Tetracore Inc. (Rockville, MD, USA). PhycoLink<sup>®</sup> Streptavidin-R-Phycoerythrin (SA-PE) was purchased from Prozyme (San Leandro, CA, USA). EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and *N*-hydroxysulfosuccinimide (sulfo-NHS), used for coupling proteins to microspheres, and the biotinylation reagent NHS-LC-LC-biotin were obtained from Pierce (Rockland, IL, USA). Phosphate-buffered saline (PBS), Tween 20 and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). EDC and sulfo-NHS used for protein immobilization onto SPR chips, as well as other reagents, buffers and materials used in conjunction with ProteON SPR, were purchased from Bio-Rad (Hercules, CA, USA).

**SdAb library construction** Immunization of one llama with BoNT A Td was done through Triple J farms (Bellingham, WA, USA). The animal was immunized five times, at three-week intervals, with 100 µg BoNT A Td per injection; the immunization protocol was essentially the same as that described previously [24]. White blood cells were isolated from 40 mL of llama blood, and total RNA was extracted using the QIAmp<sup>®</sup> RNA Blood Mini Kit from Qiagen (Valencia, CA, USA). Extracted RNA was used in an oligo-dT primed reverse transcriptase reaction to yield cDNA. The resultant cDNA was amplified by PCR using flanking primers, and DNA fragments from nonconventional IgG2 and IgG3 were cloned into the pcan21 phage display vector as previously described [16, 27, 28]. A small test library was constructed and 16 individual clones were chosen for DNA sequencing. All of the test clones were unique and had markedly different CDR lengths and sequences. The final phage-display library contained approximately  $1.3 \times 10^8$  members, as judged from the number of transformants.

**Library panning and SdAb selection** Panning of the constructed library was carried out in the same manner as reported previously [28] using BoNT A Td and BoNT Ac Td. Panning on BoNT A Tx- and BoNT A Td-coated Luminex microspheres was performed using a modified protocol. In brief, 10  $\mu\text{L}$  of phage ( $\sim 10^8$  cfu) were added to bead-containing wells of a filter plate that had been previously blocked with PBS containing 0.1% Tween-20 (PBST) and BSA (10 mg/mL) and incubated for 0.5–1 h at room temperature with shaking. After incubation, nonbinding phage were removed with ten washes with PBST and the beads were transferred to clean wells. This was followed by ten washes with PBS. Bound phage were base eluted from the beads, as previously described [28], and the beads and eluted phage were separated by centrifugation at 11000 rpm for 2 min. Monoclonal phage enzyme-linked immunosorbent assay (ELISA) was performed after two or three rounds of panning to identify individual positive clones. Luminex assay was used to further downselect positive clones by eliminating clones that displayed similar binding profiles to toxin-coated bead sets. The positive clones that were selected for sdAb protein production were then sequenced so that unique sdAb genes were identified. The MultAlin program was used to align the sdAb sequences [29].

**SdAb protein expression and purification** The production of soluble sdAb protein was performed as described previously [28]. Briefly, unique sdAb genes were subcloned into the peca45 expression vector and the resulting constructs were transformed into *Escherichia coli* Rosetta (Novagen, Madison, WI, USA). Protein was isolated from the periplasmic compartment by osmotic shock and purified by immobilized metal affinity chromatography (IMAC) followed by gel filtration on a Superdex G75 column (GE Healthcare).

**Luminex assay protocols** SdAb, conventional antibodies and toxoids were crosslinked to carboxylated Luminex microspheres (Austin, TX, USA) using the two-step carbodiimide coupling protocol provided by the manufacturer. The Luminex system allows for multi-analyte assay detection and gives a signal derived from the median fluorescence intensity of at least 100 separate microspheres.

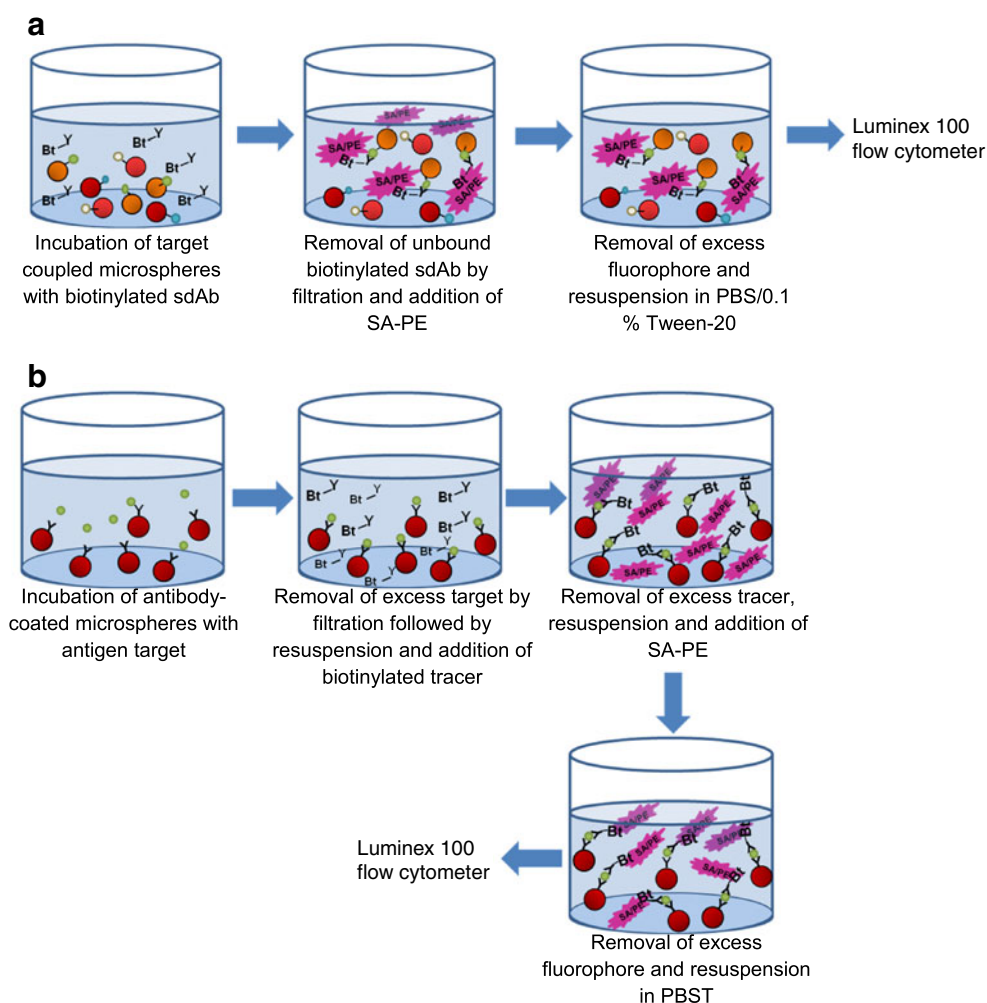
Direct binding assays were carried out as previously described [30, 31] by serially diluting biotinylated (Bt) tracer reagents in a 96-well, 1.2  $\mu\text{m}$  multiscreen filter plate (Millipore, Billerica, MA, USA) (60  $\mu\text{L}$ /well) (Fig. 1a). A mixture of protein-coated microspheres including spheres coated with target (5  $\mu\text{L}$ /well) was added to each well, and the Bt-tracer/microsphere mixture was allowed to incubate for at least 30 min at room temperature. Unbound Bt-tracer was then removed by filtration and 5  $\mu\text{L}$  of SA-PE (5 mg/L) were added to each well before the mixture was incubated for an

additional 30 min. Excess SA-PE was removed by filtration, and the microspheres were resuspended in 85  $\mu\text{L}$  of PBST and then transferred to a standard microtiter plate prior to taking measurements with the Luminex 100 flow analyzer.

For Luminex-analyzed sandwich immunoassays (Fig. 1b), selected antibody-coated microspheres were incubated in filter plate wells with different amounts of antigen for 30 min at room temperature. After excess antigen had been removed by filtration, Bt-tracer was added, and antibodies and sdAb at stock concentrations of 5 mg/L and 1 mg/L, respectively. After 30 min of incubation at room temperature, excess Bt-tracer was removed by filtration and SA-PE (2.5 mg/L) was added. The plate was allowed to incubate for another 30 min in the dark at room temperature before binding was evaluated using the Luminex instrument as described above.

**Surface plasmon resonance measurements** The SPR kinetic measurements were obtained using the ProteON XPR36 from Bio-Rad. To test the kinetics of the various anti-BoNT A sdAb, GLC chips were coated with soluble sdAb binder clones. For example, a GLC chip had one of six lanes coated with one of the following sdAbs: DA5, DF5, C1, A6, C6, and the anti-ricin sdAb G5. Each sdAb was coated at 5  $\mu\text{g}/\text{mL}$  in 10 mM acetate buffer pH 5.0 following the standard EDC coupling chemistry provided by the manufacturer. It was observed afterwards that only DA5, DF5 and NA6 as well as the G5 were active. Next, toxoided material was flowed through sdAb-immobilized channels at concentrations ranging from 0 to 100 nM at a flow rate of 50  $\mu\text{L}/\text{min}$ . An attempt was made to attach toxoided material to a GLC chip. However, the process of toxoidation resulted in a limited number of available sites and insignificant amounts of protein were immobilized on those lanes. To circumvent the lack of reactive sites that remained, the toxoided material was first biotinylated with an  $\sim 100$ -fold excess of NHS-LC-LC-biotin. Excess biotin was removed by gel filtration using a Bio-gel P-10 column (Bio-Rad, Hercules, CA, USA). The biotinylated toxoids ( $\sim 50$   $\mu\text{g}/\text{mL}$ ) were then bound to an NLC chip, which is coated with NeutrAvidin by the manufacturer. In this manner, an NLC chip was successfully coated with BoNT A Td in two lanes, and with BoNT B Td, BoNT Ac Td, BoNT Bc Td, and BoNT Ec Td each in a single lane. All experiments were performed at 25  $^{\circ}\text{C}$ . SdAb binding was evaluated by simultaneously flowing six sdAb concentrations that ranged from 0 to 30 nM at 50  $\mu\text{L}/\text{min}$  for 180 s over the toxoid-coated chips, and then monitoring dissociation for 900 s. The chip was regenerated using 50 mM glycine-HCl (pH 2.0) for 36 s, prior to testing the next sdAb. The data were analyzed with ProteON Manager<sup>TM</sup> 2.1 software, and binding constants were determined using the software's Langmuir model.

**Fig. 1** Schematic representations of the processes used for (a) direct binding assays and (b) sandwich assays by Luminex



*Circular dichroism* CD spectra were acquired using a Jasco J-815 CD spectropolarimeter equipped with a PTC-4235 single-position Peltier temperature-control system. The sdAb to be measured was dialyzed extensively against 5 mM sodium borate buffer pH 8.0 and brought to a final concentration of 30–50  $\mu\text{g}/\text{mL}$ . All measurements were made in a 10 mm pathlength quartz cuvette with a stir bar. Spectra were acquired at a temperature interval of 5  $^{\circ}\text{C}$  from wavelengths ranging from 245 to 195 nm at a scan rate of 20 nm/min. During further heating/cooling cycles, a temperature ramp rate of 5  $^{\circ}\text{C}/\text{min}$  was used, and only the value at 203 nm was monitored.

## Results

**Library construction** One llama was subjected to serial immunization with BoNT A Td. Prior to library construction, the polyclonal response to the target was evaluated by the fractionation of IgG into conventional and heavy-chain antibody subclasses to confirm the presence of heavy-chain antibodies that recognized BoNT A. The methods of

fractionation and direct binding immunoassay of IgG have been described previously [30]. Purified heavy-chain antibody subclasses from a BoNT A Td-immunized llama showed binding toward BoNT Ac Td, BoNT A Td and BoNT A Tx (not shown). On confirming the presence of anti-BoNT A heavy-chain antibody, a phage-display library of sdAb, derived from mRNA of white blood cells isolated from the immunized llama, was constructed.

**Selection of BoNT A binders** The constructed library was panned using BoNT A Td- and BoNT Ac Td-coated plates or BoNT A Td- and BoNT A Tx-coated microspheres. After the first round of panning, polyclonal phage ELISA showed the presence of BoNT A binders in both plate-panned and bead-panned amplified phage. Isolation of binders in round one was further supported by Luminex assay of amplified polyclonal phage using BoNT A Tx-, BoNT A Td-, and BoNT Ac Td-coated microspheres (Fig. S1 of the “Electronic supplementary material”). After two rounds of panning, binders were identified based on monoclonal phage ELISA and Luminex assay results. Luminex phage assays showed that most BoNT A binding phage displayed



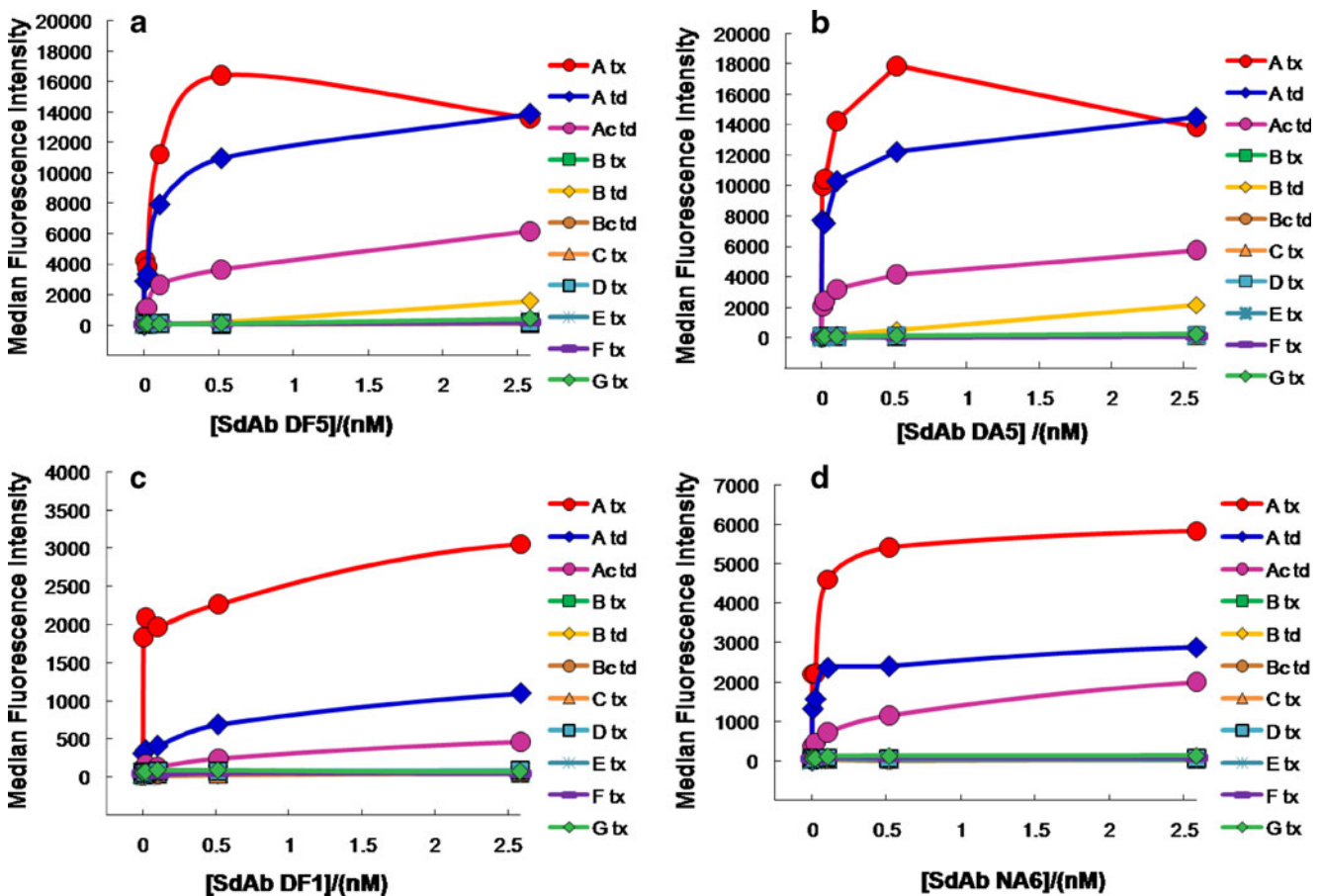
little cross-reactivity with other BoNT serotypes (B and D) and an irrelevant toxin, staphylococcal enterotoxin B (SEB), but a few clones gave high signals with BoNT B Td (Fig. S2 of the “Electronic supplementary material”). We chose 15 clones for sequencing, based on their direct binding profiles. Sequencing revealed ten unique sequences that fit into families containing related CDRs; eight clones spanning several families were moved to the expression vector for further evaluation (Fig. S3 of the “Electronic supplementary material”).

**Fluid array immunoassays** The Luminex system, which is a specialized flow cytometer for performing multiplexed fluoroimmunoassays, was used to assess binding specificity and affinity in direct binding and sandwich assay formats. In direct binding assays, the majority of the isolated sdAb showed the highest median fluorescence intensity for BoNT A Tx (Fig. 2). However, when the sdAb were tested for binding to other BoNT toxin serotypes (B, C, D, F and G), very little or no signal was observed on either toxin- or toxoid-coated microspheres. Dissociation constants for

BoNT A Tx and BoNT A Td for these binders, calculated from the Luminex equilibrium direct-binding experiments, varied from 22 to 420 pM.

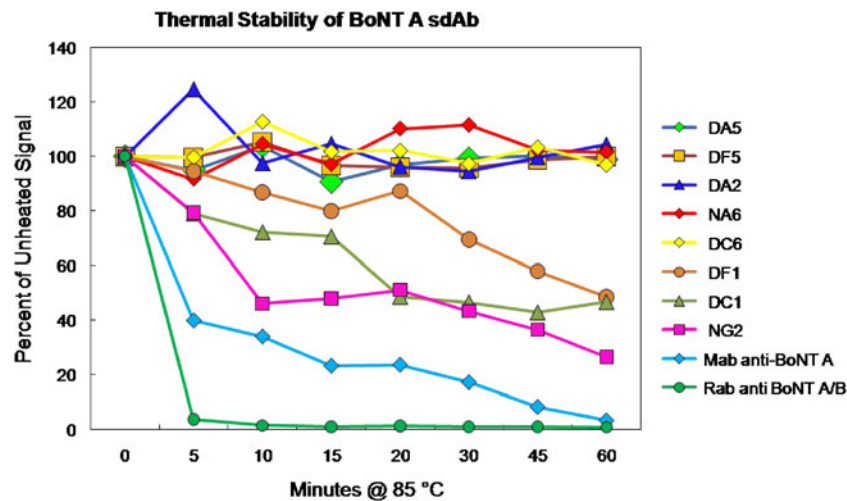
Direct-binding Luminex assay was also used to examine the thermal stabilities of the selected BoNT A binders. Aliquots of soluble sdAb binders were heated to 85 °C for periods of up to an hour, then cooled to room temperature. The sdAb were diluted to a concentration of 1 µg/mL, and their binding to BoNT A Tx- and BoNT A Td-coated microspheres was compared to the signal from unheated samples. Figure 3 shows the thermal stability results obtained using BoNT A Tx Luminex beads. After exposure to heat, the evaluated sdAb were also observed to maintain their selectivity, as no binding to BoNT B- and BoNT E-coated microspheres was observed (Fig. S4 of the “Electronic supplementary material”).

Thermal stability was evaluated in more detail by titrating BoNT A Tx-coated Luminex beads with sdAb clones DA5 and DF5 after repeated heating and cooling. Figure 4 shows the results of the direct binding assay with four cycles of heating/cooling. The data from both clones



**Fig. 2** Specific binding of selected soluble sdAb to cognate antigen. Clones DA5, DF1, DF5 and NA6 (panels A–D) are representatives of the many isolated sdAb that were highly specific for BoNT A Tx.

Homogeneous direct-binding assays were performed by incubating sdAb with microspheres coated with BoNT A toxin, BoNT A toxoid and BoNT A complex toxoid, as well as other BoNT toxin serotypes



**Fig. 3** Ability of soluble sdAb protein to bind to BoNT A Tx-coupled microspheres after exposure to heat. Both mouse and rabbit conventional antibody and sdAb were heated to 85 °C for variable

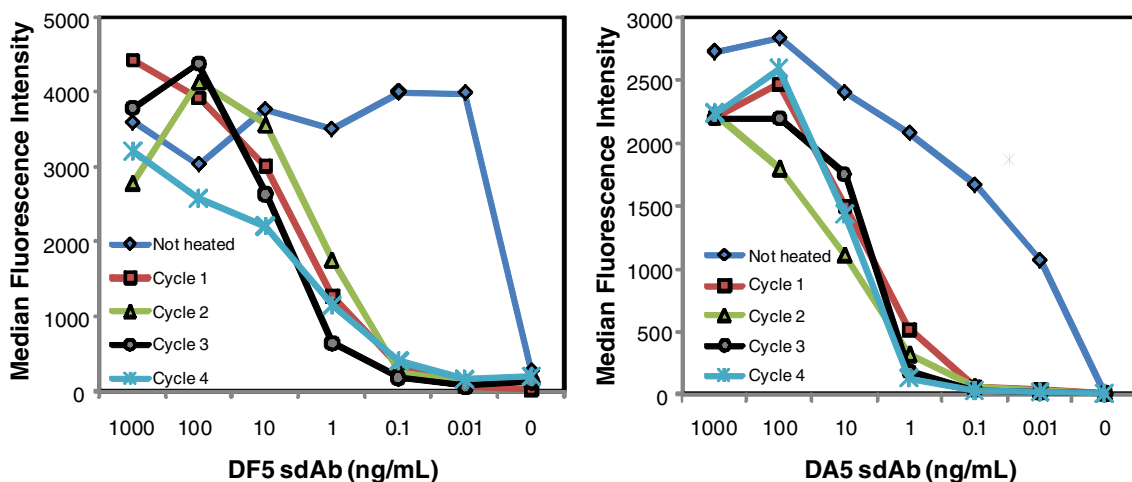
amounts of time and then cooled prior to assays. Sample assay concentrations were 1 µg/mL and 10 µg/mL for sdAb and conventional antibody, respectively

show a dramatic change in binding activity as a function of sdAb concentration after the first heating/cooling cycle. However, little change in binding activity is observed for the remaining cycles.

Eight of the anti-BoNT A sdAb were immobilized to Luminex microspheres and assessed as capture reagents in sandwich immunoassays against BoNT A Td. All of the sdAb clones were able to recognize BoNT A Td when used as captors paired with sdAb or conventional antibody tracers (Fig. S5 of the “Electronic supplementary material”). These positive results gave us the impetus to test their ability to detect live toxin.

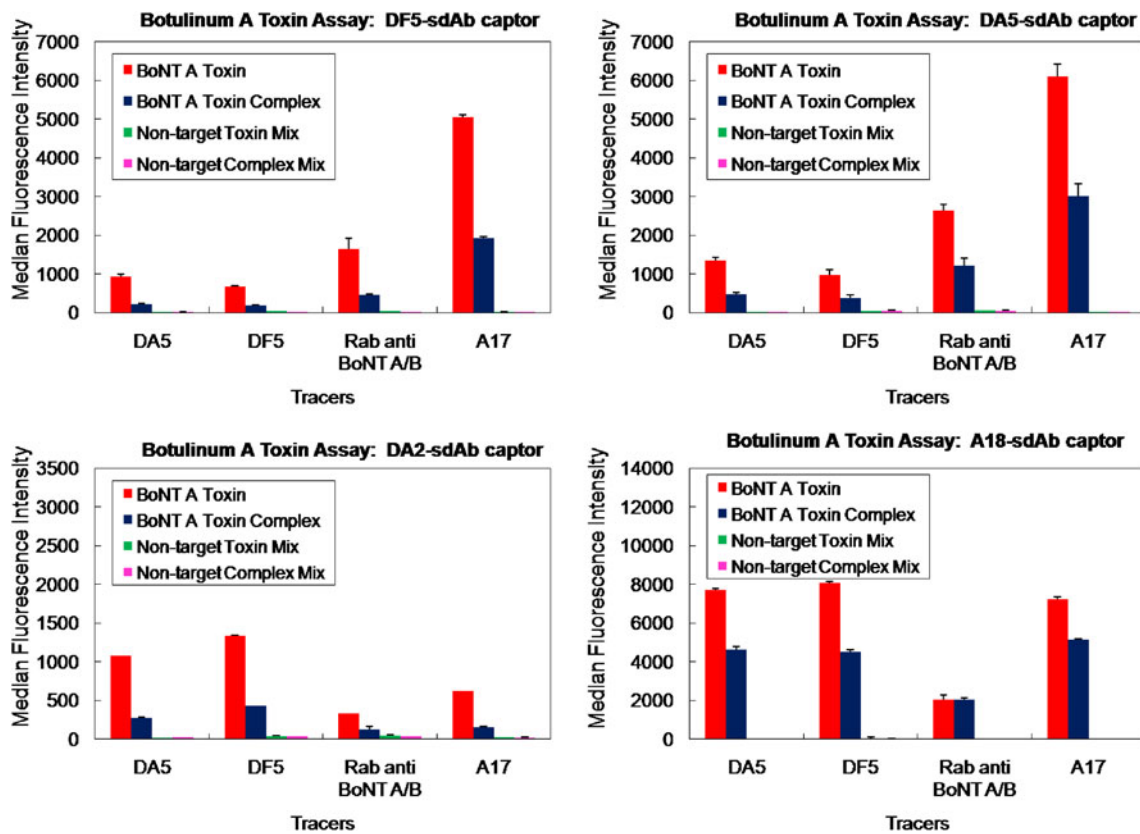
The same Luminex sandwich assay format was extended to assess the binding and specificity of sdAb-captor/tracer pairs to uncoupled BoNT A Tx and BoNT Ac Tx. The eight

sdAb isolated in this work as well as A18, an anti-BoNT A sdAb isolated by Conway et al. [24], were immobilized on microspheres and tested as capture reagents paired with each of four tracers: DF5, DA5, rabbit anti-BoNT A/B and A17, another anti-BoNT A sdAb isolated by Conway et al. [24]. Figure 5 shows the results for the DF5, DA5, DA2 and A18 captures with the four tracers. When used as capture elements in these assays, clones DC6, NA6, and DF1 gave more modest signals on the live BoNT Tx and BoNT Ac Tx, and the sdAb clones NG2 and C1 did not show binding. Cross-reactivity towards the other BoNT serotypes was assessed by challenging each captor/tracer pair with a mix of BoNT serotypes B–G each at 1000 ng/ml. Overall, the sdAb captor/tracer pairs were specific for soluble BoNT A Tx and BoNT Ac Tx.



**Fig. 4** Titration of BoNT A Tx-coupled microspheres with sdAb clones DF5 and DA5 after four successive heating and cooling cycles. For these assays, 50 µL of sdAb clones (50 µg/mL in PBS) were heated from 25 °C

to 85 °C at a rate of 6 °C/min and then held at 85 °C for 5 min and cooled back down to room temperature. Serial dilutions of cycled samples were then added to Luminex microspheres coated with BoNT A Tx



**Fig. 5** Live BoNT A toxin sandwich assays using sdAb clones DF5, DA5, DA2 and A18 as captors paired with four different antibody tracers. The captor/tracer pairs were challenged with  $1 \times 10^5$  pg of

BoNT A toxin or BoNT A toxin complex. Specificity for BoNT A toxin or A toxin complex was evaluated by challenging the captor/tracer pairs with  $1 \times 10^5$  pg each of the other six BoNT serotypes

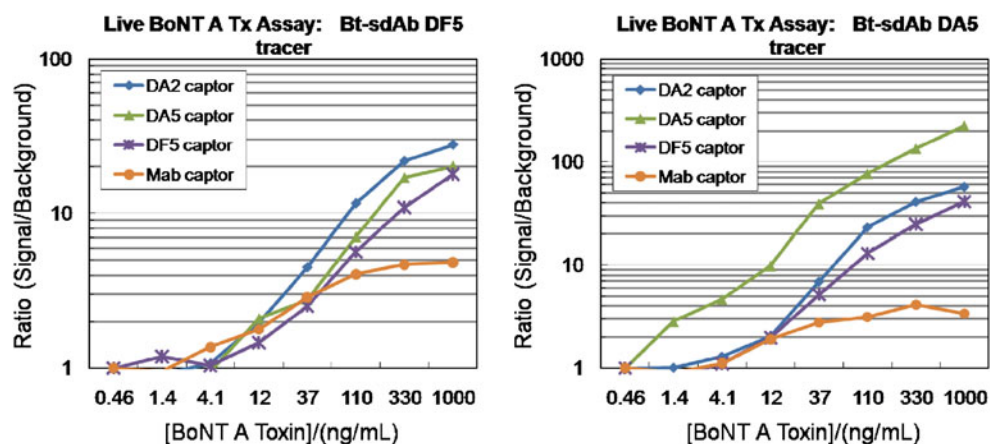
Titration of active BoNT A Tx using sdAb DF5 and DA5 as tracers were performed in order to determine the detection limits of live toxin (Fig. 6). The lower limit of detection (40 ng/ml) was established as three times the ratio of signal to background.

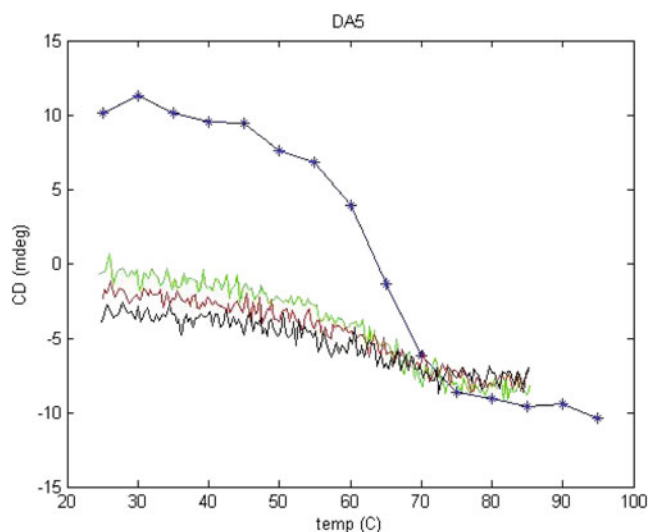
repeated cycles of heating and cooling. For these experiments, the molar ellipticity at 203 nm was recorded as a function of temperature in order to monitor sdAb backbone secondary structure (Fig. 7; Fig. S6 of the “Electronic supplementary material”). Protein concentration was 37  $\mu$ g/ml in 5 mM sodium borate, pH 8.0 buffer. Heating rate was 5  $^{\circ}$ C per minute for all scans.

*Circular dichroism* SdAb have been observed to maintain their structure at temperatures as high as 90  $^{\circ}$ C and undergo reversible thermal denaturation [15, 19]. CD was used to evaluate the secondary structure of selected sdAb upon

*Kinetics of SdAb–BoNT A toxoid binding* Surface plasmon resonance was used to determine the kinetics of the sdAb–

**Fig. 6** Live BoNT A toxin sandwich assays using sdAb clones DF5 and DA5 tracers paired with various antibody captors. The background values used were derived from signals obtained at the lowest BoNT A Tx concentration (0.46 ng/ml)





**Fig. 7** CD melting curves of sdAb DA5 during heating cycles. The blue curve is the first heating cycle, where a spectrum was collected at each step (not shown), with only the value at 203 nm shown. The green, red and black curves are the second, fourth, and seventh heating cycles, respectively, where only the value at 203 nm was monitored during the heating cycle

BoNT A Td interaction. Biotinylated BoNT A Td was first immobilized onto a NeutrAvidin-coated SPR chip. Then, to demonstrate that the chip surface was active, mouse monoclonal anti BoNT A and mouse monoclonal anti BoNT B were flowed across the chip surface in concentrations ranging from 30 to 0 nM (Fig. S7 of the “Electronic supplementary material”). Sensorgrams that show the binding response from experiments where sdAb clone DA5 was injected orthogonally into channels that contained the Bt-BoNT A Td material are shown in Fig. S8 of the “Electronic supplementary material.” The calculated  $K_D$  values for clones DF5, DA5, and DC6 are given in Table 1. All of the binders tested showed binding to immobilized Bt-BoNT A Td and Bt-BoNT Ac Td but not to Bt-BoNT B Td, Bt-BoNT Bc Td, or Bt-BoNT Ec Td.

To further validate the binding constants obtained, experiments were performed where selected sdAb were immobilized onto a SPR chip. Toxoided BoNT material was then flowed across the chip in varying concentrations. Sensorgrams showing the BoNT A Td-binding responses of the immobilized sdAb clones DA5, DF5 and NA6 are shown in Fig. S9 of the “Electronic supplementary material.” The  $K_D$  values determined by flowing BoNT A

Td with concentrations varying from 100 to 0 nM across sdAb-coated channels are reported in Table 1.

## Discussion

We compared two panning methods. In the first, BoNT Ac Td and BoNT A Td were immobilized onto wells of a 96-well plate; in the second, BoNT A Td and BoNT A Tx were immobilized onto microspheres. None of the sdAb isolated from the BoNT Ac Td panning showed good binding to BoNT A Td or BoNT A Tx in Luminex experiments. Binders to the toxin- and toxoid-coupled microspheres were identified from the other three panning strategies. Comparing the results from panning on toxin-coated beads to those from panning on toxoid-coated beads and plates resulted in the selection of several clones with identical sequences. Isolation of the same binder sequences regardless of panning method indicates that panning using toxoided material, versus bead-immobilized toxin, may be an effective means of selecting for toxin binders. Importantly, the selected binders showed signals on both BoNT A and BoNT Ac.

We chose clones spanning several different sequence families to evaluate further. Luminex direct-binding assays were used both to examine the specificity of the sdAb for the target and to determine their binding constants. Curves resulting from Luminex assays done under equilibrium conditions can be fitted to a standard binding equation to determine the  $K_D$  (Fig. 2). Binding parameters were also determined by SPR. Overall, the  $K_D$  values obtained from SPR experiments where sdAb was immobilized to a SPR chip were within the same order of magnitude as the  $K_D$  values determined from Luminex binding assays (Table 1).

Sandwich assays were performed for the detection of both live toxin and toxoided material. While all sdAb captor/tracer combinations that we examined (Fig. S5 of the “Electronic supplementary material”) showed some response to BoNT A Td, the highest signal/background response was achieved using DF5 as captor and DA5 as tracer, giving a BoNT A Td detection level of 40 ng/mL. The DF5–DA5 captor–tracer response to BoNT A Td rivaled responses observed when either mouse anti-BoNT A or rabbit anti-BoNT A/B was used as the capture antibody. Since the selected sdAb binders were isolated using BoNT A Td- and BoNT A Tx-coupled surfaces,

**Table 1**  $K_D$  values determined by fitting SPR binding and dissociation data to a Langmuir model (SPR), and Luminex  $K_D$  values determined by fitting data to the standard binding equation  $y = (B \max) \times / (K_D + x)$

sdAb	$K_D$ (SPR)/M SdAb immobilized	$K_D$ (SPR)/M Bt-BoNTA Td immobilized	$K_D$ (Luminex)/M
DF5	$1.1 \times 10^{-10}$	$1.3 \times 10^{-11}$	$1.6 \times 10^{-10}$
DA5	$6.0 \times 10^{-11}$	$5.56 \times 10^{-11}$	$2.2 \times 10^{-11}$
NA6	$8.6 \times 10^{-10}$	—	$4.2 \times 10^{-10}$
DC6	—	$1.1 \times 10^{-9}$	—



it was important to evaluate cross-reactivity and sensitivity toward uncoupled BoNT A Tx and BoNT Ac Tx. Sandwich assays for live toxin and complex showed levels of detection that ranged from 30 to 100 ng/ml for sdAb DF5 tracer/captor pairs and 20–40 ng/ml for sdAb DA5 tracer/captor pairs (Fig. 6).

In addition to testing the activity of the sdAb, we also examined their ability to regain their secondary structure and function after heating. In a first test, the sdAb, along with conventional anti-BoNT antibodies, were heated to 85 °C for various periods of time, and then their ability to bind to microsphere-immobilized toxin was determined. After heating, the sdAb were diluted to 1 µg/mL and the standard antibodies were diluted to 10 µg/mL—typical concentrations for use in standard assays, although well above the  $K_D$  values of the binders. All of the sdAb clones we tested retained more ability to bind after heat exposure than conventional IgG when tested using either BoNT A Tx- or BoNT A Td-coated microspheres. CD was then used to follow the secondary structures of several of the sdAb on heating.

The spectra of the sdAb all showed denaturation upon heating followed by partial refolding after cooling to room temperature. While the sdAb did refold measurably after heat denaturation, a significant loss in secondary structure was observed. In comparison, conventional llama IgG subjected to the same heat denaturation failed to recover any secondary structure upon cooling (data not shown). The CD melting curve results for sdAb clone DA5 are shown in Fig. 7, and are reminiscent of a previously reported two-state unfolding curve with an estimated melting point of about 66 °C [17]. Clone DA5 showed the ability to at least partially refold after heat denaturation; after the first heating/cooling cycle about 50% of the original signal at 203 nm was recovered. Significantly less of the signal was lost after repeated heating and cooling cycles, with the melting curve after the seventh cycle appearing similar to the melting curve for the second. This loss of recovery of sdAb secondary structure has been seen previously; it was suggested that it may be due in part to the aggregation and proteolysis of sdAb at elevated temperatures [15]. However, it is unclear if either of those effects are occurring, or even if the secondary structure measured by circular dichroism is directly proportional to activity.

The apparent thermal stability seen in the Luminex activity assay (Fig. 3) contrasts with the loss in secondary structure seen following a single heating cycle, as observed in Fig. 7. The most likely explanation for this is that the concentration of sdAb utilized in the Luminex direct-binding experiment was high enough that a substantial loss in antibody activity (>50% of the antibody present) was required prior to impacting the assay as conducted. To better ascertain the residual activity of the sdAb following thermal cycling, we titrated sdAb clones DA5 and DF5

after repeated heating and cooling and measured the direct binding to BoNT A Tx-coated Luminex beads. For these assays, aliquots of sdAb were heated from 25 to 85 °C at a rate of 6 °C/min, held at 85 °C for 5 min, and allowed to cool to room temperature. After cooling, each aliquot was serially diluted and its direct binding to BoNT A Tx-coated microspheres was tested. Figure 4 shows the results of the direct binding assay with four cycles of heating/cooling. The data from both clones show a dramatic change in binding activity as a function of sdAb concentration after the first heating/cooling cycle. However, little change in binding activity is observed for the remaining cycles. This is consistent with our CD data, which shows a large decrease in secondary structure following the first thermal cycle but smaller decreases with subsequent cycles. Thus, it can be concluded that, for these sdAb, the portion of the protein that refolds properly the first time tends to refold properly after subsequent heating cycles. Still unresolved is the exact nature of this decrease in activity: whether aggregation plays a role, if it is simply a failure to refold, or if it is a combination of both. Most interesting is the fact that most of the loss occurs during the first heating cycle, with the remaining material being fairly stable throughout subsequent heating cycles. This retention of a residual activity along with high affinity allow the sdAb to function effectively in immunoassays following thermal transients.

In conclusion, sdAb against BoNT A Tx, BoNT A Td and BoNT Ac Td were successfully obtained from constructed libraries that were derived from an immunized llama. Isolated sdAb showed specificity for BoNT A Tx coupled to microspheres, and the sdAb clone captor/tracer pair DA5/DF5 was effective for the detection of BoNT A Tx and Td. Most of the isolated clones tested exhibited partial thermal stability, maintaining a portion of their binding ability following exposure to heat. The sub nM  $K_D$  values observed from the selected binders provide further evidence that sdAb have the potential to replace conventional antibodies as recognition elements in immunoassay-based formats.

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