MICROFLUIDIC SENSOR FOR BOTULINUM NEUROTOXIN

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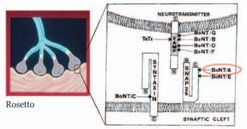
INTRODUCTION

Because of its unsurpassed potency, botulinum neurotoxin (BoNT) has been recognized by the CDC as a "category A" bioterrorism agent - a high-priority risk to national security upon accidental or intentional dissemination. BoNT type A (BoNT/A) is a protease responsible for cleaving SNAP-25 in the synaptic gap, thus blocking the release of neurotransmitters and causing flaccid paralysis associated with botulism. The oral lethal dose for an adult human is <1 mg/kg.



Possible contamination of vulnerable food supplies (i.e. milk) with BoNT warrants the development of inexpensive, routine screening devices that quickly and confidently identify the toxin. To date, the only approved means of BoNT detection is the mouse bioassay - an assay that not only requires animal sacrifice, but also trained personnel, special facilities, and tedious

We have developed multiplexed microfluidic platforms that for sensitive, on-site detection of BoNT/A in solution.

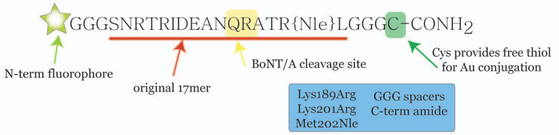
MATERIALS & METHODS

Our sensor is based on the enzymatic activity inherent to boutlinum toxin.. Two separate microfluidic assays have been developed, each using a separate means of presenting the SNAP substrate to the toxin: Au self-assembled monolayers (SAMs) and silica beads.

SUBSTRATE DESIGN

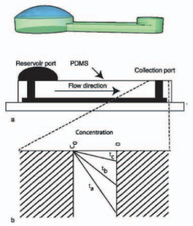
A modified version of the smallest recognizable sequence that mimics native SNAP-25 - the SNAP peptide (residues 187-203) - which includes the BoNT/A specific cleavage site Gln197-Arg198 was synthesized (Frisk, Schmidt).

The fluorescent SNAP peptide (flu-SNAP) served as a recognizable element within the microchannel, conferring specificity.

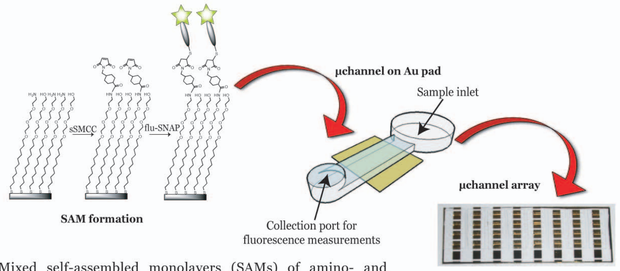


EVAPORATIVE CONCENTRATION

After toxin incubation in microchannels, cleaved flu-SNAP fragments could be concentrated at the "collection" port by evaporation. Large (10 µL) droplets were added to the "reservoir" ports. Fluid evaporation at the collection port induced fluid flow from the reservoir, thus concentrating fluorophore for detection using fluorescence microscopy.



RESULTS: Self-assembled peptide monolayers



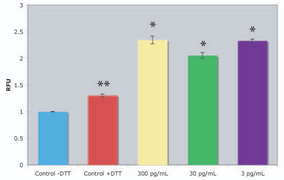
Mixed self-assembled monolayers (SAMs) of amino- and hydroxyalkanethiolates (1:4 w/w) are formed on Au surfaces. A heterobifunctional crosslinker reactive toward both amines and thiols (sulfo-SMCC) was then added to immobilize the fluorescent flu-SNAP peptide substrate.

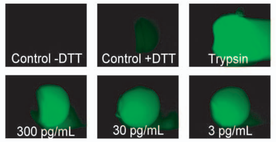
Au "pads" (10.5 mm²), each functionalized with the mixed SAMs. PDMS µchannels (5 mm x 750 µm x 250 µm) were laid atop each pad.

Response of SNAP Peptide SAMs to BoNT/A

SAMs can detect down to ~3 pg/mL. (Left) BoNT/A concentrations 3 pg/mL to 300 pg/mL showed higher fluorescence than both controls (HEPES + 1 mg/mL BSA +/- 1.3 mM DTT); *p<0.0001, **p<0.001. (Right) Fluorescent images obtained from µchannel collection port after 2.5 h incubation and 28 min evaporative concentration.

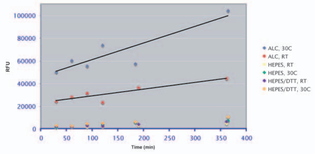
Microchannel Outlet Port Fluorescence



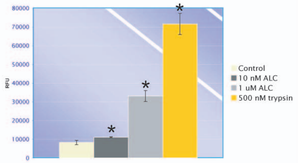


Response of SNAP Peptide SAMs to ALC

Comparison to Control +/- DTT at Varying Temperatures

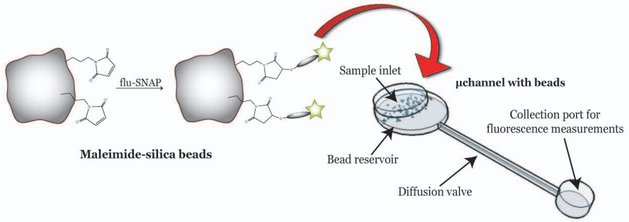


Comparison to "Less Specific" Protease Trypsin



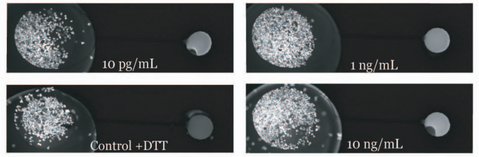
(Left) SAMs were exposed to 1 µM ALC, control (30 mM HEPES + 1 mg/mL BSA, pH 7.3), or control +DTT at either RT or 30°C. Samples were removed and fluorescence was read in a plate reader at each timepoint. (Right) SAMs were exposed to control, 1 µM or 10 nM ALC, or 500 nM trypsin at 30°C; *p<0.001.

RESULTS: SNAP-modified silica beads



Thiol-reactive silica beads were functionalized with the fluorescenct flu-SNAP peptide substrate and loaded directly into the large port of PDMS channels on glass. The PDMS channel was designed to hold the beads in a large reservoir and enhance evaporative concentration via a narrow channel connecting to the two ports called a "diffusion valve."

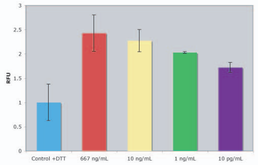
Fluorescent Images of Arrayed Microchannels with Beads

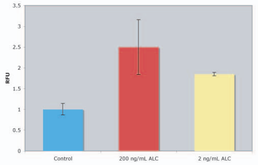


SNAP-modified silica beads can detect down to ~10 pg/mL. (Right) BoNT/A concentrations 10 ng/mL to 10 pg/mL showed higher fluorescence than the control (HEPES + 1 mg/mL BSA +/- 2 mM DTT); *p<0.001. (Above) Entire channel fluorescent images obtained after 3 h incubation and 40 min evaporative concentration.

Sample	*Collection Port (RFU)*	*Bead Reservoir (RFU)*
Control	**18.68**	24.93
10 ng/mL BoNT/A	**35.90**	36.23
1 ng/mL BoNT/A	**33.97**	35.27
10 pg/mL BoNT/A	**30.44**	23.74

Response of SNAP Peptide Silica Beads to BoNT/A and ALC





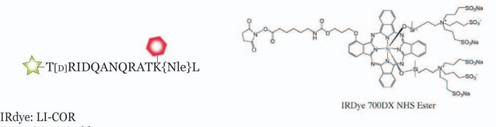
(Left) Beads were exposed to BoNT/A or control with 2 mM DTT at 30.5°C. All data were normalized to the control. BoNT/A from 10 pg/mL - 670 ng/mL showed significant fluorescent signals 1.7-2.4x the control signal, respectively. (Right) Beads were exposed to control +DTT or ALC at 30.5°C. 200 ng/mL ALC had a fluorescent intesntiy 2.4x that of the control, while 2 ng/mL ALC showed 1.8x background.

CONCLUSIONS

Both self-assembled monolayers on Au surfaces and functionalized silica beads were fabricated using a peptide derivative of BoNT/A's natural substrate - flu-SNAP. The peptide SAMs covered the bottom portion of each arrayed microchannel, while the peptide beads were confined within a large reservoir of the microchannel. In both cases, the toxin was able to recognized the fluorescent SNAP peptide by cleaving it and releasing a fluorophore-labeled fragment into solution. The combination of surface chemistry, microfluidics, and fluorescence microscopy allowed us to sense BoNT/A catalysis at or below the desired FBADS select agent concentration of 40 pg toxin per milliliter fluid.

FUTURE WORK

Future work will focus on the conjugation of IRdyes to the peptide in order to detect BoNT/A within complex mixtures without autofluorescence that is characteristic of food/drink components in the visible light region. Also, we are interested in testing other BoNT serotypes (B, D/F) using their respective peptide substrates. This will also help to determine the specificity of the different assays as well as to monitor the occurrence of false-positives (if any). Finally, we have been developing a FRET SNAP peptide for inclusion in microchannels as a quick means of measuring BoNT/A activity.



IRdye: LI-COR
FRET SNAP: Boldt

ACKNOWLEDGMENTS

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