Microfluidic Detection of Botulinum Neurotoxin Type A Utilizing Polyacrylamide Hydrogels With SNAP-25 Peptide Crosslinker

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***Abstract*— We synthesized a new botulinum neurotoxin type A (BoNT/A) sensing material consisting of a photopolymerizable polyacrylamide hydrogel incorporating a 25-mer BoNT/A recognizable peptide substrate coupled to an acryloyl-PEG-NHS crosslinker that readily reacts with free amines at pH 8–9 via the NHS moiety. The peptide substrate residues are slightly modified from synaptosomal-associated protein SNAP-25 between amino acid residues 187–203 with an additional three glycine spacers and a lysine on each end for coupling to the crosslinker via NHS-ester conjugation reactions. The alpha and epsilon amines of the N-terminal lysine and epsilon amine of the C-terminal lysine were used to couple the peptide to the crosslinker instead of previously used cysteines to avoid having to remove dithiothreitol (DTT) after sample preparation or potential false positive substrate cleavage by DTT if not removed after sample preparation. By integrating this hydrogel into a microchannel, we developed a BoNT sensor based on the morphology change of this hydrogel. The hydrogel in the microchannel can be degraded in 22 h at 45 μg/mL of light chain (LC) of BoNT/A and 90 h at the lowest concentration of 4.5 μg/mL of LC, but remains intact in Hepes buffer and DTT solutions. The results indicate that the hydrogel-based device can potentially be a portable sensor for the detection of active BoNT/A with high accuracy and specificity.**

***Index Terms*— Botulinum neurotoxin, BoNT/A, hydrogel, biosensor, SNAP-25, microfluidics.**

I. Introduction

CLOSTRIDIUM BOTULINUM neurotoxins (BoNTs), the most toxic substance known to humans with a median lethal dose (LD50) of approximately 1 ng/kg body weight, have attracted much attention in the last few decades due to their potential use as a bioterrorism agent [1]–[5]. BoNTs have traditionally been immunologically distinguished into seven serotypes (BoNT/A–G), among which BoNTs A, B, E and F are known to cause human botulism [6], [7]. An accidental or deliberate release of BoNTs in civilian population, especially in food supply, would seriously threaten human health and may cause panic resulting in large economic losses. Therefore, highly sensitive and facile assays in the early stage are essential to harness outbreak or controlled distribution of BoNTs. The current gold standard for detection of the seven serotypes of *Clostridium botulinum* neurotoxin (BoNT/A-G) is the mouse bioassay [8], [9]. Due to the high cost, low throughput and the need for highly trained personnel and large number of animal sacrifices, a practical alternative to the mouse bioassay is needed.

To date, much efforts have been made to improve technologies to sense the presence of BoNTs either in vivo (testing in animals) or in vitro, such as enzyme-linked immunosorbent assay (ELISA) [10]–[12], immune/real-time-polymerase chain reaction (PCR) [13], [14], mass spectrometry [15], cell culture assays [16], [17], as well as others [18]–[20]. These tools generally require complex instrumentation and are not suitable for in-field testing. Furthermore, some of these assays are not able to distinguish between active and inactive BoNTs. An alternative highly efficient toxin-screening approach developed by M. Frisk et al. [5] was to use a synthetic synaptosomal-associated protein of 25-kDa (SNAP-25) substrate peptide cleavage assay in a microfluidic channel to detect BoNT/A. Peptide integrated hydrogels have been used as sacrificial structures in tissue engineering by decomposition in the presence of a stimulus, such as an enzyme. SNAP-25 is one of three synaptic proteins recognized by the 50-kDa light chain (LC) of BoNT/A in the proteolytic activity leading to paralysis [21], [22]. Thus the decomposition of a hydrogel combined with SNAP-25 modified crosslinker in the presence of BoNTs provides a rapid and accurate detection for the active BoNTs [5].

Although the polyacrylamide (PAAm) hydrogel with SNAP 25-mer (sequence: CGGGSNRTRIDEANQRATR{Nle} LGG-GC) containing a crosslinker developed in the previous work [5] showed sensing capabilities with both LC and BoNT/A, the disulfide bonds in the hydrogel were not

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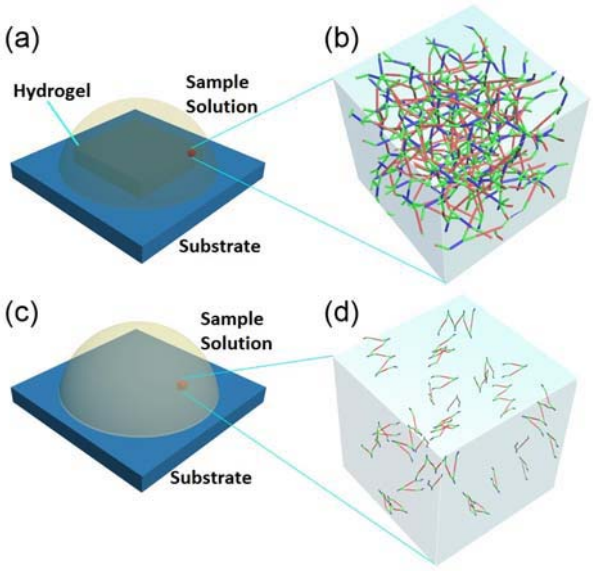

Fig. 1. Schematic of the cleavage of hydrogel by BoNT/A toxin. (a, b) The original network structure of the hydrogel. (c, d) The degraded hydrogel network in the sample solution after cleavage.

stable under reducing conditions and could cause a false positive alert, which limits its application as an on-site sensor for BoNT/A. This is particularly problematic *in vitro* since BoNT/A requires reduction for full activity in substrate cleavage assays. In addition, PAAm SNAP peptide hydrogels without disulfide bonds were investigated to detect BoNT/A. The poor degradation of gels observed was associated with the conjugation points that seemed to prevent the crosslinked portion from being cleaved by BoNT/A [5]. To develop an on-site BoNT hydrogel sensor with higher accuracy, it is important to develop a new SNAP-25 peptide hydrogel without disulfide bonds, where the SNAP-25 has optimized conjugation sites for the crosslinker such as acryloyl-PEG-NHS (APN) to anchor. We previously reported preliminary work in the synthesis of such hydrogel in [23]. Here, we present a more comprehensive study on this approach. In this work, we synthesized a modified 25-mer peptide containing PAAm hydrogel that can be specifically cleaved by BoNT/A. The hydrogel was patterned in a microchannel by an ultraviolet (UV) assisted polymerization process. The hydrogel patterns undergoing cleavage by BoNT/A displayed clear visual changes and could thus serve as a toxin readout as shown in Fig. 1. The novel 25-mer (KGGGSNRTRIDEANQRATR{Nle} LG-GGK) with three maximum conjugation sites for APN was used to improve the sensitivity and visual observation in BoNT/A detection by lowering the degree of crosslinking as shown in Fig. 2. Using this new 25-mer removes any disulfide bonds in the ultimate crosslinker, thus avoiding potential false positives due to the needed reduction by dithiothreitol (DTT) of the BoNT/A. All three conjugation sites on the new 25-mer come from the two lysines including two branch primary amino groups and one N-terminus, fewer than the 4 sites on the 25-mer used in the previous work [5]. Using the newly synthesized and photo-patterned hydrogel, we built microfluidics-based devices for sensing BoNT/A by observing

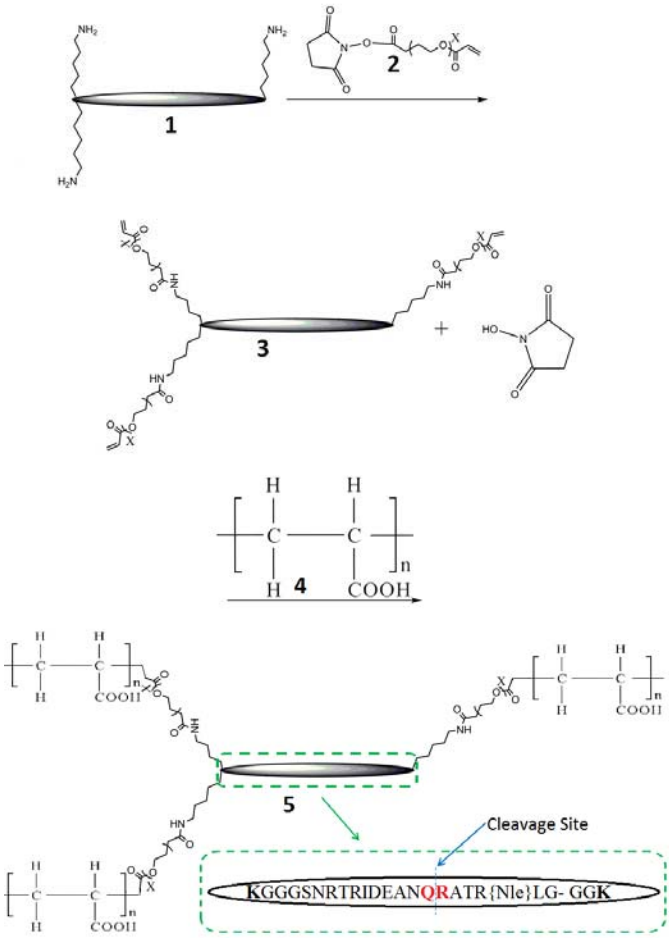

Fig. 2. Schematic of synthesis of SNAP-25 containing crosslinker for BoNT/A responsive hydrogel. The callout shows the modification of the 25-mer with lysine (K) replacing cysteine (C) at both ends. The cleavage site between glutamine (Q) and arginine (R) is also shown.

the morphology change of the hydrogels located in the polydimethylsiloxane (PDMS) microchannel. As a result, the hydrogels in the microchannel were compromised and cleaved in 500 μg/ml of trypsin in 15 minutes, and in a solution containing the LC of BoNT/A (concentration: 45 μg/mL) in 38 h. Such a device could be a promising candidate for a simple, low-cost, portable, and real-time sensor of bio-active BoNTs, such as in food packages and for on-site detection.

II. Experimental

A. Materials and Equipment

Acrylamide (AAm), 4-(benzoylbenzyl)trimethyl ammonium chloride (BP+), 2,2'-dimethoxy-2-phenylacetophenone (DMPA), triethanolamine (TEOA), Sodium bicarbonate (NaHCO3), and N-methylenediethanolamine (NMDA) were purchased from Aldrich Chemicals. N-vinyl-2-pyrolidinone (NVP) was obtained from Acros Organics (Fair Lawn, NJ). 25-mer of SNAP-25 peptide and APN were bought from Peptide 2.0 Inc. (Chantilly, VA) and Nektar Therapeutics (Huntsville, AL), respectively. BoNT/A LC (concentration: 729 μg/mL) was provided by the Johnson laboratory at the University of Wisconsin-Madison, and was further diluted to 45 and 4.5 μg/mL, respectively, with Hepes buffer.

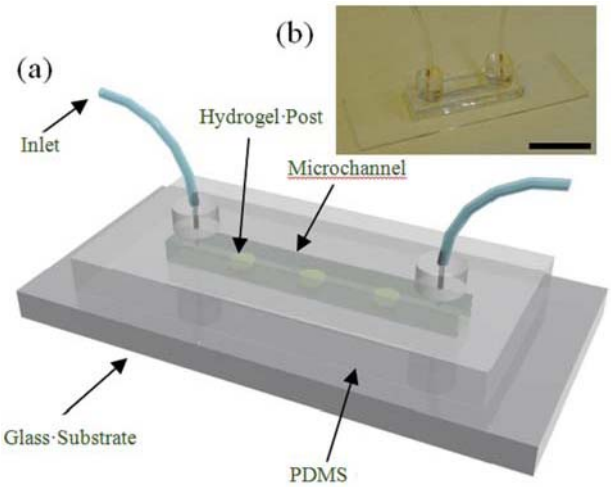

Fig. 3. Schematic of the BoNT/A sensor structure. (a) The microfluidic channel is formed between a glass substrate and a PDMS cover. Hydrogel microposts are photo-patterned in the microchannel. (b) The photo of the device. The scale bar is 2.5 mm.

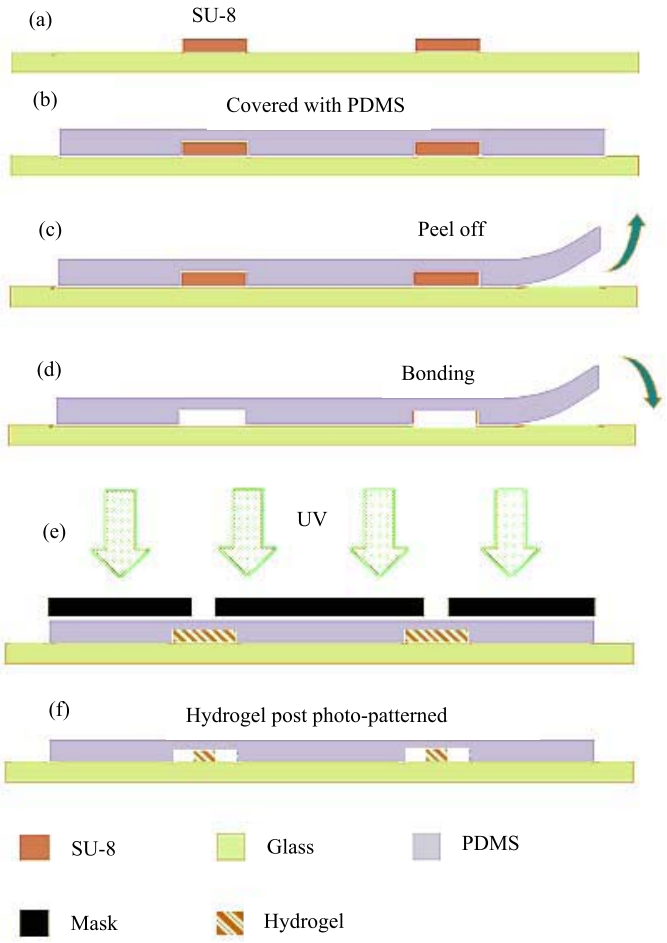

Fig. 4. The fabrication process of the device. (a) Photo-pattern SU-8 on a glass substrate as the mold. (b) Apply and cure a PDMS film over the SU-8 mold. (c) Peel off the PDMS film. (d) Form the microchannel by bonding the patterned PDMS film onto the surface of a glass slide. (e) Inject liquid pre-hydrogel into the microchannel and perform a masked photopolymerization. (f) Hydrogel microposts are defined within the microchannel.

Deionized (DI) water was used for all solutions. Lithography was operated on Omnicure series 2000. Hydrogel diameters were measured on an Olympus BX60 microscope. Figs 3 to 6 were taken on an Olympus SZX12 stereoscope using a Leica DFC 300 camera.

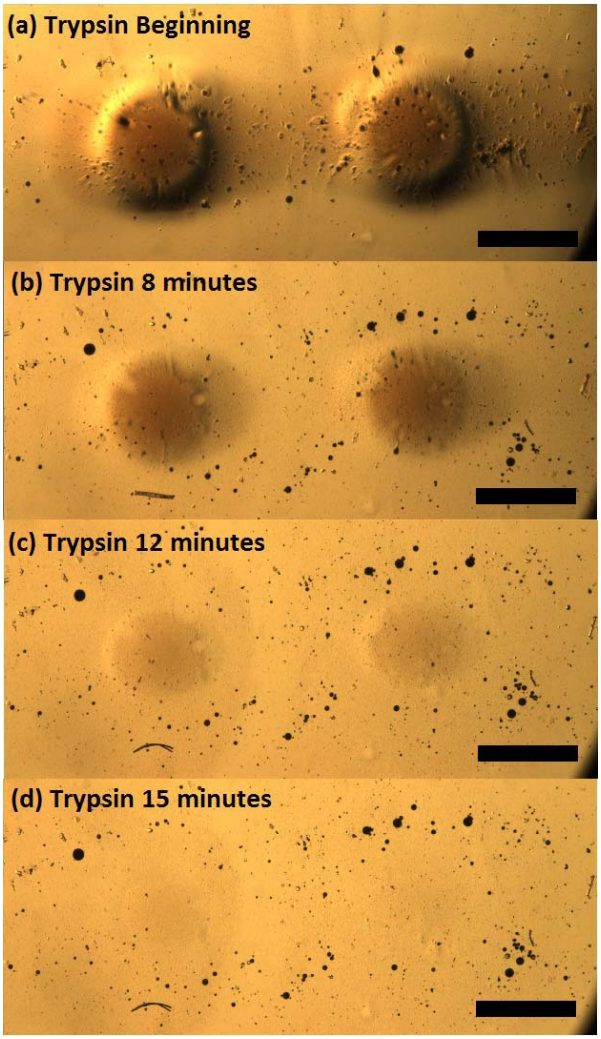

Fig. 5. Time course of testing of BoNT/A-responsive hydrogel with 500 μg/mL of trypsin. (a) Initial state. (b-c) After 8 and 12 minutes of treatment of trypsin, respectively. (d) Complete decomposition of hydrogel after 15 minutes. All scale bars represent 500 μm.

B. Synthesis of the Modified Crosslinker

To synthesize the crosslinker, 5 mg 25-mer peptide was dissolved in 5 ml 100 mM TEOA aqueous solution at pH 8.0. 3× molar excess (30 mg) APN was dissolved in 1 ml 50 mM NaHCO3 aqueous solution at pH 8.4. The two solutions were then mixed together and shaken at room temperature overnight. Acryloyl-PEG-SNAP peptide crosslinker was dialyzed against DI water using 8000-12000 MWCO Spectra/Por regenerated cellulose dialysis membranes (Spectrum), lyophilized, and stored at −20 °C until use.

C. Hydrogel Post Photopolymerization and Photo Patterning

DMPA was dissolved in NVP at a concentration of 300mg/mL (solution a); 5 M HCL was added dropwise to 100 mM TEOA aqueous solution to adjust pH value to 8.0 (solution b); 20 μL solution a was mixed with 180 μL

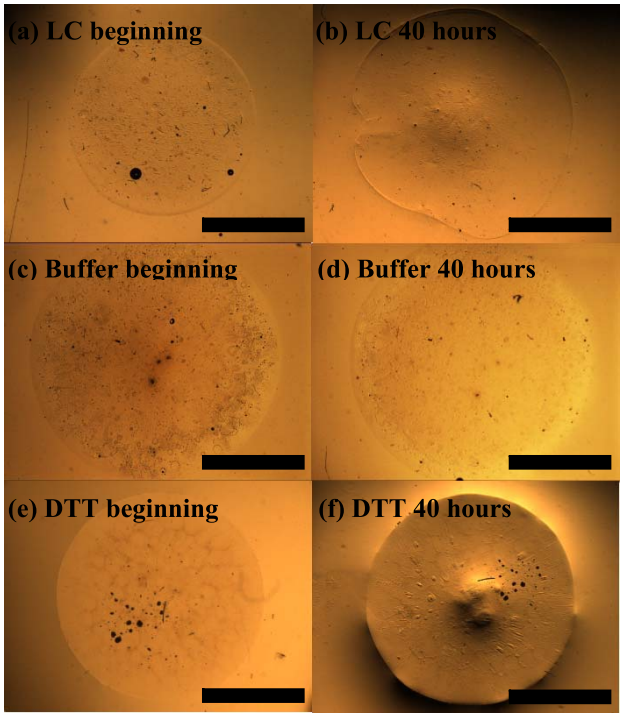

Fig. 6. Images showing the results of large-sized hydrogel posts exposed to LC of BoNT/A, DTT and Hepes buffer. Reagents and reaction times are indicated in the individual panels. (a-b) Results of the BoNT/A-responsive hydrogel treated with LC (45 μg/mL); (c-d) Results of responsive hydrogel treated with Hepes buffer (30 mM); and (e-f) Results of responsive hydrogel treated with DTT (13 mM in Hepes buffer). The hydrogel was able to be cleaved by LC of BoNT/A at 40 h post exposure, but remained intact in buffer and DTT. All scale bars are 2.5 mm.

solution b to produce solution c; 30 mg AAm, 12 mg NMDA and 6 mg BP+ were mixed together to get solution d; 200 μL solution c and 48 mg solution d were mixed together to make solution e; 4 mg acryloyl-PEG-SNAP peptide crosslinker, 20 μL solution e and 0.8 μL solution a were mixed to make BoNT/A-responsive pre-hydrogel. The pre-hydrogel was photo-patterned to form posts on glass substrates using standard lithography (exposure intensity 25 mW/cm^2, 180 s).

D. Fabrication

The schematic and the photo of the sensor structure are shown in Fig. 3(a, b), respectively. Hydrogel posts were photo-patterned in a PDMS microchannel with typical parameters of 30mm $\times$ 100 μm $\times$ 100 μm. Specifically (Fig. 4(a)), an SU-8 structure (SU-8 2100, UV light intensity: 10 mW/cm^2, 35s) was first photo-patterned to form a line on a glass substrate. Prepared PDMS was subsequently applied to cover the SU-8 line, followed by baking on a hotplate at 70 °C for 3 hours for curing (solidification, Fig. 4(b)). After solidification, the PDMS layer was peeled off and then bonded with a glass slide on a hotplate at 90 °C for 3 hours (Fig. 4(c, d)). The glass slide was pre-treated with oxygen plasma to enhance the bonding. Finally, liquid pre-hydrogel was injected into the microchannel and underwent a standard photolithography process (Fig. 4(e); UV light intensity of 25mW/cm^2 for 180s). As a result, hydrogel microposts were defined in the microchannel (Fig. 4(f)).

III. Detection Mechanism

Enzymatic cleavage sites contained in acryloyl-PEG-SNAP peptide crosslinker were introduced into the solid hydrogel matrix by a polymerization process with AAm under ultraviolet light (UV) illumination. When the crosslinker in the hydrogel matrix was cleaved by BoNT/A, the network structure of the hydrogel broke down, and the hydrogel became pasty and later totally water-soluble. The modified substrate contained residues 187 to 203 of SNAP-25 (SNKTRIDEANQRATKML) with the BoNT type A cleavage site between Q197 and R198. M202 was replaced with norleucine (Nle) to eliminate the risk of thioether oxidation, and K189 and K201 were replaced with arginines (R) for increasing peptide hydrolysis [24], [25].

Glycine spacers (GGG) were added at the termini to alleviate stress at crosslinking junctions and facilitate the recognition of BoNT/A. In this work, we added lysine (K) at the termini of the new 25-mer rather than cysteine (C) as reported in ref. 5. This modification brought about two significant advantages. First, it helped to eliminate a potential false alarm stemming from the formation of disulfide bonds in thioglycolic groups of cysteines, which can be cleaved by chemical reagents such as DTT, a necessary reducing agent required for the full activation of BoNT/A during in vitro tests. Second, the modification contained one fewer conjugating sites for APN to anchor to the modified SNAP 25-mer. Since APN was used to crosslink the modified SNAP 25-mer and AAm chains, one fewer anchor site on the modified 25-mer for APN means slightly weaker crosslinking between the crosslinker and AAm chains, which is conducive to the degradation of the hydrogel and allows it to be more easily cleaved, thus enhancing its sensitivity to BoNT/A.

IV. Results and Discussion

After the PAAm hydrogel posts were defined in the microchannel via photo-patterning, their sensing functions of BoNT/A was tested by injecting liquid samples into the microchannels.

A. Testing With Trypsin

The PAAm hydrogel containing the modified SNAP 25-mer was first tested using trypsin as a control to ensure that the 25-mer was crosslinked into the hydrogel and was accessible in the crosslinked hydrogel matrix. Because trypsin can cleave peptides on the C-terminal side of lysines or arginines, there are 5 potential cleavage sites in the new peptide substrate [26]. In Fig. 5, two hydrogel posts with 1 mm in diameter and 240 μm in thickness was treated by trypsin (500 μg/mL; reaction temperature: 37 °C). The results show that the hydrogel posts became smaller and thinner gradually and then disappeared completely in 15 minutes (Fig. 5(b–d)).

B. Testing With LC

In this work, we used the proteolytically active LC of BoNT/A to decompose the hydrogel, instead of the entire BoNT/A to avoid safety and regulatory issues. LC is the

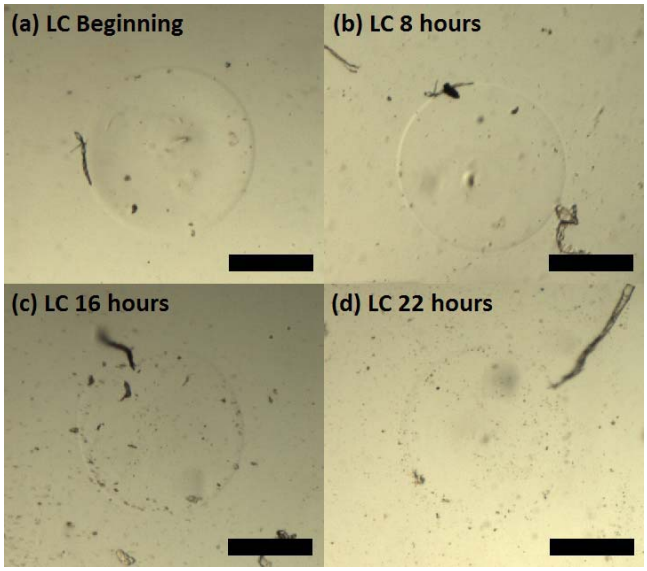

Fig. 7. Images showing the results of optimized BoNT/A-responsive hydrogel posts exposed to LC of BoNT/A (45 μg/mL). As exposure time proceeded, the hydrogel post became thinner and was nearly completely decomposed in 22 h. All scale bars represent 100 μm. Reaction time is indicted in individual panels.

catalytic part of BoNT/A and can be used to represent the toxic activity of BoNT/A. Fig. 6 shows a hydrogel post with a diameter of 5 mm and thickness of 500 μm that was treated with LC (concentration: 45 μg/mL; amount: 10 μL; refreshed every 8 hours). We observed that the hydrogel became soft, and part of the rim was cleaved by LC incubated at 37 °C in 40 h (Fig. 6(b)). An identical hydrogel post exposed to a buffer control (30 mM Hepes pH 7.4; reaction temperature: 37 °C) was used as a negative control to exclude the impact of Hepes buffer solution that was used to dilute the LC. Fig. 6(c, d) showed that there was no change in the morphology of hydrogel. The result indicated that the new modified SNAP 25-mer containing hydrogel was indeed responsive to LC of BoNT/A. We also tested the hydrogel using the DTT solution to show the addition of a reducing agent would not cause a false alarm as previously discussed. Another identical hydrogel post remained intact after being treated with DTT (13 mM in Hepes buffer solution; reaction temperature: 37 °C) for 40 h as shown in Fig. 6(e–f), confirming that the newly designed acryloyl-PEG-SNAP peptide crosslinker was stable in DTT containing buffer.

C. Reduction of Hydrogel Size to Improve the Detection Sensitivity

To enhance the sensitivity and shorten the response time of the BoNT/A-responsive hydrogel, as well as to reduce the cost associated with these hydrogels, we further improved the lithography and polymerization process to reduce the size of the photo-patterned hydrogel posts. Large-sized responsive hydrogel requires much longer reaction time to be cleaved. It also increases the cost of raw materials and requires longer UV exposure time to ensure complete crosslinking of the pre-hydrogel. It was observed that increasing the UV exposure could induce over-polymerization on the surface of hydrogels,

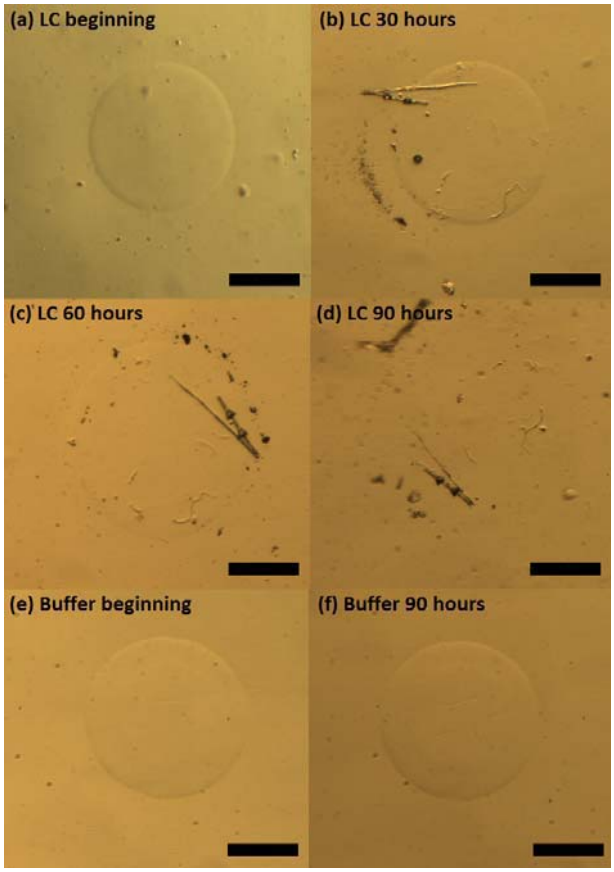

Fig. 8. Images of BoNT/A-responsive hydrogel post patterend in small size and treated with: (a-d) low concentration of LC of BoNT/A (4.5 μg/mL); (e,f) buffer solution. Reagents and reaction time are indicated in individual panels. As the reaction proceeded, the hydrogel in LC was cleaved and became thinner, and ultimately decomposed completely in 90 h. The hydrogel treated with buffer remain intact throughout the process. All scale bars are 100 μm.

which made them harder to be cleaved by LC of BoNT/A or even trypsin. In addition, the residue produced during the degradation of large gels formed a thicker blocking layer hindering further cleavage of the rest of the gel by inhibiting the diffusion of LC on the gel surface, thus lowering the sensitivity.

Fig. 7 shows the testing result of a hydrogel post with much reduced size of 200 μm in diameter and 50 μm in thickness, the smallest size achievable with our photo-patterning system. The hydrogel post was treated with the same concentration of LC of BoNT/A (concentration: 45 μg/mL; amount: 10 μL; refreshed every 8 hours) as in Fig. 6. Compared to the result in Fig. 6, the response time of this smaller sized hydrogel was dramatically shortened. Fig. 7(b–d) show that the smaller sized hydrogel post was degraded within 22 h. Identical hydrogel posts were also treated with the same Hepes buffer and DTT solutions as before as negative controls. These hydrogel posts remained stable in both Hepes buffer and DTT solutions, similar to those shown in Fig. 6(c–f).

With the reduced size of hydrogel structures, we studied the impact of LC concentration on gel cleavage. Fig. 8 shows that the rim area of the hydrogel post 200 μm in diameter and 50 μm in thickness collapsed in the presence of low concentration of LC of BoNT/A (concentration: 4.5 μg/mL; amount: 10 μL; refreshed every 8 hours) and was dissolved

by water rapidly in 60 h. The whole structure was degraded in 90 h (Fig. 8(d)). Fig. 8(e–f) indicated that the post was stable in buffer solution without shape change. As comparison, we also used this low concentration of LC (concentration: 4.5 μg/mL; amount: 10 μL; refreshed every 8 hours) to treat a slightly larger sized hydrogel post (diameter 300 μm, thickness 80 μm). The collapse of gels first took place in the thin peripheral areas within 80 h. The whole posts was degraded and dissolved into water in 160 h, 1.7 times as slow as the case in Fig. 8. This confirms our concept that smaller hydrogel structure would have shorter response time to BoNT/A.

V. Conclusion

This study developed a new microfluidics-based PAAm hydrogel sensor incorporating a modified SNAP-25 peptide for the detection of BoNT/A. The new modified SNAP 25 peptide used in the hydrogels possesses an optimized sequence and conjugation sites for the APN crosslinker to anchor. These gels could be degraded in trypsin, indicating that the modified SNAP 25 peptide was crosslinked into the hydrogel and was accessible to the enzyme. The degradation of the gels when exposed to the LC of BoNT/A depends on the concentration of LC, gel size and reaction time. The BoNT/A can be detected by direct observation of the morphology change of the hydrogel. The best response of the BoNT/A sensing hydrogel we achieved was within 22 h at 45 μg/mL of LC, and the lowest LC concentration of 4.5 μg/ in 90 h, with hydrogel post 200 μm in diameter and 50 μm in thickness. The hydrogels remained intact in both control buffer and DTT solutions. The non-response to DTT because of the removal of the disulfide bonds in the crosslinker is an improvement compared with prior art, as it eliminates potential false alarms due to cleavage of the disulfide bonds linking the peptide to the APN by DTT, a necessary reducing agent used to fully activate the BoNT/A in in vitro tests. The newly designed SNAP-25 25-mer contributed to the high sensitivity, specificity and accuracy of sensing. The result shows the promise for this hydrogel to be used to detect bio-active BoNT/A. Our future work will focus on integrating the sacrificial hydrogel-based sensor into passive sensors of BoNTs for food packages. We also plan to test our devices for long-time preservation, in different, real food matrices to study the effect of other bioactive compounds, and to validate our sensor using the holotoxin (full-length toxin rather than the LC alone).

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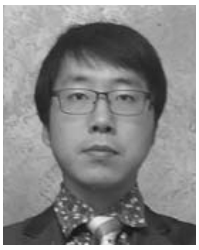

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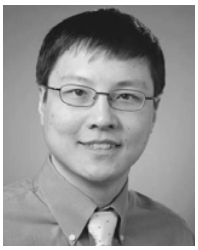

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