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RNA aptasensor for rapid detection of natively folded type A botulinum neurotoxin

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Abstract

A surface plasmon resonance based RNA aptasensor for rapid detection of natively folded type A botulinum neurotoxin is reported. Using detoxified recombinant type A botulinum neurotoxin as the surrogate, the aptasensor detects active toxin within 90 minutes. The detection limit of the aptasensor in phosphate buffered saline, carrot juice, and fat free milk is 5.8 ng/ml, 20.3 ng/ml and 23.4 ng/ml, respectively, while that in 5-fold diluted human serum is 22.5 ng/ml. Recovery of toxin from disparate sample matrices are within 91% to 116%. Most significant is the ability of this aptasensor to effectively differentiate the natively folded toxin from denatured, inactive toxin, which is important for homeland security surveillance and threat assessment. The aptasensor is stable for more than 30 days and over 400 injections/regeneration cycles. Such an aptasensor holds great promise for rapid detection of active botulinum neurotoxin for field surveillance due to its robustness, stability and reusability.

Keywords

RNA aptamer; botulinum neurotoxin; Surface Plasmon Resonance; aptasensor

Introduction

Botulinum neurotoxins (BoNT) are the most poisonous substances known [1]. BoNTs produced by anaerobic *Clostridium botulinum* are the cause of botulism, a life-threatening neuroparalytic disease. There are currently seven known serotypes of BoNTs, designated as types A to G. Serotypes A, B, E, and occasionally F have been shown to cause human botulism, and among them, type A is the most potent and has the longest lasting effect *in*

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vivo (up to 6 months). An extrapolation from primate studies estimated the median lethal dose (LD_{50}) of type A botulinum neurotoxin (BoNT/A) for an average human weighing 70 kg through intravenous, inhalation and oral routes to be 0.7–0.9 μg, 0.09–0.15 μg and 70 μg respectively [2].

Despite the average annual botulism cases reported between 2001 and 2011 only being about 140 [3], the high toxicity and relative ease in production of BoNTs create utmost fear among the population concerned with bio-terror agents [4]. Contamination of restaurants, catered or commercial foodstuffs or beverages could cause illness in large number of consumers [5]. Aerosol exposure of BoNTs does not occur naturally, but could be attempted by bioterrorists to achieve a widespread effect. A single gram of crystalline toxin, when evenly dispersed and inhaled, would kill more than one million people, although technical factors would make such dissemination difficult [2]. A more realistic scenario suggests less than one gram of BoNT, if distributed into food supply such as milk, could cause more than 100,000 casualties [4]. Currently, there is neither an effective antidote (except the equine antitoxin sera), nor a safe prophylaxis available for botulism. However, the antitoxin sera have a very narrow utility window, upon the onset of symptoms, they are unable to reverse symptoms. Therefore, rapid detection of botulinum neurotoxin is crucial for both surveillance of public health and the treatment of botulism patients. Unfortunately, the current gold standard for detection of BoNTs relies on mouse bioassay which takes up to 96 hours, involves usage of animal and requires highly trained personnel with special facilities. Despite being a highly sensitive method, the long testing time and complexity of mouse bioassays cannot meet the challenges of rapid detection and routine surveillance of BoNT. Phenomenal neurotoxicity coupled with ease in production, lack of countermeasures, no rapid detection and diagnosis methods have placed BoNTs as the only protein toxin on the tire one (the highest level) select agent list [2, 6]. To safeguard public health and homeland security, there is an imperative necessity to develop rapid detection assays for BoNT in a variety of sample conditions.

BoNTs are large proteins with molecular weight of 150 kDa. These bacterial proteins consist of a 50 kDa light chain linked to a 100 kDa heavy chain through a disulfide bond. While the heavy chain is required for binding and entry into the neuronal cells, the light chain is responsible for the observed toxicity through its endopeptidase activity on the specific SNARE proteins inside neuronal cells, eventually leading to the inhibition of neurotransmitter release and flaccid paralysis [1, 7]. The virulence factor for botulism is a protein and not the organism, thus precluding other sensitive molecular and microbiological tests, such as polymerase chain reaction (PCR) amplification or culturing based detection and identification, usually employed for bacterial and viral pathogens. Alternatively, *in vitro* detection of BoNTs has focused on immunological [8–11] and functional assays based on its enzymatic activity [12, 13]. Although the sensitivity of some immuno-assays can reach sub ng/ml level, they usually involve labor intensive / time consuming procedures and may not differentiate the functionally active toxin from an inactive (denatured) form. Furthermore, interferences from sample matrices pose significant challenges for enzymatic based functional assay, which often require time consuming and labor intensive immuno-chemical procedures to eliminate these interferences [14].

Rapid detection of BoNT/A with the innate folding and functional activity can be facilitated by employing specific RNA aptamers for the light chain of BoNT/A. Aptamers are a class of small, synthetic, multifaceted bio-molecules (DNA/ RNA/ peptides), screened by an *in vitro* iterative process called Systematic Evolution of Ligand by EXponential enrichment (SELEX), for their unique binding and high affinity to any target of interest (proteins/small molecules/cells) [15, 16]. Numerous applications of aptamers, especially RNA with complex folding (G-Quadruplex) have been established in detection [17], bio-marker identification

[18], whole cell isolation [19] etc. The ability of aptamers to form highly specific interactions offer a unique ability to discriminate targets with subtle structural differences [20, 21], thus providing the basis of selectively detecting natively folded protein with intact biological function. We have identified several RNA aptamers that not only specifically bind to the catalytic domain (light chain) of BoNT/A, but also strongly inhibit its endopeptidase activity [22]. In this study, the aptamer with the highest binding affinity (S132B-C22) was employed for the aptasensor development.

The aptasensor developed here is based on Surface Plasmon Resonance (SPR) technology. SPR is a very sensitive, affinity based technology, widely used for real-time sensing of bio-molecular binding events. The phenomenon of SPR occurs at thin metal film interfaces under conditions of total internal reflection. With a sensitivity of 1pg/mm^2, several studies have shown that SPR can be used not only as a research tool to determine binding constants and kinetics of interaction between molecules, but also as sensors by exploiting binding between molecules with strong affinity [23,24]. SPR provides a good platform for biosensors, especially for real-time, label-free, robust, sensitive and rapid toxin detection. Low sample consumption, speed of detection and the ability to detect in complex liquid samples are among the advantages of this technology.

Aptasensor performance was examined using the detoxified recombinant BoNT/A (DR BoNT/A) as the toxin surrogate. DR BoNT/A is the recombinant BoNT/A with two mutations on the enzymatic site of light chain (E224A/E262A). This detoxified protein has been confirmed to have identical structure and biological functions (binding, internalization and translocation on neuronal cells) to its wild type toxin, with much reduced endopeptidase activity [25]. Due to the absence of endopeptidase activity, the toxicity of DR BONT/A is approximately 100,000 fold less than that of native toxin, therefore providing an excellent surrogate to study BoNT without sophisticated laboratory requirements for tier one select agents. The newly developed aptasensor was used for detection of DR BoNT/A in several sample matrices, demonstrating the ability to rapidly detect structurally intact toxin within 90 minutes.

Materials and methods

Botulinum toxins, toxin domains, other proteins and chemicals

Recombinant BoNT/A LC and DR BoNT/A were isolated and purified from *Escherichia coli* transformed with pBN3 vectors harboring BoNT/A LC and the double mutant DR BoNT/A genes respectively, as described previously [25,26]. BoNT/A, recombinant BoNT/B & E LC were provided by BB Tech (North Dartmouth, MA). Streptavidin, bovine serum albumin (BSA), casein, mouse immunoglobulin G (IgG), lysozyme, tween-20, and NaOH were purchased from Sigma-Aldrich (St. Louis, MO). 10X HBS-P (containing 0.1 M HEPES, 1.5 M NaCl and 0.5% v/v surfactant P20) and surfactant P-20 (10% aqueous solution of the non-ionic surfactant Polysorbate 20) were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Phosphate buffered saline (PBS) was purchased from Life Technologies (Carlsbad, CA). Molecular biology grade dithiothreitol (DTT) was obtained from Promega (Madison, WI). Pooled normal human serum was purchased from Innovative Research (Novi, MI).

Biotinylated RNA aptamer synthesis

Previously identified BoNT/A LC-specific RNA aptamer S132B-C22, containing 2´-fluorine-pyrimidines were transcribed from ssDNA templates using DuraScribe® T7 Transcription Kit from Epicenter (Madison, WI). The sequence of S132B-C22 is GGGAGGAGGAGAGAUGUGAACUUGACAGCGUGCCUAGAAGUCCAAGCUUAAA

U AACCACGCUCGACAAGCAGAAACUCUACACUGGACUGGCG [22]. 3′-End of the purified S132B-C22 RNA was then specifically labeled with biotin16-ddUTP using a template independent recombinant terminal transferase enzyme from Roche Applied Sciences (Indianapolis, IN).

Preparation of aptasensor chip

Biacore T100 was used for detection studies with the commercially available CM5 chips (GE Healthcare Life Sciences, Piscataway, NJ). Streptavidin was first covalently immobilized to the chip surface *via* a standard amine coupling procedure [27]. Any unbound streptavidin was then removed from the surface by washing with 50 mM NaOH, containing 1 M NaCl solution. Biotin binding sites on the immobilized streptavidin were then saturated with 3′-biotinylated S132B-C22 aptamer. Flow channel containing streptavidin alone was used as control to correct for any refractive index changes and systematic noise.

Binding analysis on aptasensor surface

1X HBS-P buffer, with 0.2 % (v/v) P-20, and 10 mM DTT was used as running buffer. Analytes were injected at 30 μl/min for 2 mins followed by a 60 sec dissociation phase. After every analyte injection, the aptasensor surface was regenerated with 12.5 mM NaOH and 2% ethanol. The temperature of sensor chamber was maintained at 26°C.

Target specificity of aptasensor

Specificity of aptasensors for BoNT/A LC was examined by comparing the binding with the same concentration of BSA, casein, IgG and lysozyme. Concentration of proteins used was 1 μM.

Structure specificity of aptasensors

The ability of aptasensor to distinguish structurally intact BoNT/A proteins was examined by comparing relative binding to toxoid (formalin cross-linked proteins) and thermally denatured forms. Toxoid of BoNT/A LC and DR BoNT/A were prepared by continuous mixing of the respective proteins in 0.0125% formaldehyde at 37°C for 7 days. Insoluble material and excess formaldehyde were removed using micro-bio spin columns (Bio-Rad, Hercules, CA).The thermally denatured LC and DR BoNT/A were achieved by heating proteins at 70°C for 5 mins in the presence and absence of 0.15% SDS. SDS was added to prevent protein aggregation during the thermal denaturation. The concentration of proteins was determined using Pierce 600 nm protein assay (Thermo Fisher Scientific Inc).

Detection of DR BoNT/A and signal enrichment using immuno-pull down

Initial detection of DR BoNT/A in the running buffer was performed by simple injection of the protein at different concentrations over the aptasensor surface. Subsequently, enrichment of DR BoNT/A concentration was achieved using immuno-pull down method performed with human monoclonal antibody against heavy chain of BoNT/A (RAZ-1) immobilized on protein G or tosyl-activated dynabeads (Life Technologies, Carlsbad, CA). The beads were coated with RAZ-1 antibody as per manufacturer's protocol. Serial dilutions of DR BoNT/A were prepared separately with 1 ml of either PBST buffer (PBS buffer containing 0.2% Tween-20), or different sample matrices (undiluted carrot juice, fat free skim milk, and 5-fold diluted pooled normal human serum). During recovery study, DR BoNT/A was directly spiked into PBS buffer, carrot juice and fat free skim milk at the desired concentrations; DR BoNT/A was spiked into the 5-fold diluted human serum to study the toxin recovery from serum. 20μl of the antibody-coupled magnetic beads were incubated with the respective samples under constant agitation for 1hr at 37°C. The beads were then washed five times with 200μl of PBST, pH 7.4. DR BoNT/A was eluted from the beads using 30 μl of 10mM

glycine buffer pH 2.5 for 4 mins, immediately neutralized with 150μl of running buffer and injected over aptasensor surface for detection. The limit of detection (LOD) is determined based on the signal to noise ratio. The cut-off for LOD was 3 times the standard deviation of blank (i.e. LOD = 3*StdDev of blank/slope of the calibration curve).

Structural conformation of DR BoNT during low pH elution

The effect of low pH on protein conformation during protein elution step was investigated by studying the protein structure before and after elution using circular dichroism spectroscopy. The CD spectra were recorded at 26°C on a Jasco J715 spectropolarimeter equipped with a Peltier type temperature control system (model PTC-348W) as described previously [28]. Initially, the far UV-CD spectrum of DR BoNT/A in a running buffer (pH7.4) was collected between 200 nm to 250 nm. Similar far UV-CD measurements were then collected for DR BoNT/A subjected to pH 2.5 for 4 mins and neutralized back to pH 7.4. In order to compare the pH effect on the structural conformation, the concentration of both pH treated and non-treated (control) DR BoNT/A samples was maintained at 0.125 mg/ml.

Endopeptidase activity of BoNT during low pH elution

Enzymatically active BoNT/A was used to examine the effects of low pH elution on endopeptidase activity. BoNT/A was subjected to pH 2.5 for 4 mins and then neutralized back to pH 7.4. Both pH treated and non-treated control BoNT/A were reduced with 1.25 mM DTT at 37°C for 30 mins and assayed for endopeptidase activity using recombinant N'-His-SNAP25a-EGFP-C' (SNAG), a 55kDa recombinant protein containing the substrate for BoNT/A. The effect of acidic pH treatment on the endopeptidase activity of BoNT/A was examined by incubating 2 μM SNAG with 50 nM BoNT/A (low pH treated and non-treated) at the 37°C for 30 min in the 1X HBS-P buffer with 0.2% P-20, 1.25 mM DTT. The cleavage reaction was terminated by the addition of reducing Laemmeli sample buffer and the samples were then separated on a 12% SDS-PAGE. The electrophoresis was run using a Mini Protean II system from Bio-Rad at a constant voltage of 200 V at room temperature (25°C). The Coomassie Blue stained gels were scanned on a GEL LOGIC 100 Imager system and analyzed using the Kodak Image analysis software (Eastman Kodak Co.).

Stability of aptasensor

The activity of sensor surface was tested with 1 μM DR BoNT in running buffer during 31-day period, with 411 injections/regenerations. The conditions of binding analysis were same as above. Between uses, the aptasensor was stored at 4 °C.

Results and Discussions

A previous study from our group reported selection and binding characterization of three RNA aptamers that bind to and inhibit the activity of BoNT/A LC. The S132B-C22 RNA containing 2´- fluorine modified pyrimidines was identified as the strongest binding aptamer with a K_D of 87 ± 20nM to BoNT/A LC [22]. Based on these findings, S132B-C22 was chosen as the capture agent for the aptasensor to detect BoNT/A in different food and clinical matrices.

Aptasensor design

In order to fabricate the aptasensor and reuse the sensor surface, the aptamer was immobilized to the sensor surface. This was achieved by selectively labeling the 3'-end of the RNA aptamer with biotin16-ddUTP using terminal transferase. The biotin functionality imparts specific binding of the aptamer to streptavidin with binding energies approaching

that of a covalent bond. An electrophoretic mobility shifts assay was conducted to confirm biotinylation of the RNA aptamer (Fig.1). To assemble aptamer on SPR sensor, streptavidin was first immobilized to the CM5 chip through amine coupling procedure [27]. The biotin-labeled aptamer was then selectively captured by the streptavidin coated sensor surface. Although the biotinylation efficiency is not 100% (Fig. 1), when injected through Biacore T100 microfluidics flow system, only biotinylated aptamer was selectively captured by the streptavidin coated chip. The usage of a dideoxy nucleotide attached to biotin *via* a long linker ensured attachment of a single biotin molecule to the RNA with a long spacer. The biotin 16 dd-UTP tag not only preserved the aptamer structure after immobilization, but also provided flexibility and minimized steric hindrance to binding with BoNT/A. Earlier studies directly immobilizing BoNT/A to the sensor surface yielded very little aptamer binding [22], suggesting the improper orientation or structural changes of the protein upon immobilization have deteriorated its binding efficiency. Immobilization of biotinylated aptamers directly to the surface preserves orientation and structure while improving binding capacity for the target. Furthermore, the strong interaction between biotin and streptavidin on the sensor surface provides the basis for sensor surface regeneration and re-use.

Specificity of aptasensors

Specificity of the aptasensor was examined by comparing its binding response to 1μM concentrations of BoNT/A LC and other proteins (i.e. BSA, casein, IgG, and lysozyme). To reduce the non-specific binding, the concentration of P-20 used in the original 1X HBS-P buffer was increased to 0.2% (v/v). DTT (10 mM final concentration) was added to prevent the dimer formation of analyte. Fig. 2A shows that the aptasensor, while having strong binding to its target BoNT/A LC, did not bind other non-specific proteins (BSA and casein) even at much higher concentrations of 1mg/ml.

To study the robustness of the aptasensor, the binding between S132B-C22 aptamer and different batches of BoNT/A LC was tested. All three batches of BoNT/A LC showed strong binding to aptamer (Fig. 2B). The aptasensor also showed strong binding to DR BoNT/A, a surrogate for BoNT/A (Fig. 2B), suggesting that the aptamer can be used for detection of full length BoNT/A. Although the immobilized aptamer showed preferential binding to BoNT/A proteins (Fig. 2B), cross-reactivity to BoNT/B LC and BoNT/E LC was observed (34% and 21% binding, respectively, when compared with BoNT/A LC). Structure recognition forms the basis of aptamers binding to their targets. Thus, this cross-reactivity specific to different BoNT serotypes light chains (i.e. BoNT/B and /E) may arise from such structural similarities. While cross reaction may interfere with the typing of the serotype of BoNT, it could be used for rapid detection of several serotypes from the same sensor [29].

Structure recognition of aptasensor

Aptamers have unique specificity to distinguish the structural variations of their target [21]. Therefore, aptamers selected for a desired target could be utilized to identify the correctly folded target and discriminate the mis-folded (and presumably non-functional) counterparts. Binding of thermally denatured and formaldehyde cross-linked BoNT/A LC and DR BoNT/A was compared to their native counterparts in order to investigate structural specificity of aptasenor. Antibodies (polyclonal or monoclonal) usually only recognize unique epitopes within their target. They are highly specific to their epitopes (and thus to their targets). When proteins are denatured (and consequently nonfunctional), some of the epitopes may still be accessible and be recognized by antibodies. While conformational epitopes could be used for structural recognition between antibody and its target [30], it is not easy to identify antibodies targeting the distinct conformation of a protein, and have not been applied for detection of structurally intact BoNT. On the contrary, apatmers are selected against the structure of whole protein, thus can recognize the appropriately folded targets. Our results

showed that aptamer did not bind to inactive cross-linked proteins (Fig. 3A & B). Similarly, when proteins were heat-denatured to the unfolded state, they did not bind to aptasensor (Fig. 3A & B). These results demonstrate that the aptasensor is able to distinguish between the fully folded protein and denatured counterparts, proving that the aptasensor developed here can be used to detect the structurally native toxin. This is crucial for the homeland security surveillance, since only native toxin has the toxicity for their mass destruction.

Further, the aptamer we used can recognize both natively folded light chain, the catalytic domain of BoNT/A, and the full toxin (Figure 3). This is important, as the light chain of BoNT is responsible for the inhibition of exocytosis, and it has been demonstrated by forming the hybrid-toxin, light chain can target other types of cells [31]. While currently this has been studied for their therapeutic potential targeting a range of different diseases, it could be engineered by bioterroists to create a hybrid-version of toxin for bioattack. Our aptasensor has the ability to detect both natively folded BoNT/A and its catalytic domain, light chain, would be significant for surveillance of both native toxin and potential hybrid-toxin containing light chain.

Rapid detection of DR BoNT/A in different sample matrices

High affinity for their target, in combination with the specificity of aptamers makes them an ideal detection tool. In addition, the SPR based system offers an advantage of minimum sample processing such as removal of particulate materials, facilitating direct detection of analytes in complex and opaque liquids at rapid response time (near real-time). Therefore, exploration of the SPR-based aptasensor for rapid detection of natively folded toxin using DR BoNT/A as the surrogate was pursued. A direct binding assay by injecting sample through aptasensor is rapid (about 8 mins per sample), but yielded low sensitivity. Limit of detection (LOD) obtained from this direct binding assay in HBS-P buffer was 0.5 μg/ml (Fig. 4).

In order to improve the sensitivity, immuno-pull down of target was employed for simultaneous enrichment of protein and elimination of interferences from sample matrices. For this purpose, monoclonal human anti-BoNT/A HC (RAZ-1) was coupled to protein G dynabeads. Nanograms of DR BoNT/A spiked in 1ml of simple matrices like PBST buffer and carrot juice were effectively pulled down with these high affinity antibodies. The concentrated protein was then eluted from immuno-capture beads, in order to be detected in aptasensor. While the protein-G beads were successful in simple matrices, very high non-specific responses that masked specific identification of DR BoNT were observed in comparatively more viscous and high protein content matrices such as milk and human serum. High protein matrices may contain the species that can non-specifically bind to protein G beads, and be co-eluted out. Those species may bind to the SPR chip matrix (carboxymethylated dextran) and/or the functional group for immobilization of aptamer (streptavidin), leading to the interference. To remove the non-specific interferences, we used the tosyl-activated dynabeads, which provide covalently coupling of the antibody, and non-specific binding sites are blocked by bovine serum albumin. The tosyl-activated beads have been successfully used for capturing analytes from milk [32]. Our results showed that using tosyl-activated beads significantly decreased interferences from milk. However, human serum still showed high interferences. To further minimize interferences, human serum was diluted 5 times with PBST before spiking toxin and immuno-enrichment with tosyl-activated beads.

Fig. 5 reveals signal enhancement for DR BoNT/A from μg/ml to ng/ml range by employing this immuno-pull down step. The LOD for DR BoNT is 5.8 ng/ml in buffer, 20.3 ng/ml and 23.4 ng/ml in carrot juice and fat free milk, respectively (Table 1). High interference was observed in detection from undiluted human serum, therefore, 5-folded dilution of the serum

was used for the assay. While the relative high y-intercept was observed from the calibration curve of 5-folded dilution of human serum (Fig. 5), the calibration regression and the recoveries from samples were satisfied (Fig. 5, Table 2), suggesting that the assay is suitable for analysis of BoNT in serum when sample was diluted before assay. The LOD of DR BoNT/A in 5-folded diluted human serum is 22.5 ng/ml (Table 1). The LOD of DR BoNT/A in human serum would be 112.5 ng/ml if no dilution was made. The results shown here demonstrate that SPR-based aptasensor has the ability to detect native BoNT/A in low ng/ml in complicated food sample matrices. While the RNA aptamer used in this report can recognize both natively folded LC of BoNT/A and full toxin, using the specific antibody against HC during immuno-enrichment ensures that only full toxin will be detected. The additional capture using immuno-beads only took about 70 min (including capture and elution) making the total testing time within 90 min.

Effect of low pH elution on structure and activity of BoNT/A

Elution of the enriched protein from immuno-beads was necessary to remove interferences and detect DR BoNT/A in the SPR-based platform. For this purpose, DR BoNT/A bound to antibodies were subjected to 10mM glycine, pH 2.5 for 4 mins and immediately neutralized with SPR running buffer (pH 7.4). Therefore, the effect of this low pH elution on the structure of DR BoNT/A was investigated using circular dichroism spectroscopy. DR BoNT/A with low pH treatment followed by neutralization and that without treatment showed identical secondary structure (Fig. 6). Though low pH exposure during elution of toxin from immuno-capture beads does not alter the structure of protein, it is important to understand the effect of this process on its biological activity. To achieve this purpose, the endopeptidase activity of native BoNT/A was examined with and without low pH treatment, on a recombinant full-length protein substrate of BoNT/A, His-SNAP25a-EGFP (SNAG). The EGFP tag increased the mass differences in the cleavage products thus making it easier to detect from gel electrophoresis. The 55 kDa SNAG is cleaved by BoNT/A, yielding 28 and 26 kDa fragments (Fig. 7). The low pH treatment followed by neutralization does not reduce the endopeptidase activity of BoNT/A, as shown in Fig. 7. These results demonstrate that low pH treatment during elution step does not alter BoNT/A structure nor affects its biological activity and therefore, the SPR-based aptasensor is able to detect the active toxin after sample pretreatment.

Stability of the aptasensor

One of the advantages of aptamers over antibodies is their structural robustness. They can be regenerated after binding without sacrificing the structural integrity and stability. RNA is often subjected to chemical degradation, especially by nuclease digestion in biological samples. The advantage of using RNAse resistant 2’-F-pyrimidines modified RNA provides prolonged stability of aptamers on the chip. To evaluate the stability of the aptasensor, the sensor surface was tested for 31 days with over 400 injections/regeneration cycles. As shown in Fig. 8, the aptasensor retained strong binding affinity to DR BoNT/A during the testing period. The robustness and stability combined with reusability of the sensor make it suitable for a cost-effective field use. These characteristics are specifically important for homeland security surveillance.

DR BoNT/A recovery from different sample matrices

To investigate the accuracy of the aptasensor for detection of BoNT/A in different sample matrices, known amounts of DR BoNT/A were spiked into different sample matrices. As shown in Fig. 5, the aptasensor responses to DR BoNT/A are different when different sample matrix is used. Therefore, the calibration curve from different sample matrices was used for quantification of toxin in the corresponding matrix. Recovery of DR BoNT/A were determined within the ranges of 91% to 116% in buffer, carrot juice and fat free milk for a

wide concentration range (from 25 to 100 ng/ml) (Table 2). Considering the high matrix interferences, toxin was spiked in a five-fold diluted serum, with the recovery between 91 to 101% at different concentration ranges (25 to 100 ng/ml) (Table 2). The recoveries from toxin-spiked samples showed that the SPR based RNA aptasensor can accurately quantitate toxin in different sample matrices at low ng/ml level.

Immunological assays, including enzyme-linked immunosorbent assay (ELISA) are the most characterized *in vitro* methods currently available for BoNT toxin detection. Although the sensitivity of some of these sensitive assays reaches sub ng/ml, a level comparable with mouse bioassay, the assay detects signals from both active and denatured inactive toxin and are time consuming (6–8 hrs) [33]. Several functional assays have been developed to detect BoNT based on its endopeptidase activity. These assays suffer from protease interference from sample matrices in addition to being time consuming (several hours) and labor intensive [34].

The aptasensor developed here provides a direct way to detect the structurally intact, active toxin in food samples in low ng/ml level. It has been estimated that the oral toxicity of the most potent serotype of BoNT, type A, is about 70 μg for a 70 kg person [2]. For effective intoxication of a human through food supplies (either accidentally or intentionally), it would require at least hundreds of nanogram per milliliter level of active toxin. While the sensitivity of this aptasensor is relatively low (low ng/ml), it is well suitable for rapid detection and screening of active toxin in food supplies. More importantly, the RNA aptasensor developed here only responds to natively folded, active toxin and its catalytic domain. The immuno-capture procedure performed on the micro-beads can easily be automated, thereby increasing sample throughput and further reducing the assay time. Due to its ability to inhibit the endopeptidase activity of BoNT/A [22], the aptasensor developed here can also be integrated for simultaneous detection/decontamination of BoNT/A. While SPR provides a rapid, label free detection platform, it is not a high sensitive technique, due to their relative small surface area for capture-agent on the chip [35]. This is reflected in our results as well, with detection limit at low ng/ml level, which is comparable to those reported for other analytes [35]. While for food and environmental samples, our detection limit is adequate, for clinical diagnosis, a more sensitive detection platform will be needed to combine with the aptasensor for detection of active toxin in blood samples.

Conclusion

An SPR based RNA aptasensor has been developed for rapid detection of natively folded active BoNT/A. By using the surrogate, DR BoNT/A, the detection limit of the aptasensor was demonstrated in the low ng/ml range for disparate food matrices. To our best knowledge, this is the first sensor system for rapid detection of natively folded toxin. Our sensor is robust, has a shelf-life of at least 30 days and can be reused for at least 400 tests. The robust aptasensor developed here provides a valuable detection platform for rapid homeland security surveillance and threat assessment.

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Highlights

- We developed a unique RNA aptasensor for rapid detection of type A botulinum neurotoxin
- The RNA aptasensor can successfully differentiate natively folded and denatured toxin
- The aptasensor is robust, stable for more than 30 days, and reusable for more than 400 times
- The aptasensor can accurately detect natively folded toxin in different sample matrices at low ng/ml

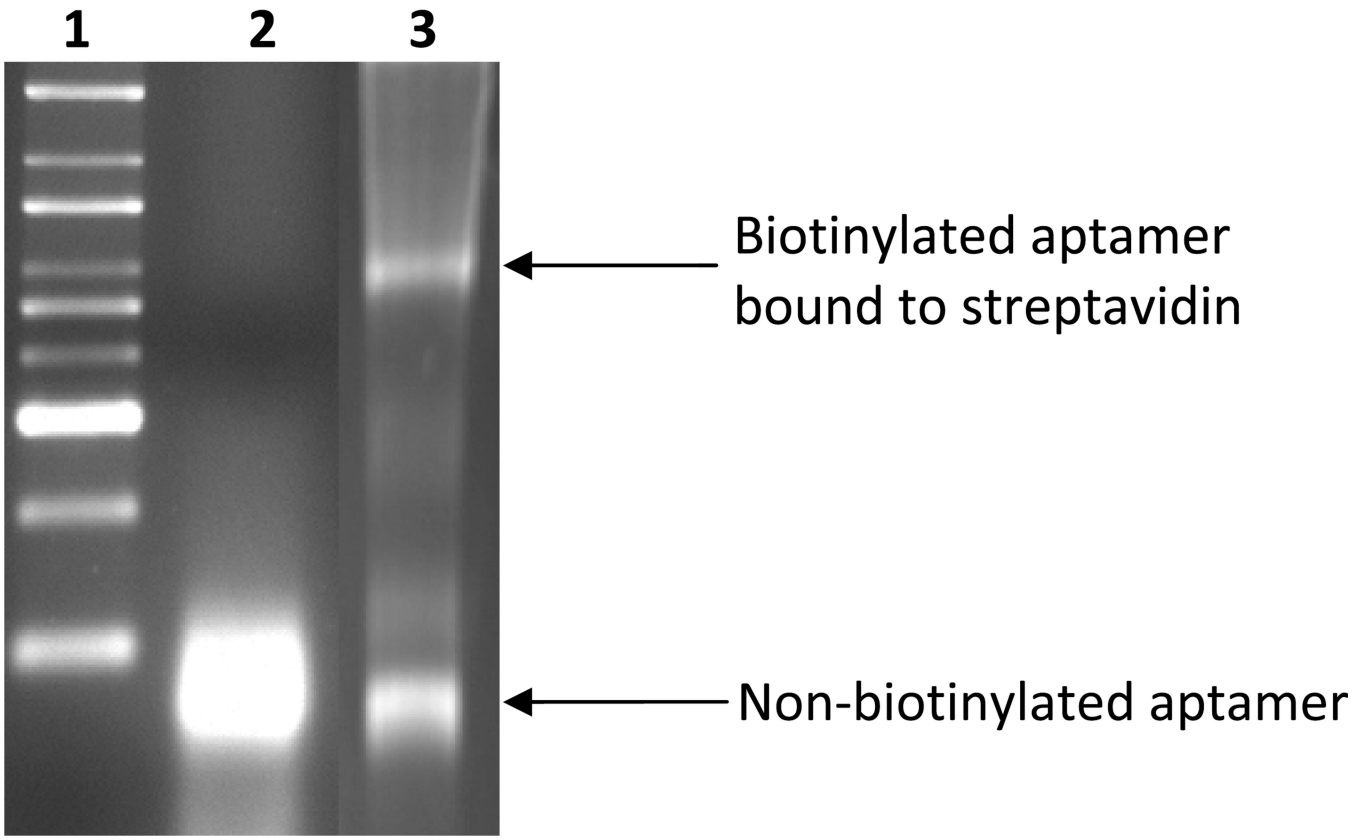

Figure 1.
Mobility shift of 3´-biotinylation of C22 aptamer on denaturing agarose gel after binding to streptavidin. Lane 1 – ssRNA low range marker; 2 – single stranded C12 RNA and 3 - 3´ biotinylated C12 RNA bound to streptavidin.

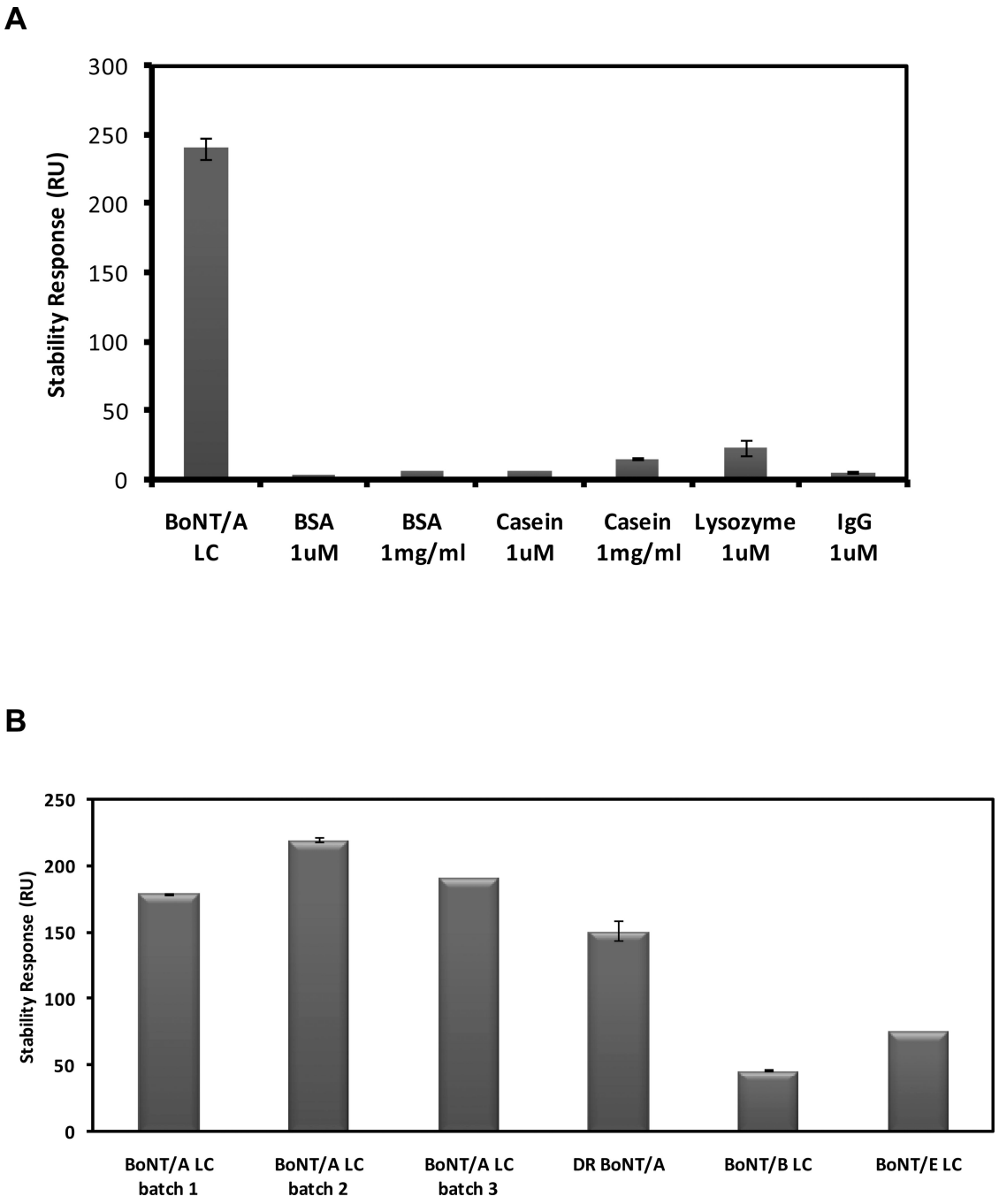

Figure 2.

Specificity of SPR based RNA aptasensor. Panel A showed the specificity of aptasensor to BoNT/A LC vs. non-specific proteins; panel B showed the response of aptasensor to DR BoNT/A, BoNT/B and /E LC, and different batches of BoNT/A LC. Concentration of all proteins is 1 μM. Data showed are the average of two independent measurements, with error bar as the standard deviation.

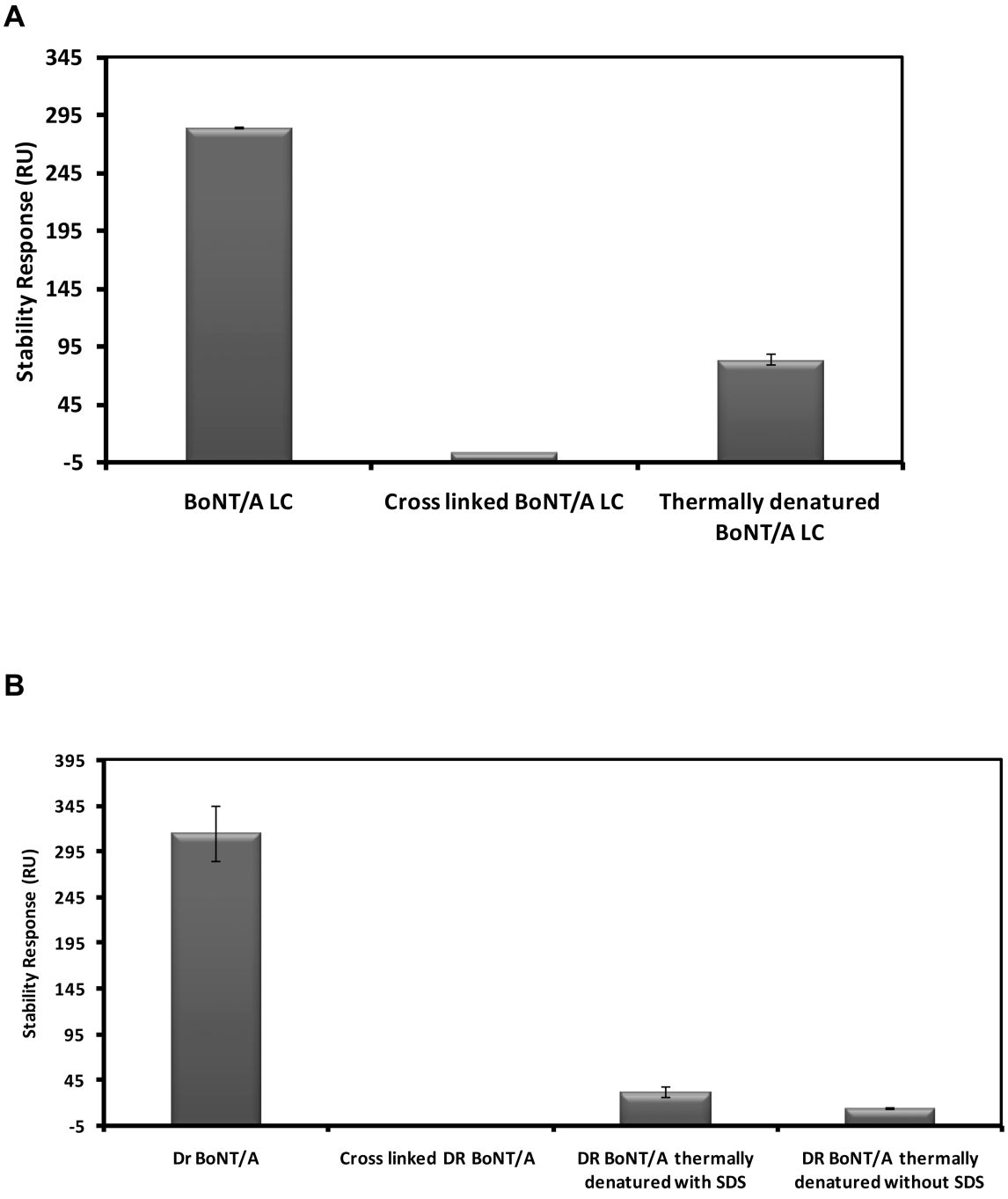

Figure 3.

Specific recognition of natively folded, active BoNT/A LC (A) and DR BoNT/A (B) by S132B-C22 aptasensor. Data showed are the average of two independent measurements, with error bar as the standard deviation.

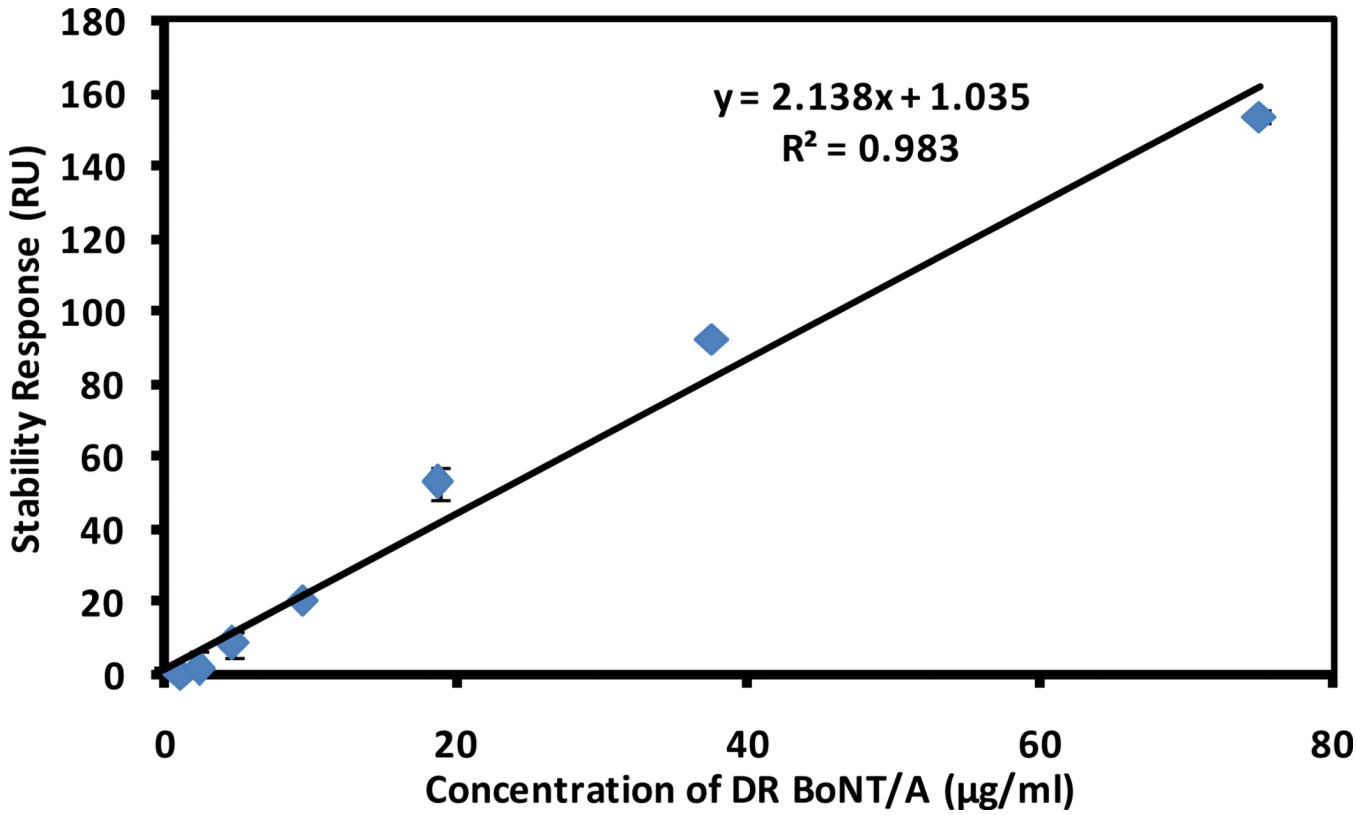

Figure 4.
Direct assay for detection of DR BoNT/A spiked in HBS-P buffer. Data showed are the average of two independent measurements, with error bar as the standard deviation.

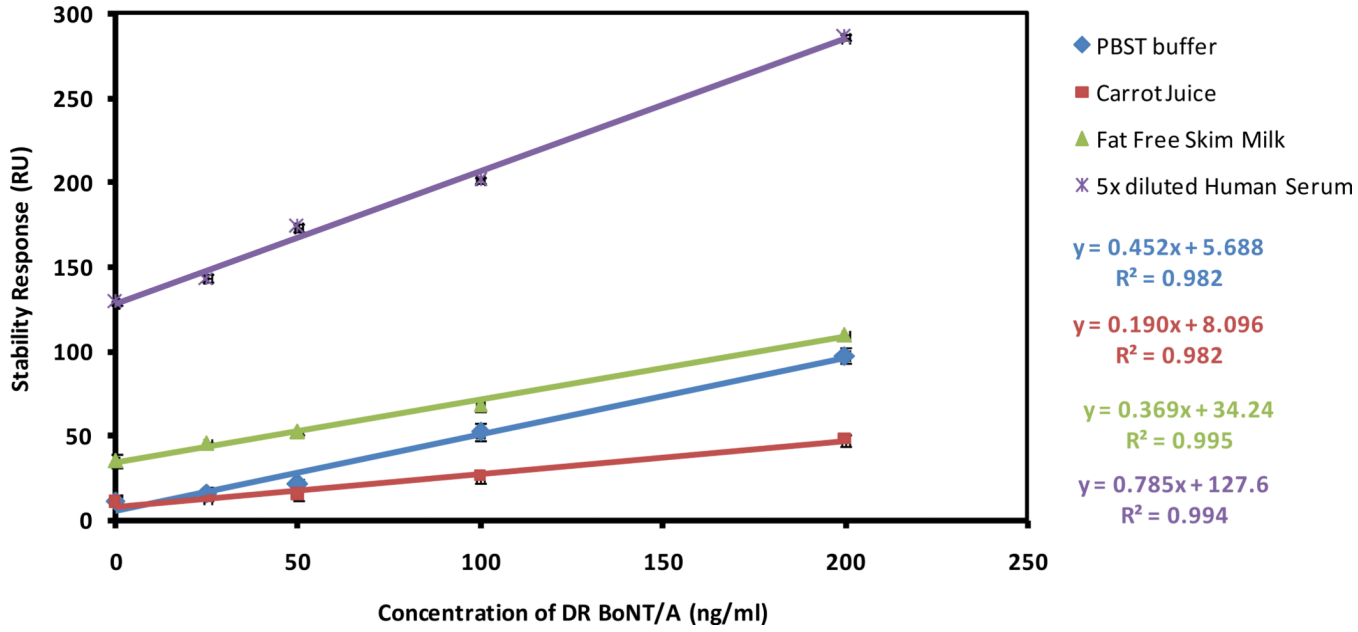

Figure 5.

Detection of DR BoNT/A spiked in PBST buffer, carrot juice, fat free skim milk and 5x diluted human serum through immuno-pull down enrichment. Data showed are the average of two independent measurements, with error bar as the standard deviation.

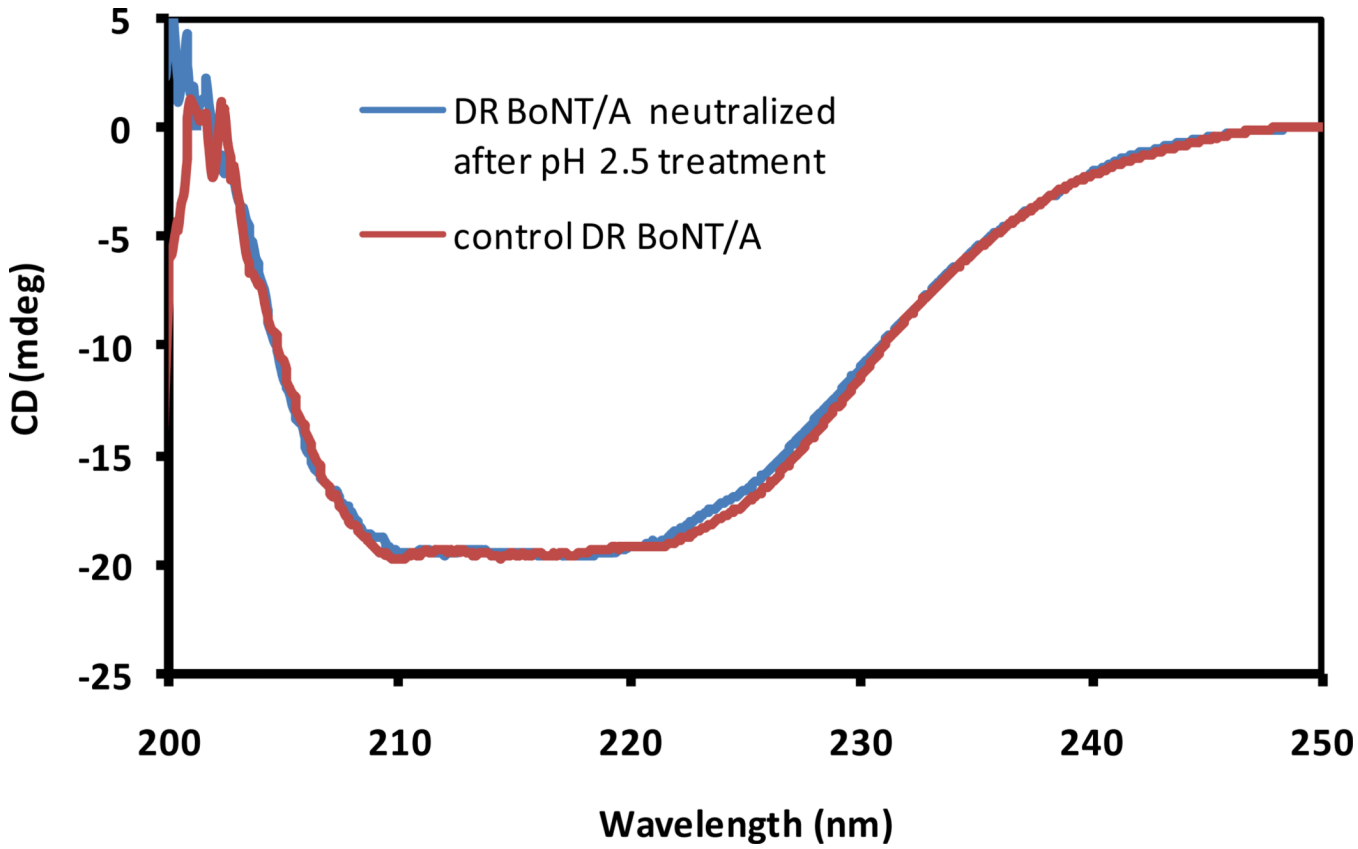

Figure 6.
Comparison of the effect of low pH elution on secondary structure of DR BoNT/A using far UV CD spectroscopy. Concentration is 0.125 mg/ml for both pH-treated and non-treated samples.

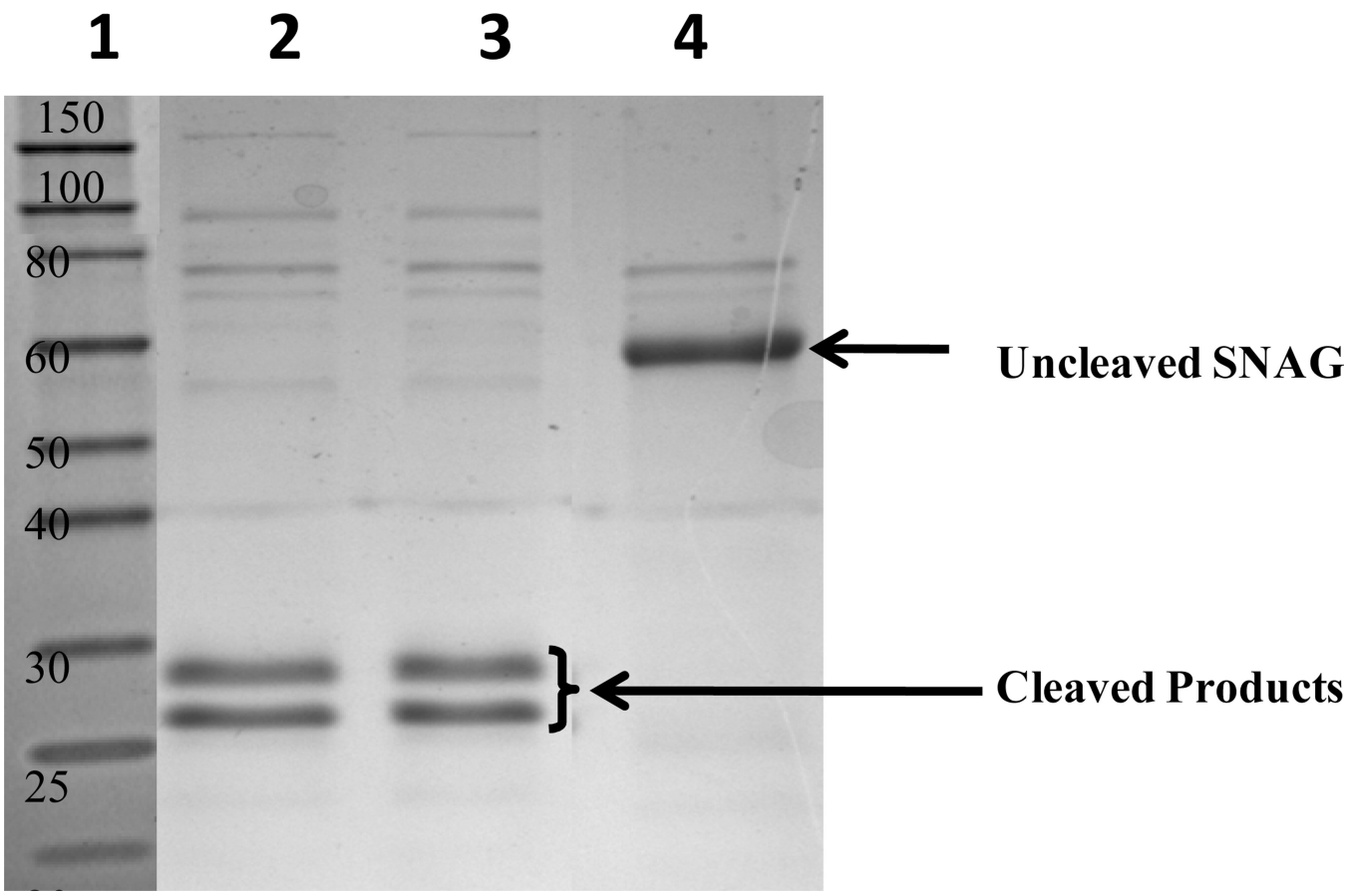

Figure 7.
SNAG-Endopeptidase assay demonstrating no effect of low pH elution on enzymatic activity of BoNT/A. Lane 1 – Broad range molecular weight marker, Lane 2 and 3 – Endopeptidase cleavage products of SNAG by non-treated (control) BoNT/A and BoNT/A neutralized after pH 2.5 treatment, respectively, Lane 4 – Uncleaved SNAG protein control.

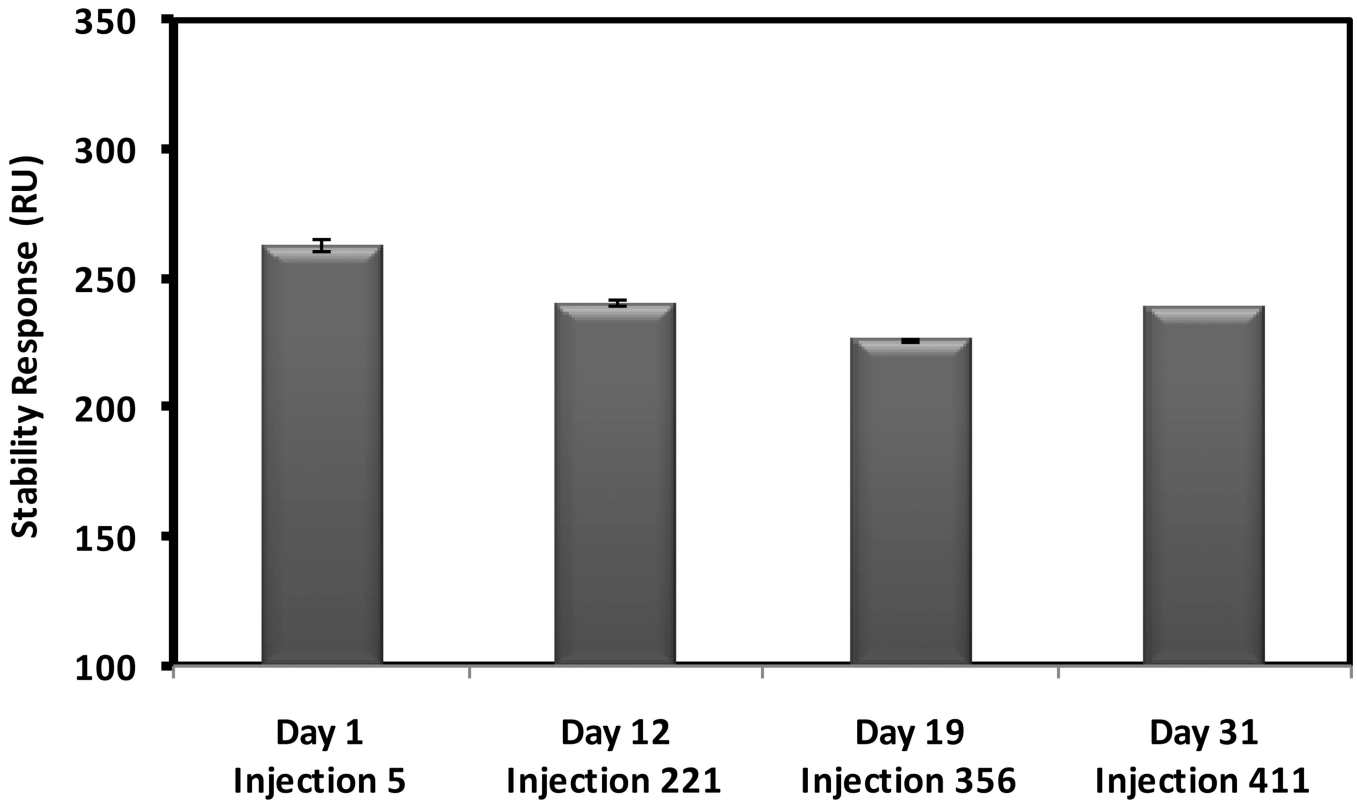

Figure 8.
Stability of Aptasensor: Representative data displaying binding response from 1μM DR BoNT/A for 31 days and 411 injections. Data showed are the average of two independent measurements, with error bar as the standard deviation.

Table 1

Limits of detection (LOD) of DR BoNT/A detected by SPR based RNA aptasensor, after immuno-pull down in sample matrices*

Sample Matrix	LOD (ng/ml)
PBST buffer	5.8±2.5
Carrot Juice	20.3±2.8
Fat Free Milk	23.4±4.4
5-Fold Diluted Human Serum	22.5±5.8

* Data represents average of three replicates ± standard deviation.

Table 2

Percentage recovery of DR BoNT/A from different sample matrices, using immuno-pull down enriched SPR aptasensor detection*[a]

Spiked Concentration (ng/ml)	PBST Buffer	Carrot Juice	Fat Free Milk	5-Fold Diluted Human Serum
25	107±13	116±7	91±16	101±14
50	102±17	109±11	100±5	97±15
100	96±6	105±8	97±12	91±11

*Data represents average of three replicates ± standard deviation.