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Synaptotagmin II peptide-bead conjugate for botulinum toxin enrichment and detection in microchannels

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ABSTRACT

This paper reports an enrichment platform for botulinum neurotoxin type B (BoNT/B) that has been realized through the fusion of bioconjugation chemistry and microfluidics. Micrometer-sized magnetic beads were conjugated to a 22mer synthetic peptide derived from the synaptotagmin II (Syt II) neuronal protein that is specific for BoNT/B binding. Exposure to BoNT/B in buffer, whole milk and fruit juices resulted in toxin capture, which was confirmed using immunofluorescence. Peptide-modified beads were integrated into arrayed, polymeric microfluidic channels, and all assay steps, from capture to detection, were performed directly in the microchannels, thereby simplifying assay utility and increasing throughput relative to existing detection methodologies. Our sensitive microscale approach required only 7 μL of intentionally adulterated sample without any pre-processing (i.e. dilution, centrifugation, filtering), and with a "hands-on" time of only 1 h to detect 16.6 pg of BoNT/B in whole milk.

1. Introduction

Botulinum neurotoxin (BoNT), produced by the bacterium *Clostridium botulinum*, is among the most potent toxins known, and is considered to be a potential biological weapon (Arnon et al., 2001). Seven BoNT serotypes exist (A–G), each comprising a 100 kDa heavy chain and a 50 kDa light chain. The heavy chain facilitates entry into neurons via cell surface binding and receptor-mediated endocytosis, whereas the light chain is the catalytic component that, once inside the cell, cleaves neuronal proteins necessary for neurotransmitter release (Schiavo et al., 2000). BoNT potency stems from its enzymatic activity within the cell, where as little as 70 μg delivered orally would be lethal to an adult human (Schantz and Johnson, 1992).

The gold standard for BoNT detection is the mouse bioassay in which suspect samples are injected intraperitoneally into mouse subjects. Infected animals are then observed for 2–4 days for signs of botulism or death. The assay itself is very low throughput, requires further immunological testing to determine BoNT serotype, relies on highly trained personnel for conducting testing, and frequently results in animal sacrifice. Alternate methods of BoNT detection range from endopeptidase assays (Schmidt and Stafford, 2003; Rasooly and Do, 2008; Frisk et al., 2009) to various immunoassays (Gatto-Menking et al., 1995; Ferreira et al., 2003; Sharma et al., 2006; Han et al., 2007; Grate et al., 2009) to PCR (Lindstrom et al., 2001; Chao et al., 2004; Fenicia et al., 2007), which are reviewed in detail elsewhere (Scarlatos et al., 2005). Cleavage assays are advantageous for detecting the amount of catalytically active toxin present in a sample, but potentially suffer from non-specific substrate cleavage by proteases present in food or bodily samples (Rasooly et al., 2008). Variations of the traditional ELISA have emerged as faster alternatives to the mouse bioassay, with comparable detection limits (~10 pg toxin) through the use of novel fluorescent and chemiluminescent labels. However, most of these are sandwich immunoassays that require two serotype-specific antibodies – one for toxin capture, one for recognition after capture – as well as a detection antibody (e.g. enzyme- or fluorophore-conjugated), thus adding to the cost and complexity of the assay (Hoofnagle and Wener, 2009). Additionally, assays that employ immobilized antibodies risk variable efficiency and functionality and an increased propensity for cross-reactivity (Angenendt, 2005).

A significant drawback to many of these assays is the inability to detect low quantities of BoNT (<10 pg) without additional pre-processing steps that remove the toxin from complex sample matrices, such as food or stool. Integrating a robust solid phase for BoNT capture would not only pre-concentrate the toxin for downstream detection, but also allow for washing away interfering agents, such as nonspecific proteases and auto-fluorescent proteins and colloids abundant in food and clinical samples. Antibody-laden

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beads have been used to capture and enrich attomolar concentrations of BoNT type A before incubating with a FRET peptide substrate for fluorescent readout of enzyme activity (Bagramyan et al., 2008). Similarly, antibody beads have been employed to capture BoNT types A, B, E and F from serum and stool (Kalb et al., 2006); in this case, captured toxin was subsequently exposed to peptide substrates and cleaved products were detected using mass spectrometry (MS), leading to sensitivity in the femtomolar range. In addition to FRET and MS detection, immunomagnetic separation has been demonstrated with many visual outputs and variable sensitivity (Weimer et al., 2001; Kwon et al., 2008). Novel solid phases, such as ganglioside-functionalized liposomes and beads, have been explored for enriching toxin samples prior to detection with either PCR (Desai et al., 2008) or a flow-strip immunoassay (Ahn-Yoon et al., 2004), respectively. A cyclic peptide-polymer conjugate has also been developed for BoNT/A capture and detection of 1 pg/mL BoNT/A using a chemiluminescent substrate (Ma et al., 2006).

Although the abovementioned methods (MS, PCR, ELISA, etc.) can be highly sensitive, they are often expensive, require experienced end-users for their operation, and frequently cannot handle large batches of sample (i.e. low throughput). There is a high demand for sensors that capture botulinum toxin in contaminated samples in a rapid, specific and facile manner, with potential for on-site usage. Here, we describe both the creation of an antibody-free solid phase to recognize and bind BoNT type B (BoNT/B) from various matrices, and the testing of this enrichment method in liquid food samples. A synthetic 22 amino acid peptide (P22) was derived from residues 40–60 of the transmembrane neuronal protein, synaptotagmin II (Syt II), which is the known *in vivo* binding site for the BoNT/B heavy chain (Dong et al., 2007) (Fig. 1a). Syt II P22 was immobilized on 5 μm magnetic beads (Fig. 1b) for capturing BoNT/B in liquid foods (milk and juices) that are considered to be likely targets of intentional toxin contamination (Wein and Liu, 2005). BoNT/B capture using the Syt II P22 peptide-bead conjugate will be described, as well as confirmation of bound toxin using various fluorescence detection methods, which altogether demonstrate the practicality of this enrichment platform for upstream use in conjunction with endopeptidase- and immuno-based detection methods. Toward high-throughput, parallel sensing, our BoNT/B capture assay is demonstrated using arrayed microchannels, with all steps – from sample introduction to signal detection – performed directly within a single channel.

2. Materials and methods

2.1.1. Syt II peptide immobilization on magnetic beads

A 22mer Syt II peptide (P22) was designed and synthesized to represent residues 40–60 of the BoNT/B heavy chain binding site on the Syt II neuronal protein (Dong et al., 2003) (Fig. 1a), with a cysteine residue added to the C-terminus for thiol-specific conjugation to beads. Syt II P22 was synthesized by Peptide 2.0 (Chantilly, VA) at 90% purity with an acetylated N-terminus (Ac) and the resulting sequence: Ac-GESQEDMFAKLKEKFFNEINKC. Free primary amines of diaminodipropylamine (DADPA)-functionalized 5 μm magnetic beads (BcMag®, Bioclone Inc., San Diego, CA) were converted to maleimide head groups by exposure to a solution of sulfo-SMCC (succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate) (Pierce) in 50 mM triethanolamine (TEOA) buffer, pH 8.0, for 35 min at room temperature (RT) with intermittent vortexing. Beads were then incubated overnight at 4 °C with Syt II P22 dissolved in 1× PBS, pH 7.4, with 3.0% (v/v) 1 M NaOH added to dissolve the peptide. This resulted in the Syt II P22 peptide-bead conjugate with a ~30 Å spacer (Fig. 1b). Syt II P22 beads were washed with 1× PBS containing 0.1% Tween-20, resuspended at 16 mg/mL in 1× PBS, pH 7.4, and stored at 4 °C until use.

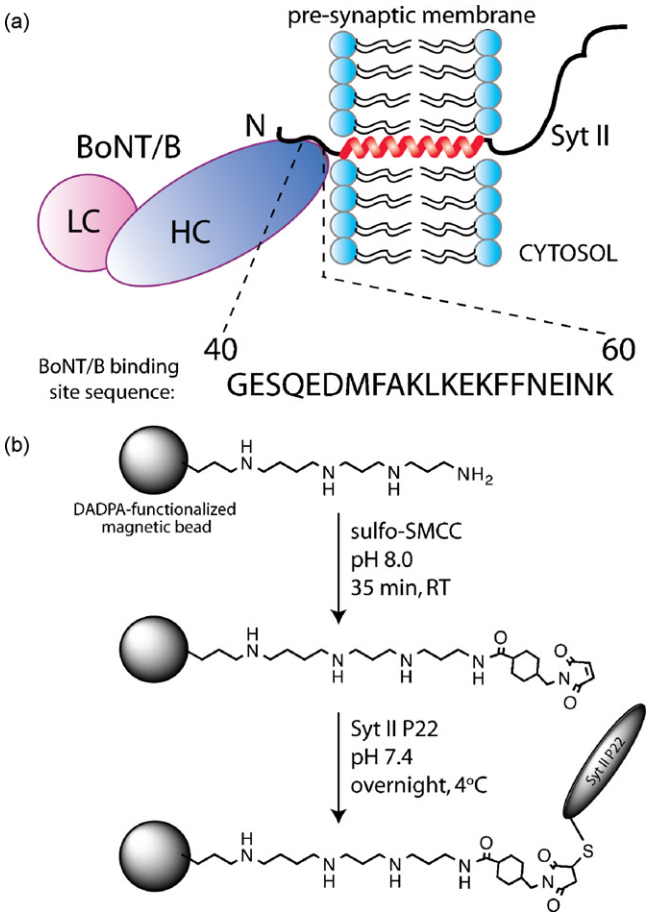

Fig. 1. (a) Botulinum neurotoxin type B (BoNT/B) recognizes and binds extracellular residues 40–60 of the synaptotagmin II (Syt II) transmembrane neuronal protein for translocation into the cell *in vivo*. (b) The Syt II 22mer peptide (P22) was synthesized to contain Syt II residues 40–60, in addition to a C-terminal cysteine for thiol-specific conjugation to magnetic beads via the heterobifunctional crosslinker sulfo-SMCC.

2.1.2. Confirmation of BoNT/B binding using western blot and ELISA

Western blot (WB) analysis was performed to first confirm the ability of the Syt II P22 beads to bind BoNT/B in food and buffer matrices. Five microlitres of bead suspension was mixed with 0.5 μg BoNT/B in 50 μL of either 1× PBS (pH 7.6), whole milk or orange juice in microcentrifuge tubes for 1 h. Beads were washed with three 200 μL aliquots of TBST (Tris-buffered saline containing 0.1% Tween-20), pH 7.6, to minimize nonspecific binding. The beads were then boiled for 5 min in SDS loading buffer with 0.1 M DTT to reduce the bound, whole toxin to its heavy and light chain components, effectively releasing the toxin from the beads. Beads were centrifuged, and the supernatant analyzed by WB. The samples were separated in NuPage 4–12% Bis–Tris SDS-PAGE gel (Invitrogen) and then transferred onto a PVDF membrane. WB analysis was performed according to Invitrogen kit manual. Polyclonal rabbit anti-BoNT/B IgG (raised in the Johnson Laboratory at the University of Wisconsin-Madison) was used as the primary detection antibody at a working titer of 1:400. An alkaline phosphatase (AP)-conjugated anti-rabbit secondary IgG (Santa Cruz Biotechnology) was used at a working titer of 1:2000.

ELISA was used to assess the extent of BoNT/B capture by Syt II P22 peptide-bead conjugate. After toxin capture, beads were exposed to an AP-conjugated antibody to BoNT/B, followed by incubation with the colorimetric reagent *p*NPP (*p*-nitrophenyl