Substrate-Modified Hydrogels for Autonomous Sensing of Botulinum Neurotoxin Type A

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Botulinum neurotoxin type A (BoNT/A) is the most poisonous substance known1,2 and is thus considered one of the most likely toxins to be used as a bioweapon, especially within food supplies.3–5 Deliberate or accidental contamination of food or drink with microbial toxins like BoNT/A is not only a form of a biological attack, but also a “global public health problem”,6 considering the oral lethal dose for a human is 1 µg per kg of body weight.7 The mouse bioassay is the only accepted method for BoNT/A detection, with ELISA typically serving as a quick, preliminary screening tool;8,9 however, both methods suffer from respective drawbacks,10–13 preventing them from being decisive, on-site toxin-screening vehicles. In an effort to improve early BoNT/A detection, we have developed toxin-responsive hydrogel sensors. The responsiveness of the hydrogels relies on toxin enzymatic activity and is therefore specific, as BoNT/A has a substrate cleavage site unique to its type.14 The autonomous BoNT/A sensor was generated by housing toxin-sensitive hydrogels within microfluidic channels, requiring less than 20 µL of contaminated fluid for visual output indicating the presence of BoNT/A. We report herein peptide-modified hydrogels for sensing enzymatic activity of BoNT/A. Preliminary testing with both the BoNT/A catalytic light chain and live BoNT/A will be highlighted, and implications of our work and future explorations will be discussed.

BoNT/A is a disulfide-linked dichain (heavy and light) 150 kDa polypeptide that operates exclusively via enzymatic cleavage of its target substrate, the synaptosome-associated protein SNAP-25. BoNT/A cleaves SNAP-25 in the synaptic cleft, blocking exocytosis of neurotransmitters. The toxin has high substrate specificity, recognizing not only the residues around the cleavage site but also the tertiary structure of the target sequence.15 To develop a BoNT/A sensor, we incorporated modified peptide substrates derived from the SNAP-25 cleavage site into hydrogel structures. Peptides and polypeptides have been integrated into hydrogels for tissue engineering,16,17 drug delivery,18,19 small molecule sensing,20,21 and microfluidic22,23 applications. More importantly, peptide-modified hydrogels can act as sacrificial structures by dissolving in the presence of a stimulus, such as a reducing agent or an enzyme; this response mechanism has been demonstrated effectively by Plunkett et al. with an α-chymotrypsin-sensitive peptide cross-linker24 and by Lutolf et al. with a collagenase-sensitive peptide cross-linker when forming synthetic extracellular matrices.16 Peptides can be incorporated into hydrogels as pendant factors on the main chains constituting the hydrogel, as the cross-linker, or as soluble factors entrapped within the hydrogel matrix. Because of ease of handling, simple fabrication steps, and design and application versatility, peptide-modified hydrogels are practical candidates for sensing toxins that possess enzymatic activity, such as botulinum neurotoxin type A. Toward a BoNT/A sensor, SNAP-25-derived peptide substrates were designed for cross-linking into hydrogel matrices. BoNT/A recognition and cleavage of the cross-linked substrate resulted in visually evident gel dissolution, indicating toxin contamination of a given fluid.

The smallest BoNT/A-recognizable sequence contains residues 187–203 of SNAP-25, SNKTRIDEANQRSTKML, and includes the BoNT type A cleavage site between Q197 and R198. For the peptic SNAP substrate, this sequence was first modified to replace M202 with norleucine (Nle), as this has proven to be a suitable analog without the risk of

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thioether oxidation. Cysteine residues were also added at the termini to facilitate thiol-specific conjugation of functional groups that permit inclusion of the following peptide into a hydrogel matrix via photopolymerization: CSNKTRIDEANQRATK\{Nle\}LC. This 19mer was, in fact, not recognized by BoNT/A within a hydrogel (data not shown). Attributing lack of BoNT/A cleavage and/or recognition to unfavorable tertiary conformations adopted by the peptide upon hydrogel polymerization, glycine spacers (GGG) were added at the termini to alleviate stress at cross-linking junctions. Additionally, K189 and K201 were shown). Attributing lack of BoNT/A cleavage and/or recognition to unfavorable tertiary conformations adopted by the peptide upon hydrogel polymerization, glycine spacers (GGG) were added at the termini to alleviate stress at cross-linking junctions. Additionally, K189 and K201 were shown. The final BoNT/A peptide substrace sequence was: CGGGSNRTRIDEANQRATK\{Nle\}LGGCC (herein referred to as the “SNAP peptide”). SNAP peptide was reacted with PDTEMA (N-[2-(2-pyridyldithio)ethyl methacrylamide] at pH 2.65 to induce a thiol–disulfide exchange at the termini (see Scheme S1 in the Supporting Information), resulting in a dimethacrylated SNAP peptide cross-linker. The SNAP peptide cross-linker was cophotopolymerized in situ with acrylamide (AAm) using long-wavelength UV (25 mW/cm², 30–75 s) and a transparency mask to form 300–500 µm (d) hydrogel posts within 125 µm (h) microfluidic channels.

Substrate-cross-linking hydrogels were used to detect BoNT/A enzymatic activity in microchannels. Because of the toxin’s highly poisonous nature, pilot studies were performed with BoNT/A light chain (ALC) instead of the live toxin. ALC is the 50 kDa subunit that provides catalytic activity, but is unable to enter the cytosol without the 100 kDa BoNT/A heavy chain. ALC is nontoxic; however, its usefulness as a mimic of toxin activity has been asserted during inhibitor screening and for understanding BoNT/A catalysis in vivo.

For initial testing in buffer, ALC (45 µg/mL in 30 mM HEPES, pH 7.4) was added to the microchannels and incubated at 30 °C. Hydrogel degradation was monitored every 1.5–2 h for 13 h by gently removing the SNAP peptide cross-linker after 48 h hydrogel degradation (see Figure S2 in the Supporting Information). In the presence of ALC, SNAP peptide cross-linkers were cleaved, leading to total gel degradation. Because milk products are likely media for mass distribution of the toxin, hydrogel responsiveness was also tested using ALC in cream. Degradation was observed after a one-time 70 h incubation period after which 1× PBS was flowed through to wash out insoluble cream components/residue for visualization (Figure 1). The control for the cream experiments consisted of the SNAP peptide hydrogel exposed to ALC-free solution; lack of degradation of the control post suggests that enzymes present in cream did not cause nonspecific cleavage and, in effect, a “false positive.” The SNAP peptide hydrogels were finally exposed to live, activated 15 g/mL BoNT/A in cream with 1 mg/mL BSA. Caution: Botulinum neurotoxins are highly poisonous and may be handled only by trained researchers in qualifying BSL-2 laboratories. Posts were incubated with less than 20 µL of live toxin for 48 h at 30 °C, whereupon the cream solution was removed and posts gently washed with PBS. It is evident from Figure 2 that SNAP peptide hydrogel posts showed signs of degradation in the presence of live BoNT/A, acting successfully as autonomous biosensors.

Disulfide-linked dichain BoNT/A must be reduced to release ALC for enzymatic activity. In vivo, the toxin is reduced and the heavy and light chains are uncoupled, in vitro, however, BoNT/A requires activation with a reducing
agent such as dithiothreitol (DTT). Thus, despite the sensing capabilities of the SNAP peptide hydrogels with both ALC and BoNT/A, the substrate was cross-linked by means of disulfide bonds (see Scheme S1 in the Supporting Information) and was therefore not ideal for detecting BoNT/A under reducing conditions. With the goal of on-site detection within a microfluidic device, it is impractical to have tedious sample preparation steps, including removal of DTT after toxin reduction (which typically requires gel filtration). As such, we sought to create BoNT/A-responsive hydrogels that did not rely on disulfide linkages. An 8.5 kDa recombinant polypeptide comprised of residues 141–206 of SNAP-25 (rSNAP) was chosen to more accurately mimic the actual in vivo BoNT/A target substrate. rSNAP was conjugated to photopolymerizable moieties using a heterobifunctional cross-linker, acryloyl-PEG-NHS (APN), that readily reacts with free amines at pH 8–9 via the NHS moiety and adds an inert 5 kDa PEG spacer (see Scheme S1 in the Supporting Information). The acryloyl-PEG-rSNAP cross-linking scheme avoids disulfide bonds, thus allowing DTT to activate live BoNT/A and remain within the sample. Simple reaction schemes, rapid in situ photopolymerization, and inexpensive materials are advantageous aspects to these hydrogel biosensors. Using microfluidics, we were able to simply introduce ~20 µL of toxin-laden solution and observe degradation without the necessity for detectors, pumps, or valves, thus making microfluidic-based biosensors promising as toxin-screening vehicles. Toward on-site detection capability, we are currently interfacing hydrogel walls with electrodes for signal transduction upon cleavage to further enhance sensitivity, as demonstrated previously.

The combination of hydrogels and microfluidics has allowed us to generate autonomous sensors that simply require sample addition for a visual readout. To the best of our knowledge, this is the first application of substrate-cross-linked hydrogels for toxin detection. This versatile hydrogel platform can be extended to create sensors for any biological agent that acts through enzymatic cleavage, including tetanus toxin and other BoNT serotypes.

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Supporting Information Available: Construct design and expression of recombinant SNAP-25; detailed syntheses and polymerization of the SNAP peptide and rSNAP cross-linkers; and microfluidic device fabrication (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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EXPERIMENTAL

TOPO TA PCR cloning kit, Gateway pDest14 cloning kit, Gateway pDONOR 221 cloning kit, IPTG, NuPage SDS-PAGE gel system, S.N.A.P MINI prep kit and Probond protein purification system were purchased from Invitrogen. Oligers were purchased from IDT. Codon/plus BL21 competent cells were purchased from Stratagene. Acrylamide (AAm), 4-(benzoylbenzyl)trimethyl ammonium chloride (BP+), 2,2'-dimethoxy-2-phenylacetophenone (DMPA), isobornyl acrylate (IBA), N-methylenediethanolamine (NMDA), N-methylenebisacrylamide (NMBA), and trifluoroacetic acid (TFA) were purchased from Aldrich Chemicals. Acetonitrile (ACN), bovine serum albumin (BSA), dithiothreitol (DTT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phosphate buffered saline (PBS, 10X), and sodium phosphate monobasic dihydrate (NaH₂PO₄) were purchased from Fisher Scientific. Sodium bicarbonate (NaHCO₃), N-vinyl-2-pyrrolidinone (NVP), and thioanisole were obtained from Acros Organics. Ethanedithiol (EDT) was obtained from Alfa Aesar. Tetra(ethylene glycol) dimethacrylate (TeEGDMA) and triethanolamine (TEOA) were obtained from Fluka. (N-[2-(2-pyridyldithio)]ethyl methacrylamide) (PDTEMA) was synthesized in the Moore lab at University of Illinois at Urbana-Champaign according to a previously published protocol¹ and then generously provided for our experiments. Botulinum neurotoxin serotype A holotoxin and light chain fragments were provided by the Johnson laboratory at University of Wisconsin-Madison (the former handled only in said laboratory). Deionized (DI) water was used for all solutions. Hydrogel diameters were measured on an Olympus BX60 microscope. Figures S1, 1, and 2 were taken on an Olympus SZX12 stereoscope using a Leica DFC 300 camera. Figure 3 was taken on a Zeiss Axioscope 2 Plus using a Zeiss AxioCam HRc camera.

Peptide Synthesis

All peptides were synthesized on a Symphony® instrument (Protein Technologies, Inc.) in the UW-Madison Peptide Synthesis Facility using standard solid phase syntheses methods on either Rink Amide resin or NovaPEG Rink Amide resin (Novabiochem). All peptides were N-terminal acetylated and C-terminal amidated. Peptides were deprotected for 4 hrs using a TFA cocktail containing 5% (v/v) thioanisole and 2.5% (v/v) EDT. Peptides were purified using reverse phase HPLC on a preparative scale C-18 column using a 15-50% gradient of 0.08% TFA in ACN against 0.1% TFA. Lyophilized peptides were stored at -20ºC until use.

Recombinant SNAP-25 (rSNAP)

Generation of tagged partial SNAP-25 expression construct. Gateway and TOPO technology from Invitrogen were used. Partial SNAP-25 from amino acids 141-206 containing cleavage site between amino acids 197 and 198, a 6-histidine (His) tag on N-terminus, and a cysteine (Cys) on each terminus (Figure S1) was generated using the following steps:

First, PCR amplified Cys-6His-SNAP-25 (141-206)-Cys from plasmid DNA that contained full length SNAP-25 gene. The primers used were: 5’ SNAP 25 6HIS for TOPO clone: 5’-ATG TGT CAT CAC CAT CAC CAT CAC GCC CGA GAA ATG GAT GAA AAC C-3’ (italic indicates start codon, bold indicates Cys, underline indicates 6-His); 3’ SNAP 25 6HIS for TOPO clone: 5’-TTA ACA ACC ACT TCC CAG CAT CTT TGT TGC ACG TTG G-3’ (italic indicates stop codon, bold indicates Cys). The PCR product was inserted into TOPO TA PCR cloning vector as described by manual.

¹ Supporting Information
Second, in order to put ribosome binding site (RBS) in front of the start codon, a pair of primers, which contained RBS, was designed for use with PCR. The primers were: 5′-AGAAGGAGATATACATATGTGTCATC-3′ (bold indicates RBS); 3′-SNAP25 6HIS for TOPO clone: 5′-TTA ACA ACC ACT TCC CAG CAT CTT TGT TGC -3′. The PCR product was TOPO cloned into TOPO TA vector.

Third, in order to use Gateway technology, a pair of primers was designed for PCR amplification of the partial SNAP-25 gene as described above with B1/B2 adaptors on both sites. Primers were: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGTCATC-3′ (italic indicates Gateway B1 site) and 3′-GGGGACCACTTTGTACAAGAAAGCTGGTTA ACA ACC ACT TCC CAG CAT CTT TGT TGC-3′ (italic indicates Gateway B2 site). PCR product was Gateway cloned into pDONOR 221 by using BP reaction as described in manual to create pEntry vector. The pEntry vector with all the elements was LR crossed with pDest 14 as described in manual to generate the final Gateway expression vector, named pDest 14 SNAP25. All recombinant colonies were verified by sequencing.

Expression and purification of tagged partial SNAP-25. Positive recombinant pDest14SNAP25 DNA was transformed into codon/plus BL21 competent cells. One colony was inoculated into 2 mL LB medium containing 100 µg/ml ampicillin and shaken overnight at 37°C. 500 µl of the overnight culture was inoculated into 50 mL LB medium with 100 µg/ml ampicillin and shaken at 37°C until OD/550 reached between 0.5-0.6. IPTG was then added to a final concentration of 1 mM to induce the expression for 3 hours. Cell pellets were collected by centrifugation at 5000 rpm for 15 min. Resulting protein was purified by using Probond protein purification system as described by manufacturer. Purified recombinant SNAP-25 was verified by SDS-PAGE (Figure S1).

Crosslinker Synthesis

SNAP peptide. SNAP peptide (CGGGSNRTIDEANQRATR[Nle]LGGGC) was reacted with 2.5-5 molar excess PDTEMA in 1:9 ACN:pH 2.65 50 mM NaH₂PO₄ overnight at 4°C. The resulting dimethacrylated SNAP peptide crosslinker was dialyzed against DI water using 1000 MWCO Spectra/Por® Biotech cellulose ester dialysis membranes (Spectrum), lyophilized, and stored at +4°C until use.

rSNAP. rSNAP was dissolved at 1 mg/mL in 100 mM TEOA, pH 8.0. 6X molar excess acryloyl-PEG-NHS (Nektar Therapeutics) was dissolved separately in 50 mM NaHCO₃, pH 8.35. The two solutions were mixed together and shaken at RT for 1.5 hours. rSNAP crosslinker was dialyzed against DI water using 3500 MWCO Spectra/Por® regenerated cellulose dialysis membranes (Spectrum), lyophilized, and stored at -20°C until use.

Microfluidic device

Channels. Polycarbonate cartridges (Grace Bio-labs) were sealed to glass slides (1” x 3” x 0.04”, Corning) via 125-µm thick adhesive gaskets and placed on a hot plate for 30 minutes to seal. Cartridges were filled with IBA prepolymer consisting of 1.9:0:1.0.06 IBA:TeEGDMA:DMPA. Photomasks were designed in Adobe Illustrator and printed on transparency film with a high-resolution printer (3000 dpi, Imagesetter, Madison, WI). For photolithographic formation of channel networks, a photomask was placed on top of the cartridge and exposed to long-wavelength UV (365 nm) for 17 sec at 8.7 mW/cm² (EFOS Acticure A4000). Channels were rinsed with DI water and air-dried.

Ports. At channel inlets and outlets, PDMS connectors (poly(dimethylsiloxane), Dow Corning Sylgard 184) were attached for introducing and removing fluid according to online protocol².
Hydrogel post photopolymerization

**SNAP peptide.** 3-5 µL of a solution containing 5.5-11% (w/v) SNAPDMA crosslinker in an AAm prepolymer (62.5:25:12.5 AAm:NMDA:BP+, 1:9 ACN:pH 2.65 50 mM NaH₂PO₄) was introduced into a microchannel and let settle for 3 minutes. A photomask (Imagesetter) was placed on top and the device was exposed to 365 nm UV light for 30-70 s at 25 mW/cm² (EFOS Acticure A4000). Unpolymerized material was gently removed via suction and structures were washed with DI water. Hydrogel structures were swelled in 1X PBS for 24 hours and diameters were measure before use.

**rSNAP.** 3-5 µL of a solution containing 9-14% (w/v) rSNAP crosslinker in an AAm prepolymer (62.5:25:12.5 AAm:NMDA:BP+, 1:9 ACN:pH 8.0 100 mM TEOA) with 2-4% (v/v) 300 mg/mL DMPA in NVP was introduced into a microchannel and let settle for 3 minutes. A transparency mask was placed on top and the device was exposed to 365 nm UV light for 120-240 sec at 25 mW/cm². Unpolymerized material was gently removed via suction and structures were washed with DI water. Hydrogel structures were swelled in 1X PBS for 24 hours and diameters were measured before use.

**Sample preparation**

*Note 1:* For consistency, all ALC solutions were 45 µg/mL and all BoNT/A solutions were 15 µg/mL. In addition, this allowed for direct comparison of conditions and results to other assays reported in a number of our cited publications. No other concentrations were employed for this particular manuscript. Future experimentation will focus on limits of detection and will therefore investigate a range of concentrations of both ALC and BoNT/A.

*Note 2:* BSA was not added to any ALC samples, as it has not been shown to affect (positively or negatively) the catalytic activity of the light chain. However, Schmidt et al. have shown that 1 mg/mL BSA increases both catalysis and binding affinity for BoNT/A holotoxin; this has been attributed to simple stabilization of a consensus sequence in zinc metalloproteases, but more recent experiments by others may provide more detail regarding this phenomenon. As such, all BoNT/A assays included BSA.

**Controls.** Control hydrogels for SNAP peptide experiments contained NMBA crosslinker; however, some experiments were controlled by exposing SNAP peptide hydrogels to ALC-free solution. Control hydrogels for rSNAP experiments contained unrecognizable peptide crosslinker, CSNKTRIDEANQRATK{Nle}LC.

**ALC in buffer.** Stock solutions of ALC were diluted to 45 µg/mL in 30 mM HEPES, pH 7.4. Diluted solutions were stored frozen at -20°C until use.

**ALC in milk product.** Half & half creamer (Glenview Farms) was centrifuged at 10,000 rpm for 10 minutes and the fatty supernatant was discarded. Stock solutions of ALC were diluted to 45 µg/mL in centrifuged cream and used immediately.

**BoNT/A in buffer.** Concentrated toxin was reduced with 50 mM DTT at RT for 30 min. Stock solutions of BoNT/A were diluted to 15 µg/mL in 30 mM HEPES with 1 mg/mL BSA, pH 7.4. For experiments that required the absence of DTT, BoNT/A stock was gel filtered on a Biorad PD10 desalting column; for experiments that could be completed in the presence of DTT, the final DTT concentration was 1.3 mM.

**BoNT/A in milk.** Concentrated toxin was reduced with 50 mM DTT at RT for 30 min. Stock solutions of BoNT/A were diluted to 15 µg/mL in 30 mM HEPES with 1 mg/mL BSA, pH 7.4. BoNT/A stock was gel filtered using Biorad PD10 to remove DTT. Half & half creamer (Glenview Farms) was centrifuged at 10,000 rpm for 10 minutes and the fatty supernatant was discarded. Stock solutions of BoNT/A were diluted to 15 µg/mL in centrifuged cream and used immediately.
Scheme S1. Cysteine-terminated SNAP peptide 1 (CGGSRTRIDEANQRAT\{Nle\}LGGGC) was reacted with PDTEMA 2 at pH 2.65 overnight at 4°C to form dimethacrylated SNAP peptide crosslinker 3 and 2-thiopyridone, the latter of which was removed during dialysis. Recombinant SNAP-25 polypeptide 4 (rSNAP) was reacted with acryloyl-PEG-NHS 5 at pH 8.35 at RT for 2 hrs to form multi-acrylated rSNAP crosslinker (maximum of four sites shown – 3 Lys, 1 N-terminus) and N-hydroxysuccinimide, the latter of which was removed during dialysis.

Figure S1. (above) Recombinant SNAP-25 construct. (below) SNAP-25 was purified under native conditions: S – supernatant, F – flow through, W – wash fraction, E – elution fraction, M – marker.
Figure S2. 45 µg/mL ALC degrades SNAP peptide hydrogel posts in buffer.  a) initial 525 µm SNAP peptide post in air, t = 0; b) air bubbles were introduced during solution replacement, t = 8.5 hrs; c) completely degraded hydrogel in air, t = 13 hrs; d) 475 µm control post in air after 13 hr exposure to ALC. Corresponding images below a-c are edge outlined using ImageJ software for better visualization of degraded hydrogels. Scale bars = 150 µm.