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 \(\mu\)L 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.

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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 pg 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; Crate 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...
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-GESQEDMFAKLKEFFNEINKC. Free primary amines of diaminodipropylamine (DADPA)-functionalized 5 μm magnetic beads (BcMag®, Bioclon 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.](image-url)
2.1.3. Microfluidic channel fabrication

Straight or “wedge” microchannels were fabricated using standard soft lithography and PDMS [poly(dimethylsiloxane), Sylgard 184, Dow Corning]. Molds (also known as masters) were created by coating SU-8 100 photoresist (Microchem) onto a silicon wafer. The first layer (250 μm) defined the channel and the second layer (200 μm) defined the ports. The master-making process is described in detail elsewhere (Jo et al., 2000). PDMS was poured over the resulting master, covered with transparency film, and weighted with ~20 lb to ensure complete port formation without thin films covering them. This stack was baked on a hot plate at 80 °C for 3 h, after which the PDMS was peeled off the master and placed in a plastic petri dish for later use.

2.1.4. Toxin capture

Aqueous buffer (30 mM HEPES with 0.1%, w/v BSA, pH 7.3) and liquid food (whole milk, orange and peach juices) samples were spiked with concentrations of BoNT/B ranging from 100 pg/mL to 10 ng/mL. Orange juice, whole milk and peach juice (Naked Juice Co.) were used as received. Peptide-bead suspension was added to each sample at a 5% (v/v) ratio and incubated for up to 90 min at 37 °C with intermittent mixing. Beads were removed from the sample and washed 3 × with 1 × PBS, pH 7.4, containing 0.1% (v/v) Tween-20.

2.1.5. Detecting bound toxin

Bound BoNT/B was confirmed using immunofluorescence. Washed beads were first blocked with a 1% (w/v) BSA solution in 1 × PBS for 20 min at RT. Beads were then incubated with the polyclonal rabbit anti-BoNT/B IgG diluted 1:400 in 1 × PBS for 1 h at RT. Lastly, beads were washed 3 × with a 1% (w/v) BSA solution and incubated with a donkey secondary antibody to rabbit IgG, conjugated to either Alexa488 dye (Invitrogen) or a near-infrared 800CW fluorophore (Rockland), diluted 1:250 in 1 × PBS, for 1 h at RT. If the capture portion of the assay was performed “off-chip” (i.e. in microcentrifuge tubes), 2 μL of washed bead suspension was introduced into arrayed PDMS microchannels for detection. If the capture assay was performed within the microchannels, the beads were washed and the resulting fluorescent signal was quantified in situ. Fluorescent beads were detected using either fluorescence microscopy at 30 × or a near-IR scanner at 21- or 42-μm resolution. To detect the amount of catalytically active toxin, beads were incubated with 7.5 μM VAMPtide8 (List Biological Laboratories) – a FRET peptide derived from the toxin’s in vivo intracellular protein substrate, VAMP-2. A DMSO-based stock solution of VAMPtide8 was reconstituted in 50 mM HEPES containing 0.05% (v/v) Tween-20, pH 6.3. After incubation at 37 °C for 2–24 h, fluorescence output was collected using a Biotek plate reader at 321/418 (ex/em).

2.1.6. Data processing

Fluorescence output was quantified using ImageJ (NIH), and sample averages were calculated and normalized to a Syt II P22 peptide-bead conjugate control sample that was not exposed to toxin, but had been incubated with both of the primary and secondary antibodies. For all graphs, individual data points are averages of three or more independent trials (n ≥ 3), and y-axis error bars represent ±1SD. Limits of detection (LODs) were determined using the normalized control + 3SD and best-fit trendlines to the sample data.

3. Results and discussion

3.1. Confirmation of BoNT/B binding to Syt II P22 beads

BoNT/B recognition of and binding to the Syt II P22-modified beads was first confirmed with both ELISA and WB. ELISA was used to roughly determine the extent of BoNT/B capture by the peptide-bead conjugate in buffered solution. Absorbance outputs were compared between free and bound toxin samples at 10- and 100-ng quantities. By comparing the bound toxin signal to a separate, free BoNT/B signal (BoNTbeads/BoNTfree), a maximum capture of 88 ng BoNT/B was determined for the Syt II P22 beads (data not shown), which is three orders of magnitude lower than the oral lethal dose for an adult human.

The ability of the peptide-bead conjugate to capture BoNT/B in various liquid foods and buffer was assessed using WB (Fig. 2). Beads were exposed to excess toxin (0.5 μg) in 1 × PBS (P), whole milk (M) or orange juice (J). Control beads were DADPA (amine)-functionalized magnetic beads with or without Syt II P22 conjugation. WB analysis indicated that the peptide-bead conjugate readily captured BoNT/B in all sample matrices. There was a weaker signal resulting from BoNT/B capture from orange juice. The acidic pH of the juice (~4.6) may have lessened toxin recognition and binding of the Syt II P22, which normally occurs at physiological pH; or, removal from beads under reducing conditions may have been hampered at an acidic pH. Improved BoNT/B detection in acidic liquid foods could be achieved by diluting into a more basic buffer prior to capture. The control beads without Syt II P22 showed a very weak signal for the BoNT/B heavy chain, but this could be attributed to sample remaining in the microcentrifuge tube from incomplete washing, as other WB results did not show nonspecific BoNT/B binding to the amine beads (Figure S1). The other control sample (amine-functionalized beads with toxin addition) did not show errant capture or nonspecific adsorption.

3.1.2. Quantifying BoNT/B capture

Syt II P22 beads were incubated with BoNT/B spiked into both 30 mM HEPES buffer, pH 7.3, and liquid foods (milk, orange juice and peach juice) at concentrations ranging from 100 pg/mL to 10 μg/mL (Fig. 3). Captured BoNT/B was quantified using immunofluorescence microscopy, and all points greater than the normalized average ±2SD were considered detectable. Linear or logarithmic trendlines fit to the data along with the respective control values and standard deviations were used to determine the relative LOD for each of the four matrices. Normal–normal or
normal-log linear ranges were considered best-fit if $R^2$ exceeded 0.990, and therefore representative of a quantifiable region for BoNT/B in a given sample matrix (Table 1).

Magnetic bead enrichment using the Syt II P22 substrate allowed for capture and detection of 16.6 pg BoNT/B in 40 μl whole milk, which corresponded to 111 amol toxin in an adulterated sample. LODs for other matrices were slightly higher (Table 1), with the thickest matrix, peach juice, producing an LOD of 312 ng (83.3 pmol) BoNT/B; this was most likely due to excessive pulp that aggregated the beads, which often complicated bead removal from solution with an external magnet. Our magnetic bead capture and detection assay demonstrated an LOD of 42.1 ng/mL BoNT/B in a HEPES buffer sample – three orders of magnitude lower than the 75 μg/mL detection limit in buffer previously reported using the Syt II peptide (Dong et al., 2007). LOD disparity between different samples suggests variable levels of recognition between the Syt II peptide and the toxin in certain matrices. BoNT/B in milk, for example, had the lowest LOD, possibly indicating higher stability of the toxin (i.e. closer to native conformation), and therefore greater recognition and binding to Syt II P22. It has been noted that BoNT is fairly unstable in aqueous solutions, especially pure water, which could explain a higher LOD in HEPES buffer versus milk. Milk could also act as a natural blocking buffer, thereby lowering the background signal.

### 3.1.2.1. Evaluating post-capture detection method

Imaging fluorescent beads with microscopy can be tedious and time-consuming. At 30×, the field-of-view was 0.09 mm² and contained 1100 ± 80 magnetic beads. With a channel area of 2.65 mm², one image displayed <4% of the beads present in the microchannel, assuming a homogeneous distribution. Quantifying individual beads using fluorescence microscopy can lead to variability among data sets based on bead-size heterogeneity or even user bias. In order to acquire a representative fluorescence measurement for a sample, 4–5 images were averaged. This could be especially problematic for sensing lower concentrations of BoNT/B where only a few beads might encounter and capture the toxin; even if those beads fluoresce, they could be either lost outside the field-of-view or appear to be “noise” among toxin-free beads. To determine whether or not this is a valid drawback to our current means of quantifying fluorescence, we examined the differences in fluorescence output and quantification if all magnetic beads present in a microchannel were: (i) concentrated in the large outlet using an external rare earth magnet and imaged via fluorescence microscopy, or (ii) imaged in their entirety using a fluorescence scanner. Antibody-free detection methods were also explored and are discussed herein.

Concentrating all magnetic beads resulted in a 3–4-fold increase in fluorescence intensity for BoNT/B concentrations above 30 ng/mL, suggesting greater assay sensitivity upon bead concentration (data not shown). However, we observed a large amount of inter-assay error amongst data sets, likely owing to differences in the quantity of beads added to a microchannel, which makes this an unreliable method of detection and quantification. Bead-related variability has been shown to be a factor in both fluorescent (Bagramyan et al., 2008; Frisk et al., 2008) and colorimetric (Moorthy et al., 2004) outputs for BoNT detection assays.

As mentioned, imaging several areas within a channel to achieve a representative average of fluorescence intensity can lead to a lengthy and variable data acquisition process. We therefore explored the use of a scanner for collecting bead fluorescence. The scanner produced a single image of the entire microchannel array and allowed for quantification of the whole channel-of-interest, significantly reducing assay and data analysis time. Syt II P22 beads were incubated with BoNT/B in HEPES buffer as before. Fluorescence images of the microchannels and corresponding data normalized to toxin-free controls are shown in Fig. 4. Fluorescent signals obtained from the scanner correlated well with the microscopy data (Fig. 4), even providing slightly higher outputs at all BoNT/B concentrations. When fluorescence was collected and quantified with the scanner, the assay LOD in HEPES buffer was 1.60 ng/mL (64 pg) BoNT/B – an order of magnitude lower than that achieved with microscopy – potentially owing to minimized measurement error (independent of user). The ability to scan entire microchannel arrays at 21-μm resolution in less than 10 min, and subsequently quantify without user bias, demonstrates the ease with which this bead-based concentration method can be used in high-throughput toxin sensing.

Both the visible fluorescence microscopy and near-IR scanning detection methods utilized the short Syt II peptide to capture the neurotoxin, with a serotype-specific antibody for labeling toxin after capture and a species-specific secondary antibody for fluorescence detection. The use of a 22mer peptide for capture and enrichment versus a BoNT-specific antibody avoids the complexity and potential cross-reactivity that is typical of sandwich immunoassays, including ELISAs. The next step in optimizing the microfluidic bead-based BoNT/B enrichment platform will be the use of antibody-free, downstream detection methods, such as FRET, to measure bound toxin enzyme activity. Because the use of two antibodies for detection purposes is not ideal, we preliminarily investigated the use of a FRET peptide to detect bound BoNT/B catalytic activity. Using the commercially available VAMPTide® FRET substrate, we were able to detect 10 ng/mL captured BoNT/B (Figure S2), thus demonstrating the feasibility of detecting live toxin following a magnetic bead enrichment step. However, the time required to achieve a signal at that concentration was 22 h, which is not practical for rapid on-site detection, and the linear range spanned only two orders of magnitude. Furthermore, VAMPTide® is an expensive reagent with limited published kinetic data. Efforts have been underway (Boldt et al., 2006; Purcell and Hoard-Fruchey, 2007) to develop robust, peptidic substrates for

### Table 1

<table>
<thead>
<tr>
<th>Samples matrix</th>
<th>LOD (ng/mL)</th>
<th>LOD (ng)</th>
<th>Linear range (μg/mL)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>42.1</td>
<td>1.69</td>
<td>0.10–10</td>
<td>0.993</td>
</tr>
<tr>
<td>Whole milk</td>
<td>0.414</td>
<td>0.0166</td>
<td>0.01–1</td>
<td>0.999</td>
</tr>
<tr>
<td>Orange juice</td>
<td>88.9</td>
<td>3.56</td>
<td>0.001–5</td>
<td>0.997</td>
</tr>
<tr>
<td>Peach juice</td>
<td>312</td>
<td>12.5</td>
<td>0.001–5</td>
<td>0.990</td>
</tr>
</tbody>
</table>

Fig. 3. Syt II P22 magnetic beads capture and concentrate BoNT/B holotoxin (1 ng/mL to 10 μg/mL) in intentionally contaminated HEPES buffer, whole milk, orange juice and peach juice. (Note: some error bars are within the data point itself.)
BoNT endopeptidase assays, the resulting products of which could be useful antibody-free methods of detection to couple with our bead-based microfluidic capture platform.

3.1.2.2. Reducing assay time

Suspect food samples require rapid screening in order to effectively neutralize a biological threat. It is therefore necessary to decrease the time for individual assay steps without compromising assay integrity and sensitivity. In previous experiments, Syt II P22 magnetic beads were incubated with BoNT/B-contaminated samples for 90 min to ensure complete binding. Toward rapid toxin pre-concentration for downstream detection, a shortened time course was considered for BoNT/B capture. Syt II P22 beads were exposed to a 5 μg/ml toxin solution in 30 mM HEPES buffer, pH 7.4; aliquots were subsequently removed from the solution every 15 min for 90 min total, followed by the standard anti-BoNT/B primary and a fluorophore-labeled secondary antibody incubation for 45 min each. We found that as little as a 15 min incubation period is sufficient to achieve a 30-fold higher signal than control (Figure S3). Our rapid capture platform integrated into a BoNT sensor could reduce total sensing time to ~2 h, with significantly less hands-on time, thus greatly improving upon the time constraints of the mouse bioassay and the standard ELISA, among others.

3.1.3. Wholly integrated microfluidic BoNT enrichment and sensing

The ultimate goal is to have an increased-throughput assay for rapidly determining the presence of botulinum toxin in suspect liquid food and clinical samples. A microfluidic channel (1–2 μL volume), rather than 96-well plates (100–250 μL per well) or microcentrifuge tubes (0.1–1.0 mL), is a more efficient technology for bioanalytics because one can seamlessly incorporate multiple assay components to create a “lab-on-a-chip”. Total toxin sensor integration has been achieved by interfacing waveguides directly on-chip (Rowe-Taitt et al., 2000), as well as by incorporating flow strips into microfluidic cells for a chromatographic readout (Han et al., 2007). Sophisticated imaging equipment has also been coupled to an electrochemiluminescence biosensor (Sapsford et al., 2008).

To avoid complicated device fabrication steps and mixed media components, we have formatted the magnetic bead-based BoNT capture assay for the microscale by using simple, straight microchannels and external magnets placed below to immobilize or mix beads as needed. Microchannels can be incorporated into any fluorescence or absorbance detection scheme, as demonstrated previously with a standard plate reader for counting cells in arrayed channels (Yu et al., 2007). With even a simple channel, fluid can be manipulated easily using passive pumping, which relies on differences in surface tension between input and output port droplets (Walker and Beebe, 2002). The use of passive pumping avoids complicated external tubing and valving networks typical of previously developed microfluidic BoNT sensors (Sun et al., 2009; Mangru et al., 2005; grate et al., 2009). When incorporating several fluid control components during chip design, the device becomes increasingly less accessible for a variety of end-users, and could require extra training for operation — both of which should be avoided when developing on-site toxin sensors. Importantly, bead washing and “treatments” (e.g. antibody solution and blocking buffer) in microchannels using passive pumping involves fluid replacement via laminar flow rather than total fluid aspiration characteristic of assays in microtiter wells and microcentrifuge tubes. Laminar flow ensures nearly 100% solution replacement after washing with only two “channel volumes” (Warrick et al., 2007).

For on-site toxin sensing, instrumentation should be ubiquitous and inexpensive. A standard plate reader coupled with absorbance or fluorescence output is therefore an obvious candidate, and has been used previously with microfluidic channels (Yu et al., 2007). To test the utility of a plate reader with absorbance readout for BoNT/B detection, microchannels were spaced identically to a 96-well format and placed within polystyrene Omnitrays (Nunc). Syt II P22 beads and BoNT/B were mixed together in individual channels of the array, with all assay steps performed directly in-channel. An AP-conjugated secondary antibody and the pNPP reagent were chosen for a colorimetric readout. Although able to detect down to 1 pg/ml BoNT/B in microchannels using this method, there was significant background signal, presumably due to the presence of the magnetic beads (Figure S4). As a result, the absorbance detection method required bead removal, which was a tedious task for multi-channel arrays. Additionally, aligning the channels to mimic the placement of wells can lead to inter-channel error for larger arrays, where slight misplacement of one row or column can adversely affect the entire readout.

A more consistent method for detecting fluorescence in a microchannel array was the near-IR because it detects fluorescent signals within an entire area, rather than discrete, predefined 96- or 384-well locations, and produces a single image for easy channel-to-channel comparison and quantification. The entire assay, from BoNT/B capture to fluorescence detection, was performed in-channel. "Wedge" channels (Fig. 5) were chosen because of their increased surface area, which allowed easy visualization of beads. Each channel received 7 μL of an intentionally contaminated HEPES buffer and 0.75 μL bead suspension in 1 × PBS, pH 7.4. Following capture, Syt II P22 beads were incubated sequentially with an anti-BoNT/B primary IgG and a species-specific secondary IgG conjugated to an IR dye. BoNT/B capture and detection in a microchannel array showed a log-linear range that spanned 100 ng/mL to 10 μg/mL (R² = 0.990), and demonstrated an LOD of 23.6 ng/mL (165 pg) in HEPES buffer, which is more sensitive than

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**Fig. 4.** (a) Fluorescent scanning (shown for the near-IR) is less subjective than fluorescence microscopy, and offers an image of the entire microchannel array in <10 min. Furthermore, microscopy requires manual counting of individual bead frames and image-stitching to achieve an entire channel view. Shown here is a representative image of BoNT/B (10 ng/mL to 10 μg/mL) captured on Syt II P22 beads within straight microchannels; control channel is outlined for ease of visualization. (b) The use of a fluorescent scanner appeared to be a more sensitive method for detecting and quantifying fluorescent beads within whole microchannels, providing a detection limit of 64 pg BoNT/B in HEPES buffer. Control signal (solid line) + 1 SD (dotted line) are plotted.
our “macroscale” version of the assay in microcentrifuge tubes (Fig. 5).

The only drawbacks noted for performing the entire BoNT/B capture assay in microfluidic channels was the presence of larger background signals and increased channel-to-channel variability (i.e. greater standard deviation). This was most likely the result of ineffective washes during the antibody treatments, which could be eradicated through a more efficient microchannel washing protocol. We found that the constant pumping of one aliquot of 30 μL wash buffer through each channel, rather than the consecutive application of three channel volumes (5–10 μL) removed nearly 100% of the background fluorescence (data not shown). The only disadvantage of this procedure is that it requires constant removal of the output droplet, usually achieved via vacuum suction, thus making the amended wash protocol potentially difficult to perform in the field.

Significant advantages of the microfluidic capture method are minimal sample and reagent consumption (5–10 μL per channel) and ease of magnetic bead washing and treatments via passive pumping. Automatic pipettors for channel washes and treatments decreased hands-on time to <1 h, thus limiting user contact with toxin-contaminated samples and reducing total assay time to 3 h from the point of sample addition to the final readout. In the case of BoNT/B, this microfluidic magnetic bead capture method provided a positive readout for quantities more than five orders of magnitude below the oral lethal dose of 70 ng for an adult human. Importantly, we demonstrated that this BoNT enrichment platform could be interfaced with a variety of absorbance, fluorescence (e.g. FRET) and immunofluorescence detection methods, depending on the instrumentation and reagents available, thus making it a versatile component of any BoNT sensor.

4. Conclusion

A magnetic bead-based capture platform has been presented for botulinum toxin type B (BoNT/B) that uses a synthetic 22mer binding peptide, Syt II P22, derived from the synaptotagmin II neuronal protein, which is specific for the BoNT/B heavy chain. The assay was first evaluated on the “macroscale” using intentionally contaminated buffer, whole milk, orange juice and peach juice, with immunofluorescent readouts quantified within straight microchannels using microscopy. Toward an on-site toxin sensor, the entire BoNT/B capture assay – from sample addition to fluorescent readout – was then performed directly within arrayed microfluidic channels, requiring only 7 μL of sample. The assay produced LODs for BoNT/B in buffer of 42.1 ng/mL, when using fluorescence microscopy and 1.60 ng/mL with a whole-channel fluorescent scanner. The LOD in whole milk was 0.4 ng/mL, possibly reflecting greater BoNT/B stability and, in turn, recognition of the Syt II P22.

The Syt II P22 beads have proven capable of capturing trace amounts of BoNT/B directly from intentionally contaminated buffer and liquid food, thereby serving as a quick, solid phase enrichment step both for increased downstream detection sensitivity and for removal of extraneous, interfering food components. Furthermore, our capture method employs a short peptide substrate, offering the advantages of low cost and high specificity, which are in contrast to the broad, capture properties of gangliosides and the potential cross-reactivity and expense associated with antibodies. The versatile, inexpensive bioconjugation chemistry coupled with a microchannel array comprises a platform that could be used widely in the field of select agent detection and quantification, especially at the point of contamination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.06.035.

References