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 known[1,2] 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 microfluidic[22,23] 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-linker[24] 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 pendent 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, SNKTRIDEANQRATKML, and includes the BoNT type A cleavage site between Q197 and R198. For the peptidic 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.[25,26] 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 replaced with arginines, as as this has experimentally been shown by others to increase peptide hydrolysis[27]. The final BoNT/A peptide substrate sequence was: CGGGSNRTRIDEANQRATR{Nle}LGGGC (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/cm^2, 30–75 s) and a transparency mask to form 300–500 μm (*d*) hydrogel posts within 125 μm (*h*) microfluidic channels.

Substrate-cross-linked 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[28] and for understanding BoNT/A catalysis in vivo.[29–32] 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 solution and imaging with a stereoscope. Solution replacement at each time point helped to remove cleaved material for better assessment of the extent of gel degradation; however, this also introduced air bubbles that were attracted to the posts, but did not appear to interfere with 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,[5] 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 μg/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 qualifitying 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;[14] in vitro, however, BoNT/A requires activation with a reducing

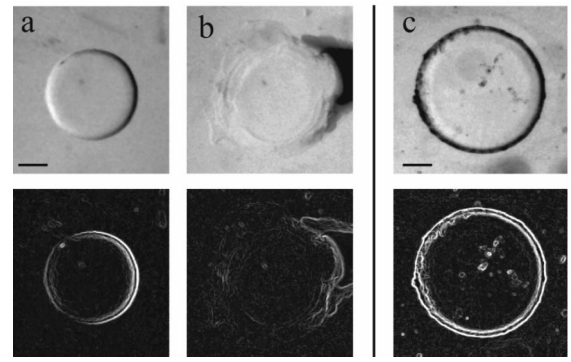

Figure 1. Forty-five micrograms per milliliter ALC degrades posts in cream. (a) Initial 310 μm SNAP peptide post in 1× PBS, $t = 0$ h; (b) after 70 h exposure to ALC in cream; (c) control 425 μm SNAP peptide post after 70 h exposure to cream only. Corresponding images directly below a–c are edge-outlined using ImageJ software for better visualization of degraded hydrogels. Scale bars = 100 μm.

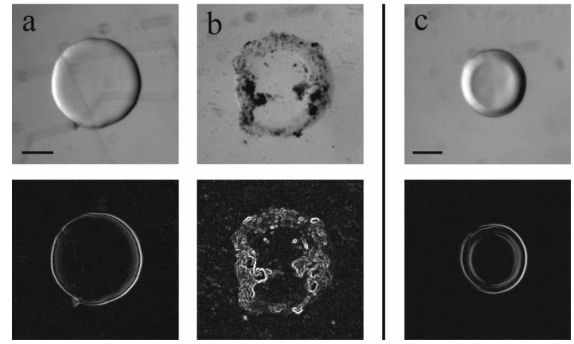

Figure 2. Fifteen micrograms per milliliter BoNT/A degrades posts in cream. (a) Initial 425 μm SNAP peptide post in 1× PBS, $t = 0$ h; (b) after 48 h exposure to BoNT/A in cream; (c) 325 μm control post with BoNT/A-insensitive cross-linker after 48 h toxin exposure. Corresponding images directly below a–c are edge-outlined using ImageJ software for better visualization of degraded hydrogels. Scale bars = 150 μm.

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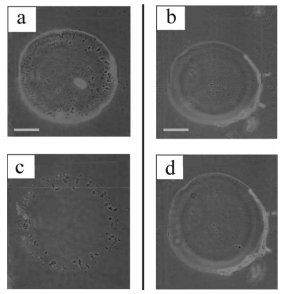

Figure 3. Fifteen micrograms per milliliter BoNT/A degrades rSNAP posts in HEPES. (a) Initial 400 μm rSNAP post in 1X PBS, $t = 0$ h; (b) initial 375 μm control post with BoNT/A-insensitive cross-linker in 1X PBS, $t = 0$ h; (c) after 2 h exposure to BoNT/A with 1.3 mM DTT and 1 mg/mL BSA; (d) control after 2 h of toxin exposure. Area surrounding posts has been false-colored grey to remove dimension markers automatically inserted by camera software. Scale bars = 100 μm.

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-linker was cophotopolymerized with AAm under conditions similar to those of the dimethacrylated SNAP peptide cross-linker to form hydrogel posts within microfluidic channels.

rSNAP hydrogels were exposed to 15 μg/mL BoNT/A with 1 mg/mL BSA and 1.3 mM DTT at 30 °C. Preliminary results indicate that hydrogel posts containing the rSNAP cross-linker degraded in less than 3 h, whereas control posts remained intact (Figure 3). Visual evidence of cleavage suggests that rSNAP was integrated into the hydrogel structure as the cross-linker and that a majority of the APN conjugation points occurred on either side of the BoNT/A cleavage site. Degradative responsiveness supports autonomous BoNT/A sensing capabilities of rSNAP hydrogels without any sample preparation steps, such as removal of DTT. However, controlling the sites of APN attachment could be critical, as some gels resisted degradation, presumably because of conjugation points that excluded the cleavage site from the cross-linked portion; therefore, the cross-linked portion would have remained intact despite BoNT/A cleavage. By controlling conjugation sites, a larger percentage of BoNT/A-recognizable cross-linker should be present within the gel. Optimization of polymerization conditions (time, intensity), site-specific APN conjugation to rSNAP, and BoNT/A sensing tests in other media (milk, orange juice) are in progress to find the optimal hydrogel composition for timely, sensitive detection of BoNT/A.

Both SNAP peptide- and rSNAP-cross-linked hydrogels have shown promise as sensors for the most poisonous toxin known. Using modified forms of BoNT/A's own biological substrate as the hydrogel cross-linker confers a high level of specificity. In addition, visual readout within a microfluidic channel provides that these hydrogels can potentially be used with any contaminated fluid, as demonstrated with milk, without the need for fluorescence, electrochemical, or other detectors typically coupled to biosensors. 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.[33]

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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