Supporting Information

Centrifugal Microfluidic Platform for Ultra-sensitive Detection of Botulinum toxin

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Supplementary Materials

Materials

Carboxylic acid-functionalized silica microparticles were purchased from Bangs Laboratories, Inc (Fisher, IN). 2-(N-morpholino)proanesulfonic acid (MOPS), poly(ethylene glycol) bis(amine) (PEG bis(amine)), phosphate buffered saline (PBS), succinic anhydride, sodium borate, sodium bicarbonate, Sephacryl S400HR, Percoll, bicinchoninic acid kit (BCA), N-hydroxysuccinimide (NHS), glycine (Gly), bovine serum albumin (BSA), Pluronic F127, sodium azide, Tween-20, n-dodecyl β-D-maltoside, and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Zeba desalting columns, were purchased from Thermo-Fisher Scientific Pierce (Rockford, IL). Quantum dots were purchased from Invitrogen (Carlsbad, CA). Horizon whole milk, canned meat, carrot juice, Dr. Pepper, peanut butter, mayonnaise, canned green beans, honey, and raspberry vinaigrette were purchased from Safeway (Pleasanton, CA). BoNT/A holotoxin and complex were purchased from Metabiologics (Madison, WI). BoNT/A complex was also purchased from List Biological Laboratories (Campbell, CA).

Methods

Monoclonal antibody development (Western Regional Research Center, USDA). Mouse anti-BoNT/A monoclonal antibodies (F1-2, F1-40, F1-51 and F2-43) used in this study were generated following immunization of Balb/c mice with BoNT toxoid (Stanker et al. 2008). Animal protocols adhere to institutional guidelines approved by the Animal Care and Use Committee of the U.S. Department of Agriculture, Western Regional Research Center.

Antibody-microparticle conjugation. Conjugation of the capture antibody to the microparticle proceeded via standard carbodiimide chemistry. 10 mg of silica microparticles pre-functionalized with carboxylic acid groups were activated with 0.5 mmoles of EDC and 0.5 mmoles of NHS at pH 6.4 in 1 mL of 100 mM MOPS to form the succinimidyl ester. The particles were washed with MOPS and PBS. The capture antibody was added at a concentration of 4 μM and the solution was raised to pH 8.15 with 1M $NaHCO_3$ and reacted at 4 °C for two hours. Any remaining activated ester was quenched with 200 mM glycine and washed in PBS three times. The particles were then twice blocked with 1% BSA for 30 minutes at 4 °C. The particles were then washed in wash buffer (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 0.05% (w/v) Tween-20, 0.05% (w/v) Pluronic F127, 0.05% (w/v) n-dodecyl β-D-maltoside, 7.6 mM NaN_3, 0.1% (w/v) BSA) and resuspended in wash buffer to a concentration of 5% solids.

Antibody-quantum dot conjugation. Detection antibodies were labeled with quantum dots using standard EDC/NHS carbodiimide chemistry. Carboxylic acid-terminated quantum dots were diluted in 100 mM sodium borate to a concentration of 600 nM. Detection antibodies added to this mixture to a final concentration of 500 nM. To this mixture 1 nmol of EDC/NHS was added and the reaction proceeded at room temperature for 30 minutes with stirring. The reaction was spun through a desalting column made of Sephacryl S400HR and the first fraction was taken as the quantum dot-antibody conjugate. Degree of labeling was determined using the published value for UV absorption of the quantum dot and the protein concentration was determined using BCA.

SpinDx^{TM} immuonassay protocol. Immunoassays were performed in triplicate. Standard curves were collected by diluting BoNT/A in 50% FBS as the sample matrix. To 7 µL of a 5% solids suspension of capture particles was added 1 µL of a 300 nM solution of quantum dot-labeled detection antibody. To this suspension was added 7 µL of the BoNT/A-spiked FBS to yield 20 nM final concentration of detection antibody. The suspension was incubated with mixing for 20 minutes at room temperature. Each channel of the disk was preloaded with 3 µL of a density medium consisting of 90% Percoll in PBS with 0.05% Tween 20, 0.1% BSA, and 0.1% F127. After incubation, 4 µL of the suspension was added to the channel and the disk was spun at 8000 rpm for 45 s. The bead pellet was analyzed on an Olympus IX-70 fluorescence microscope with 405 nm excitation and 705 nm emission, a CoolSnap HQ interline CCD camera (Roper Scientific, Trenton, NJ) and Image-Pro Plus imaging software (MediaCybernetics, Bethesda, MD). The average fluorescence of each bead pellet was measured and compared with calibration curves generated in parallel with standard dilutions to quantify the target analyte.

Mouse Intranasal Intoxication and Sample Collection (UMass Dartmouth). Swiss-Webster female mice (22 to 25- g) were purchased from Charles River Laboratories, Inc (Wilmington, MA). Intranasal toxin application was carried out by first lightly anesthetizing mice with isoflurane (Isothesia, Abbott Laboratories North, Chicago, IL). Toxin was administered by single application of 20 µL solution to the nares at doses of 50-ng, 500-ng, and 5-µg. The heads of animals was maintained in an upright position to minimize drainage into the posterior pharynx. Blood samples were collected at 30-min and 60-min post-intoxication via retro-orbital bleeding. Serum was separated by centrifugation at 1665xg for 16 min, stored at -20°C, and shipped to Sandia National Laboratories for analysis. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee, University of Massachusetts Dartmouth (2003).

Mouse Intravenous and Oral Intoxication and Sample Collection (Western Regional Research Center, USDA). For oral dosing, 4-5 week old female Swiss-Webster mice were treated with 100 µl of BoNT/A complex (dose levels 500, 5000 or 50,000 ng/ml) or with the same volume of control phosphate gelatin buffer via gavage using Popper feeding needles. Surviving mice were monitored for at least 7 days following experiments for signs of intoxication. For intravenous mouse treatments, mice were injected with 100 µl of BoNT/A holotoxin (500, 5000, or 50,000 pg/ml) or with same volume of phosphate gelatin buffer control via the lateral tail vein. Blood was collected at the submandibular site at indicated time points using serum collection tubes (BD Biosciences). Samples were incubated on ice for at least 30-min before centrifugation at 3000 *g* for 10 min to separate sera from the cellular fraction. Samples were then aliquoted and stored at -80 °C before analysis. Animal protocols adhere to institutional guidelines approved by the Animal Care and Use Committee of the U.S. Department of Agriculture, Western Regional Research Center.

Live-Mouse Bioassay (Western Regional Research Center, USDA). Samples consisting of serial dilutions of BoNT/A holotoxin in phosphate gelatin buffer were prepared, blinded, and shared with Sandia National Laboratories for parallel analysis via both SpinDx^{TM}, ELISA and the live mouse bioassay on the same day. Random groups of 10 Swiss Webster mice (females 4-5 weeks old) were injected with 500 µl of each dosage level intraperitoneally. Animals were monitored for 7 days for signs of intoxication (wasp-waist phenotype, labored breathing and paralysis) or death. Moribund animals were humanely euthanized and counted as dead.

ELISA Protocol. The capture ELISA used here was previously described (Stanker et al. 2008) but modified as follows. Briefly, mAb F1-2 (2 µg/mL) in 0.1 M carbonate buffer (pH 9.6) was absorbed on the surface of microtiter wells and served as the capture antibody. Plates were blocked with 0.1 M Tris-HCl buffer (pH 7.4) adjusted to 0.5% tween-20 and 3% non-fat dry milk powder (TBS-T-NFM). Toxin standards dissolved in TBS-T-NFDM were added and the plate incubated at 37 °C for 1 h followed by 6X wash with TBS-T. A mixture of biotin-labeled mAbs F1-40, F1-51, and F2-43, each at 0.5 µg/mL, was added and the plates incubated at 37 °C for 1 h. The plates were then washed as above and streptavidin-HRP (0.1 µg/mL) was added and the plates incubated at 37 °C for 1 h. The plates were then washed 9X as above and a luminerscent substrate (SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce) was added and incubated for 3 min at room temperature with gentle agitation. Luminescent counts were recorded using a Wallac Victor 3 Multilabel Counter (PerkinElmer Inc., Waltham, MA). Results from a typical analysis of standards are shown in Figure S1.

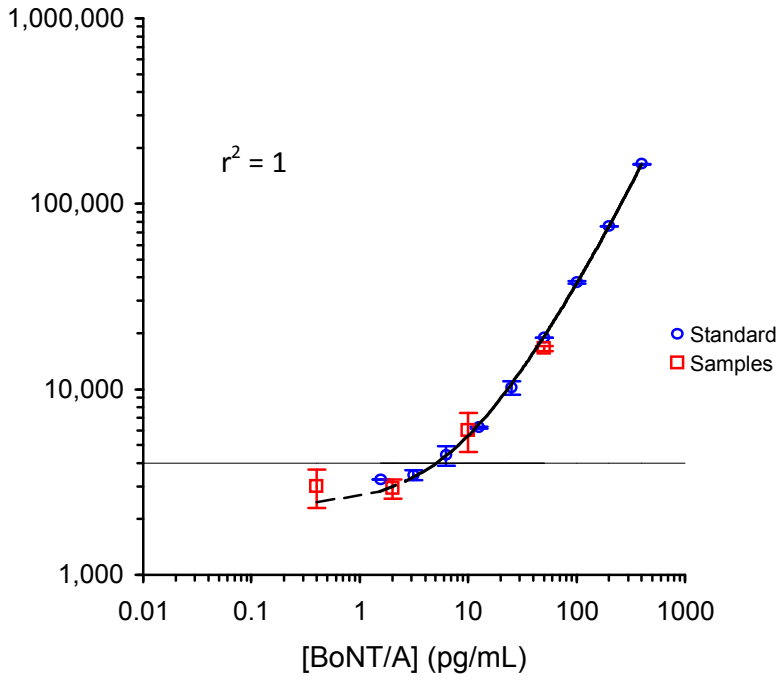

Fig. S1. Typical competition ELISA. Solid line represents average of the blank plus three standard deviations. N=3, bar ± one standard deviation. LoQ is ~6.25 ng/mL.

Detection of BoNT/A Complex

Figure S2 below shows SpinDx™ quantification of BoNT/A complex spiked in FBS. Experimental details are identical to previously described protocol, with substitution of antibodies MCS 80-33-1-1 and MCS 83-71-1-3 as the capture and detection antibodies, respectively, targeting complex protein hemagglutinin 70 (HA70).

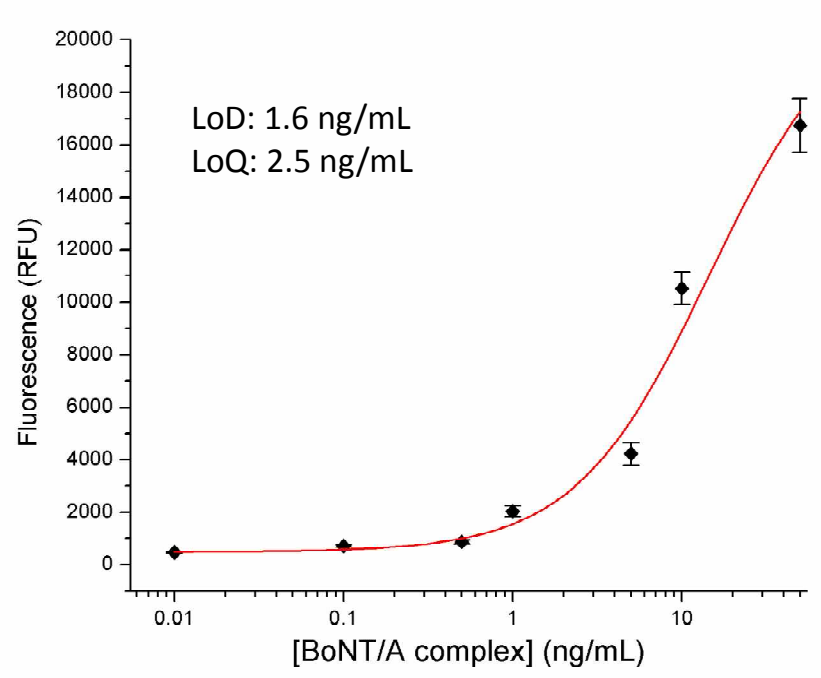

Fig. S2. Dose-response quantification of BoNT/A complex spiked in FBS. The assay targets hemagglutinin 70 (HA70) – a protein within the BoNT/A complex.