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Evaluation of Chemical Degradation of a Trivalent Recombinant Protein Vaccine Against Botulinum Neurotoxin by LysC Peptide Mapping and MALDI-TOF Mass Spectrometry

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Abstract

Vaccines utilizing recombinant protein antigens typically require an adjuvant to enhance immune response in the recipients. However, the consequences of antigen binding to adjuvant on both the short- and long-term stability of the protein remain poorly defined. In our companion paper (Vessely C, *et al.*, 2007), we characterized the effects of binding to adjuvant on the conformation and thermodynamic stability of three antigen variants for botulinum vaccines: rBoNTA(H_C), rBoNTB(H_C), and rBoNTE(H_C). In the current study we evaluated the effect of binding to adjuvant (AlhydrogelTM, aluminum hydroxide) on chemical stability of these antigens during long-term storage in aqueous suspension. We developed methods that employ LysC peptide mapping in conjunction with MALDI-TOF mass spectrometry. Peptide maps were developed for the proteins for a vaccine formulation of rBoNTE(H_C), as well as a trivalent rBoNT(H_C) vaccine formulation. This method provided high sequence coverage for the proteins in part due to the implementation of a post-digestion elution fractionation method during sample preparation, and was also successfully utilized to evaluate the chemical integrity of adjuvant-bound rBoNT(H_C) protein antigens. We found that all three of the rBoNT(H_C) proteins were susceptible to degradation via both oxidation and deamidation. In many cases, such reactions occurred earlier with the adjuvant-bound protein formulations when compared to the proteins in control samples that were not bound to adjuvant. Additionally, some chemical modifications were found in the adjuvant-bound protein formulations but were not detected in the unbound solution controls. Our studies indicate that binding to aluminum-based adjuvants can impact the chemical stability and/or the chemical degradation pathways of protein during long-term storage in aqueous suspension. Furthermore, the methods we developed should be widely useful for assessing chemical stability of adjuvant-bound recombinant protein antigens.

Keywords

Botulinum neurotoxin; recombinant vaccine; trivalent vaccine; stability; aluminum salt adjuvant; mass spectrometry; deamidation; oxidation

Introduction

The importance of adjuvants in vaccine development was recognized as early as the 1920s with the observation that diphtheria toxin bound to an aluminum salt adjuvant elicited a much stronger immune response compared to the soluble antigen alone¹. Though the precise mechanism behind such an effect remains unclear, it is generally observed that the antigen of interest must be bound to the adjuvant to stimulate a strong immune response²⁻³. An adjuvant is therefore routinely incorporated into the formulation to enhance the immune response of the target antigen in contemporary vaccines⁴⁻⁷. Aluminum hydroxide and aluminum phosphate are currently the only adjuvants used in vaccines approved by the US FDA⁸⁻¹⁰.

Recombinant protein antigens for botulinum neurotoxin serotypes A, B and E (rBoNTA(H_C), rBoNTA(H_C) and BoNTE(H_C), respectively) have been created by the US Army Medical Research Institute for Infectious Diseases (USAMRIID) for the development of a trivalent vaccine against the toxins from the three most common strains of the bacterium^{4,11}. These protein antigens are 50 kDa portions of the C-terminal domain of the heavy chains of the molecule, and there is no neurotoxicity associated with this portion of the molecule¹¹. In our companion paper¹², the effects of binding of three protein botulinum antigens to adjuvant (AlhydrogelTM, aluminum hydroxide) on the protein's structure and conformational stability were characterized. Protein stability is a critical parameter in the development of protein-based therapeutics and vaccines because the integrity of the molecule is often directly related to both product efficacy and safety¹³⁻¹⁴. In spite of the prevalent use of aluminum-based adjuvants in vaccines, little is understood in regards to the impact of these adjuvants on the short- and long-term stability of proteins.

Protein molecules are both structurally complex and chemically reactive, which results in susceptibility to physical and chemical degradation¹⁴⁻¹⁵. Physical instability can manifest in loss of native protein structure and lead to reduced drug potency. Oxidation and deamidation are the most commonly encountered routes of protein chemical degradation¹⁵. Such modifications may occur during production, purification, processing, and storage of protein molecules. The Asn and Gln residues are most susceptible to deamidation reactions. Numerous factors affect deamidation rates, including primary sequence, pH, buffer species, and protein conformation. Oxidation may arise from a variety of sources: trace levels of contaminating metal ions and peroxides, ultraviolet radiation, or simply molecular oxygen dissolved in the aqueous formulation¹⁵. Though the protein residues prone to oxidation vary somewhat according to the mechanism of oxidation, Met, Cys, Trp, Tyr, His, and Phe are typically found to be most sensitive¹⁵⁻¹⁷. As the integrity of specific amino acids is often coupled to the biological activity of the molecule, chemical modifications can also translate into loss of the desired therapeutic or prophylactic effect. It is important to note that physical and chemical instabilities may be linked. A greater degree of conformation flexibility due to partial unfolding of the molecule can result in increased rates of chemical degradation^{11,18}. Conversely, chemical changes such as deamidation impact the net charge of the molecule and may result in an increase in protein-protein interactions including aggregation¹⁹⁻²⁰.

Our hypothesis is that the protein conformational perturbations due to binding to adjuvant will accelerate the chemical degradation of bound protein antigens. Binding of protein antigens to the surface of the adjuvant can result in structural changes to the protein including loss of the native structure and reduced thermal stability^{12,21}. Increased conformational flexibility and solvent exposure of the structurally-perturbed molecule could lead to increased degradation rates. Charges on the surface of the adjuvant particles may lead to microenvironments in which the pH is quite different from that of the bulk solution, affecting chemical degradation rates as well²².

A variety of analytical techniques are employed to detect, monitor and characterize the chemical degradation of protein molecules²³⁻²⁴. For example, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) can detect large changes in protein mass and disulfide cross-links. Reverse-phase and ion exchange chromatography methods are useful in determining oxidation and deamidation, respectively. The application of mass spectrometry to the field of protein chemistry has proven to be invaluable in the detection of chemical changes in protein molecules^{17, 25}. Peptide mapping combined with mass spectrometry is commonly employed in the pharmaceutical industry to detect and characterize chemical modifications of specific amino acid residues. The protein is first digested with one or more enzymes to produce a specific set of peptides based on the cleavage sites in the primary sequence. These peptides can then be analyzed by mass spectrometry directly (i.e. matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) or after chromatographic separation (i.e. liquid-chromatography- mass spectrometry, LC-MS). Changes in the mass to charge ratio (m/z) of the peptides can be indicative of a chemical modification, which can be further explored by other analytical techniques such as tandem mass spectrometry (MS/MS)²⁶.

In the current study, we used peptide mapping coupled with mass spectrometry to characterize the chemical degradation of a monovalent rBoNTE adjuvant-bound vaccine and a trivalent rBoNTABE adjuvant-bound vaccine, as well as the respective adjuvant-free control samples containing unbound protein, during storage at 4 and 30 °C. We first developed a method to desorb the protein antigens from the surface of the adjuvant after which the proteins were subjected to digestion by endoprotease LysC for peptide mapping. Since biochemical similarities between the three protein antigens made separation by conventional methods (i.e. size exclusion chromatography, SEC, reverse-phase, high-performance liquid chromatography, RP-HPLC) extremely difficult, simultaneous digestion of all three proteins was required. We developed a fractionation method during preparative desalting and concentration steps to improve the sequence coverage of the peptide map of the trivalent rBoNT(H_c). This approach was then utilized to characterize and detect the chemical degradation the adjuvant-bound protein antigens and unbound proteins during storage in aqueous suspension.

Materials and Methods

Materials

Alhydrogel™ 2.0% (aluminum hydroxide adjuvant, ALOOH), made by Brenntag Biosector, was purchased through E.M. Sergeant Pulp & Chemical Co, Inc (Clifton, NJ). Succinic acid, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma Chemical Company (St. Louis, MO). Trehalose (high purity, low endotoxin) was purchased from Ferro Pfanstiel (Cleveland, OH), and polysorbate 20 (Tween®20, low carbonyl and peroxides) was purchased from Pierce (Rockford, IL). Protein 200 Plus LabChip® kits were purchased from Agilent Technologies (Palo Alto, CA). The rBoNTA(H_c) and rBoNTB(H_c) proteins were provided by Cambrex (Hopkinton, MA), and the rBoNTE(H_c) protein was provided by the Biological Process Development Facility at the University of Nebraska, Lincoln. The mass calibration standard kit used for internal calibration of MALDI-TOF spectra was purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile and HPLC-grade water were purchased from Fisher Scientific (Pittsburgh, PA). Trifluoroacetic acid was obtained from Pierce Biotechnology, Inc. (Rockford, IL). C₁₈ ZipTips® as well as Centricon® and Microcon® devices were obtained from Millipore Corporation (Belford, MA). Sequencing-grade LysC was purchased from Wako Chemicals USA Inc. (Richmond, VA). The MALDI-TOF matrix α -cyano-4-hydroxycinnamic acid was from Agilent Technologies Inc. (Palo Alto, CA).

Preparation of Formulations

Formulations were prepared by first dialyzing rBoNTA(H_c) (~0.8 mg/mL) and rBoNTB(H_c) (~0.8 mg/mL) into 15 mM succinate (pH 4). A rBoNTE(H_c) stock solution was obtained from the supplier in 15 mM succinate (pH 4) at a concentration of ~0.2 mg/mL. In order to achieve the appropriate concentrations of each protein, rBoNTE(H_c) was concentrated to ~0.46 mg/mL using a 10 kDa cut-off Centricon® device. Buffers, excipient solutions and protein solutions were sterile filtered in a laminar flow hood under aseptic conditions prior to mixing. For the study of the trivalent rBoNTABE(H_c), the proteins were mixed together prior to sterile filtration. Alhydrogel™ adjuvant was received as a sterile suspension, and sterility was not compromised prior to sample preparation.

Excipient solutions were added to the monovalent and trivalent rBoNT(H_c) protein solutions and mixed gently to create a bulk solution. This bulk was aliquotted into three portions: one for solution control samples without adjuvant, a second for adjuvant samples and a third for the time 0 samples. For the solution control samples, water was added at 10% of the total final volume. For the adjuvant-containing samples, 10 vol % adjuvant suspension was then added, to yield a final adjuvant concentration of 0.2%. All samples were aliquotted into 15 mL centrifuge tubes in previously determined volumes as necessary to complete the analysis at each time point. The final protein concentration for each sample was ~0.1 mg/mL of each protein. Final formulations are as described below.

Formulation Composition and Stability Conditions

For the rBoNTE(H_c) monovalent stability study, three formulations were prepared as follows: formulation 1 contained 0.1 mg/mL rBoNTE(H_c) with 0.2% Alhydrogel™ in 10 mM succinate, 10 mM sodium phosphate (pH 4); formulation 2 contained 0.1 mg/mL rBoNTE(H_c) with 0.2% Alhydrogel™ in 10 mM succinate, 10 mM sodium phosphate, 7.5% trehalose (pH 4); and formulation 3 contained 0.1 mg/mL rBoNTE(H_c) with 0.2% Alhydrogel™ in 10 mM succinate, 10 mM sodium phosphate, 7.5% trehalose, 0.01% polysorbate 20 (pH 4). A solution control, which did not contain adjuvant, was also prepared with 0.1 mg/mL rBoNTE(H_c) in 10 mM succinate, 10 mM sodium phosphate (pH 4).

Trivalent samples that contained all three rBoNT(H_c) proteins were also prepared in various formulations as follows: formulation 1 contained 0.1 mg/mL of each rBoNT(H_c) protein with 0.2% Alhydrogel™ in 25 mM succinate, 15 mM sodium phosphate (pH 4); formulation 2 contained 0.1 mg/mL of each rBoNT(H_c) protein with 0.2% Alhydrogel™ in 25 mM succinate, 15 mM sodium phosphate, 7.5% trehalose (pH 4), and formulation 3 contained 0.1 mg/mL rBoNTA(H_c), 0.1 mg/mL rBoNTB(H_c), and 0.1 mg/mL rBoNTE(H_c) with 0.2% Alhydrogel™ in 25 mM succinate, 15 mM sodium phosphate, 7.5% Trehalose, 0.01% polysorbate 20 (pH 4). An adjuvant-free solution control was also prepared with 0.1 mg/mL of each rBoNT(H_c) protein in 25 mM succinate, 15 mM sodium phosphate (pH 4).

Time 0 samples were analyzed immediately while the remaining aliquots were incubated at two storage temperatures: 4°C for real-time stability studies and 30°C for accelerated degradation studies.

Desorption

At the given time-points, rBoNT(H_c) samples were diluted with an equal volume of desorption solution (250 mM succinate, pH 3.5). This desorption solution was shown in preliminary studies to promote ~100% desorption of the proteins with no apparent changes to protein conformation¹². Samples were centrifuged for 5 minutes at 2000 x g to pellet the adjuvant. Supernatants were then subjected to LysC peptide mapping as described below.

During the incubation study, it became increasingly difficult to desorb rBoNTE(H_c) from the adjuvant in the monovalent formulations. In order to obtain sufficient protein concentrations for the analysis, an alternate desorption protocol was developed. First, adjuvant-bound samples were incubated for 24 hr in 4 M urea (pH = 7.5) at room temperature under gentle agitation. The adjuvant was then pelleted by centrifugation (2,000 *xg*) and the supernatant collected. Solution control samples underwent the same incubation and centrifugation processes. The urea concentration in the supernatant was then lowered to 1.2 M with reaction buffer (1 M potassium phosphate, pH 7.5) and LysC digestions were conducted as described below.

LysC Peptide Mapping and MALDI-TOF Analysis

Desorbed rBoNT(H_c) protein solutions (1 ml) were concentrated to approximately 50 μ l using a YM-10 Microcon® device (20,000 *xg* at room temperature). The concentrated sample was diluted with an equal volume of reaction buffer and LysC was added at a 1:25 mass ratio of LysC to rBoNT(H_c) protein. Samples were incubated for 4 hr at 37°C, after which the digestion was quenched by the addition of guanidine hydrochloride (GdnHCl) to a final concentration of 4 M. If not assayed immediately, samples were stored at -20°C.

Digested samples were desalted and concentrated using C₁₈ ZipTips® following the manufacturer's instructions. For rBoNTE(H_c) samples that did not contain polysorbate, the elution buffer from the ZipTip was 0.1% trifluoroacetic acid (TFA) in 90:10 acetonitrile (ACN):H₂O. The eluted peptides were then mixed with an equal volume of α -cyano-4-hydroxycinnamic acid containing picomolar levels of mass standards (ranging from 757 to 2465 *m/z*). The mixture was spotted onto the MALDI-TOF sample plate and air dried. Mass spectra were collected using a Bruker MALDI-TOF (Bruker Daltonics, Inc, Billerica, MA) mass spectrometer operated in the positive ion and reflectron modes.

Samples that contained polysorbate showed substantial interference in the MALDI-TOF spectrum that masked the rBoNT(H_c) peptides. For this reason, the polysorbate-containing samples were processed using a fractionation elution method. Rather than eluting the digestion peptides as well as the polysorbate in a single fraction, the ACN concentration was increased incrementally in steps (10, 20, 30, 50, and 90%) during consecutive elution from the ZipTip®. Each fraction was then plated separately to improve detection of the peptides. All trivalent rBoNT(H_c) samples were eluted using the fractionation procedure to enhance the sequence coverage of each protein, which is discussed in more detail below.

Results and Discussion

Development of LysC Peptide Maps of rBoNT(H_c) Proteins

We first developed LysC peptide maps of each of the individual rBoNT(H_c) proteins. LysC was selected as a digestion enzyme for the rBoNT(H_c) proteins based on three criteria: (i) the specificity of LysC to cleave only on the carboxy terminal of lysine (Lys, K) residues, (ii) the theoretical digestion of rBoNT(H_c) proteins resulted in most peptides within the range of 500 to 5000 *m/z* (assuming singly charged species), and (iii) the majority of peptides were separated by at least 10 *m/z* to improve the resolution between peptides. The theoretical LysC peptide maps were anticipated to yield 97.7, 92.5, and 96.0% sequence coverage for rBoNTA(H_c), rBoNTB(H_c), and rBoNTE(H_c), respectively, assuming that all peptides were detected.

Peptide maps for each of the unbound rBoNT(H_c) proteins were first developed independently by optimizing various digestion parameters and evaluating the digestion efficiency based on the extent of sequence coverage. Parameters investigated included rBoNT(H_c) protein mass (10 to 100 μ g), ratio of LysC to rBoNT(H_c) protein (1:10 to 1:100),

digestion buffer pH (pH 7.5 to 9.0), digestion time (4 to 24 hr), digestion temperature (ambient vs. 37 °C), and the addition of a denaturing agent (i.e. GdnHCl or urea) during digestion.

The details of the optimized LysC digestion protocol are given in the Methods section above. The LysC peptide maps are shown in Tables I through III for each of the rBoNT(H_C) proteins. In all cases, the singly charged species, [M+H]⁺, was considered due to the nature of MALDI-TOF ionization. We routinely achieved sequence coverage of at least 79.0, 74.0, and 77.9% for rBoNTA(H_C), rBoNTB(H_C), and rBoNTE(H_C), respectively, utilizing this LysC digestion method.

We then attempted to digest the unbound trivalent rBoNT(H_C) formulation using the same LysC digestion method as for the individual proteins. It was observed that the sequence coverage of each protein was reduced in the trivalent sample. For example, the sequence coverage of rBoNTE(H_C) when digested alone was 82.9% but dropped to less than 50% when digested with the other two rBoNT(H_C) proteins. A similar trend was observed with the sequence coverage of rBoNTA(H_C), which dropped from 80.9 to 66.7%, as well as rBoNTB(H_C), which dropped from 73.9 to 59.2%. It is important to consider the extent of sequence coverage in peptide mapping as chemical modifications to the protein of interest may be difficult to detect if the sequence coverage is low. To improve the sequence coverage of the trivalent rBoNT(H_C) formulation, we employed a fractionation procedure during the elution of the peptides from the C₁₈ ZipTip®. Rather than eluting all of the peptides in a single step with 90:10 ACN:H₂O containing 0.1% TFA, the peptides were fractionated consecutively from the ZipTip® with increasing amounts of ACN (10, 20, 30, 50, and 90%). Each fraction was then plated and analyzed by MALDI-TOF separately, which produced 5 individual MALDI-TOF spectra for each sample. The resulting peptides from each spectrum were combined and analyzed as a single peptide map data set. The fractionation procedure greatly increased the sequence coverage of each rBoNT(H_C) protein in the trivalent sample, giving sequence coverage of 93.3, 87.8, and 95.6% respectively for rBoNTA(H_C), rBoNTB(H_C), and rBoNTE(H_C). The peptides that were previously undetected with the one-step elution, but then were found by the using the fractionation method, are indicated in the respective LysC peptide maps for each rBoNT(H_C) protein (Table I through III).

We also found that the sequence coverage of the rBoNTE(H_C) was greatly decreased in the presence of polysorbate 20. This was due to interference of polysorbate 20 in the mass spectrum. The surfactant bound to the C₁₈ ZipTip® and co-eluted with the peptides in the *m/z* range of 1000 to nearly 2000 (Figure 1A, 1C). The rBoNTE(H_C) peptides peaks were completely masked by the interfering polysorbate 20 peaks within this range, leading to a decrease in peptide detection (i.e. peptides E24 through E15, Table III) and a corresponding drop in the sequence coverage from 77.9 to 58.5%. By applying the same fractionation elution method developed for the trivalent rBoNT(H_C) formulation, the rBoNTE(H_C) peptides with masses in the *m/z* range of 1000 to 1800 were once again detectable in the polysorbate 20 formulation (Figure 1B, 1D). This led to rBoNTE(H_C) sequence coverage consistently greater than 75%, which was similar to the LysC peptide maps generated in the absence of polysorbate 20. Other formulation excipients (i.e. trehalose) did not noticeably impact the sequence coverage of the rBoNTE(H_C) formulation or any of the rBoNT(H_C) proteins in the trivalent formulation (data not shown).

Chemical Degradation during Storage of Monovalent rBoNTE(H_C) Formulations

The three rBoNTE(H_C) adjuvant-bound formulations and the unbound solution control were incubated at 4 and 30 °C. We found that rBoNTE(H_C) degraded at both temperatures, and chemical changes were detected as early as two weeks into the study (Table IV and V). The

chemical degradation of rBoNTE(H_c) at 4°C, which included both oxidation and deamidation, is summarized in Table IV. The amino acid residues that are most susceptible to oxidation reactions are methionine (Met, M), cysteine (Cys, C), tryptophan (Trp, W), tyrosine (Tyr, Y), and histidine (His, H); oxidation can be detected as mass shifts in multiples of +16.0 *m/z*. Protein deamidation is also a common degradation pathway with asparagine (Asn, N) and glutamine (Gln, Q) being sensitive to the formation of aspartate and glutamate, respectively, which manifests as a mass shift of +1.0 *m/z*. We found at least four modified peptides characterized by a shift of ca. +16.0 *m/z* suggesting the addition of a single oxygen atom (Table IV, peptides E2, E4, E11, and E15). Deamidation was also noted for peptides E22, E20, E17, E4, and E2 (Table IV). Peptide E20 showed three sites of deamidation due to a mass shift of +3.1 *m/z* when compared to the native peptide (Figure 2). In some cases, the change in mass compared to the intact peptide suggested that multiple degradation events had occurred in a single peptide. For example, a mass shift of +49.1 *m/z* was observed for peptide E4 (IVNVNNEYTIINC MRDNNSGWK). As peptide E4 contains four possible sites of oxidation (Y, C, M, and W) it is likely that the observed shift in mass is due to the addition of three oxygen atoms (+48.0 *m/z*). The additional shift of +1.1 *m/z* could arise due to the simultaneous deamidation of one of the many N residues found in peptide E4.

Chemical modifications of the protein were found in all three rBoNTE(H_c) formulations with some modifications specific to a given formulation (i.e. W oxidation of peptide E15) and others somewhat more ubiquitous (i.e. modifications to E11). In most cases, chemical modifications detected in the rBoNTE(H_c) formulations were also observed in the solution control. As a general trend, however, the degradation could be detected in the adjuvant-free solution control at a much later time point (i.e. 9 weeks) in the storage study compared to the formulations that contained adjuvant.

Many of the chemical modifications of rBoNTE(H_c) that were found during incubation at 4°C were also detected at 30°C, as summarized in Table V and also shown in Figure 3. We observed various cases of oxidation (i.e. W oxidation of E15), deamidation (i.e. two sites of deamidation on E20), as well as both forms of degradation on the same peptide (i.e. E4). Similar to the results obtained from the 4°C incubation, the majority of modifications were found early in the 30°C incubation in the three rBoNTE(H_c) formulations. Many of these modifications were also detected in the solution control but not until week 9, which indicates that the adjuvant-bound formulations degraded more rapidly than the unbound control at both storage temperatures. Overall, rBoNTE(H_c) formulation 1 appeared to be the most resistant to chemical degradation during incubation at both temperatures since this formulation displayed the fewest number of chemical modifications.

It became increasingly difficult to obtain high sequence coverage of rBoNTE(H_c) after incubation at both incubation temperatures as the study progressed. For example, the sequence coverage of rBoNTE(H_c) formulation 1 decreased to ~45% by week 4 and less than ~10% by week 9 (data not shown). We hypothesized that the loss in sequence coverage resulted from poor desorption of the protein from the adjuvant¹². Thus, alternate methods of desorbing the protein from the adjuvant were tested: boiling for 15 min, boiling for 15 min in the presence of 1% SDS, and incubation in 4 M urea for up to 24 hr at room temperature. We found that incubating the adjuvant-bound material with 4 M urea at room temperature for 24 hr substantially improved the sequence coverage to over 70%. Therefore, in order to obtain adequate sequence coverage of the protein, rBoNTE(H_c) samples were subjected to such conditions at week 9 of the study. It is worth noting that the deamidation detected for peptide E22 (Table IV and Table V) may be an artifact of exposing the protein to these conditions since this modification was found only in the samples subjected to this process.

Chemical Degradation during Storage of Trivalent rBoNT(H_C) Formulations

Formulations containing all three rBoNT(H_C) proteins were also prepared and incubated at 4 and 30°C as described in the Methods section. LysC peptide mapping and MALDI-TOF mass spectrometry also successfully detected a number of chemical modifications on each of the three rBoNT(H_C) proteins in the trivalent formulation. A summary of the chemical modifications are presented for the incubation at 4 and 30°C in Tables VI and VII, respectively. A large number of the modifications detected early in the study (i.e. 2 week time-point). For example, peptide A11 of rBoNTA(H_C) formed a degradation product with a shift in mass of +16 *m/z* in the MALDI-TOF mass spectrum (Figure 4). This modification is likely due to the oxidation of one of three susceptible residues in peptide A11 (Tyr and Met). Because Met is generally more susceptible to oxidation than Tyr²⁶⁻²⁷, the observed degradation may be due to Met oxidation. Other examples of oxidation in all three of the rBoNT(H_C) proteins include the single oxidation of rBoNTA(H_C) peptides A5 and A6 (Figure 5 and 6, respectively), single oxidation of rBoNTB(H_C) peptide B20 (Figure 7), and the single oxidation of rBoNTE(H_C) peptide E6 (Figure 8). The oxidation of peptides A5, A6, and B20 are presumably the result of Met oxidation in each case whereas the oxidation of the E6 peptide is most likely due to Tyr modification. In addition, there also appeared to be multiple deamidation products of rBoNTB(H_C) peptide B4, which contains 2 Asn and 1 Gln residue, as evidence by the observed mass shift of +3.1 *m/z*.

For the trivalent rBoNT(H_C) formulations, no single formulation stood out as the most resistant to chemical degradation. Formulation 1 had fewer modifications compared to the other formulations during the incubation at 4°C whereas formulation 2 performed better under the conditions of 30°C. In both incubation studies, it appeared that formulation 3 had the greatest degree of chemical degradation. The location of the chemical modifications detected in the trivalent vaccines for rBoNTA(H_C), rBoNTB(H_C), and rBoNTE(H_C) are shown in Figures 9 to 11.

Of the three rBoNT(H_C) proteins, rBoNTE(H_C) had the fewest number of detected degradation sites compared to the other two proteins in the trivalent vaccine formulation. It is interesting to note that rBoNTE(H_C) degradation appeared to be much less severe in the trivalent vaccine when compared to the rBoNTE(H_C) only vaccine (Tables IV to VII). The cause for this effect is not known.

Similar to the results obtained from the rBoNTE(H_C) formulations, in the trivalent formulations many of the degraded peptides were detected at an earlier time point in the adjuvant-bound formulation compared to the unbound solution control sample. Also, many of the chemical changes that were observed in the adjuvant-bound formulation were not detected in the control sample. Thus, it appears that binding of protein to adjuvant increased susceptibility to chemical degradation. Finally, it is interesting to note that no examples of complete conversion to the modified peptide were observed.

Modifications of Unknown Origin

We also detected new masses during the incubation studies that were problematic in regards to assigning the new mass as a modification to a known peptide. For example, a new *m/z* signal of 2889.6 was found in the rBoNTE(H_C)-only formulation incubated at 30°C at the 9 week time-point (Table V). The closest mass to this new *m/z* is 2849.3 (peptide E2) based on the observed peptides in the LysC peptide map (Table 3). It is unlikely that modifications to peptides E3 (2663.4) or E1 (4449.5) would produce such a large shift in mass to explain the new mass of 2889.6 *m/z*. Thus, we have tentatively assigned this new mass as a modification to E2 though the origin of this potential modification is not clear based on the primary sequence of the peptide (ISSSGNRFNQVVVMNSVGNNCTMNFK). The other

masses of unknown origin, with their speculative assignment to respective nearest peptides, are also listed in the chemical degradation tables (Table IV to VII).

Causes of Enhanced Chemical Degradation for Adjuvant-Bound Proteins

Binding of protein to adjuvant may result in structural perturbations that facilitate chemical degradation. Investigation into the impact of binding of three model proteins to adjuvant showed a substantial loss in native protein structure upon binding²¹. In our companion paper¹², structural perturbations were observed upon binding of the three rBoNT(H_C) proteins to the adjuvant. A change in conformation, specifically partial unfolding, might not only result in the exposure of previously buried residues to the solvent (including water molecules as well as molecular oxygen and reactive oxygen species) but also might result in a greater extent of conformational flexibility, which could increase chemical reactivity due to more energetically favorable spatial arrangements.

Additionally, the surface properties of the adjuvant itself may influence protein degradation. Wittayanukuluk et al.²² found that the acid-catalyzed hydrolysis of adjuvant-bound glucose-1-phosphate was substantially reduced when compared to hydrolysis of the enzyme in bulk solution. This difference was attributed to an increase in the microenvironment pH (of up to 2 units) at the surface of the adjuvant, a result of the attraction of anions to form a Stern layer²² around the positively-charged adjuvant particle. The deamidation of Asn residues, which was observed in the current study, is highly dependent on pH with regards to both reaction kinetics as well as reaction mechanism¹⁵. Differences between the bulk and surface pH, as well as charge heterogeneity of the adjuvant surface, could result in a range of pH microenvironments to which the adjuvant-bound proteins are exposed and that could contribute the differences in chemical degradation observed for bound vs. unbound formulations. This may explain the apparent reduction in the chemical degradation of rBoNTE(H_C) when bound to adjuvant compared to the solution control under both storage conditions for the buffer only formulation (Table IV and Table V).

Potential Impact of Chemical Degradation on Vaccine Efficacy and Potency

The dramatic impact of botulinum proteins on neurological signaling is a concerted effect of functionally distinct domains of the molecule. Each botulinum protein is composed of a light chain (~50 kDa) linked via a disulfide bond to a heavy chain (~100 kDa), which is further divided into an N-terminal and C-terminal domains. Two binding sites, located in the C-terminal of the heavy chain, allow the botulinum proteins to first dock onto the surface of the cell²⁸. The N-terminal domain of the heavy chain then facilitates the translocation of the protein into the cytosol by receptor-mediated endocytosis. Finally, the zinc-metalloprotease of the light chain catalyzes the cleavage of cytosolic proteins to block the release of the neurotransmitter acetylcholine and eventually induce flaccid paralysis²⁹.

In the current study, the three antigens of interest are C-terminal domains of the respective heavy chains and therefore contain synaptotagmin and ganglioside binding sites. Blocking or disrupting the interaction between the botulinum proteins and their binding partners on the cell surface may prove to be advantageous in the development of vaccines against the neurotoxin. Synthetic peptides derived from the C-terminal domain of the BoNTA heavy chain produced antibodies in two mice strains, and elicited responses during *in vitro* toxin challenge³⁰. A comparison of the degradation-prone BoNTA peptides found herein with the dominant epitope peptides defined by Rosenberg *et al.*³⁰ revealed a region of overlap containing Trp⁸⁴, which may oxidize during storage under real-time and/or accelerated storage conditions (Peptide A6, Tables VI and VII). However, as peptide A6 also contains two Tyr residues as well as a Met residue, the observed shift in *m/z* cannot be distinctly attributed to oxidation of the Trp residue found to be part of the antibody epitope. In a

separate study, neutralizing antibodies against the BoNTE serotype have been identified to interact specifically with the C-terminal domain of the heavy chain³¹. The epitope sequences determined by Kubota *et al.*³¹ did not overlap with any of the susceptible BoNTE heavy chain residues found in the current study. Collectively, the BoNT residues that were found to have a propensity to degrade do not appear to be associated with any known antibody epitopes and may therefore not result in a negative impact to the potency or efficacy of the vaccine. Animal studies are required to more clearly access the relationship between chemical antigen stability and vaccine performance.

Summary and Conclusions

The goal of the current work was to develop a method to detect the chemical modifications of an adjuvant-bound trivalent botulinum vaccine by mass spectrometry during long-term storage. We utilized a desorption technique to first remove the protein antigens from the adjuvant followed by LysC peptide mapping using MALDI-TOF mass spectrometry. Optimization of the LysC digestion as well as preparative procedures provided conditions in which the three protein antigens were simultaneously digested and analyzed reproducibly. We have demonstrated that not only does such an approach provide high sequence coverage of each of the protein antigens in the trivalent formulations but also provides the sensitivity to detect and monitor chemical changes to the molecules over time, including both deamidation and oxidation. This study demonstrates that the chemical integrity of proteins bound to adjuvant may be studied effectively with peptide mapping and mass spectrometry. We found that all three of the rBoNT(H_C) proteins were susceptible to both oxidation and deamidation reactions, and these reactions were often accelerated in the presence of the adjuvant. Thus, it appears that adjuvant-bound proteins in vaccine formulations may chemically degrade more rapidly than unbound proteins.

Acknowledgments

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Figure 1.

Polysorbate 20 interference in the rBoNTE(H_c) LysC peptide map. Formulations that contained Tween 20 showed a substantial loss in rBoNTE(H_c) sequence coverage (A) specifically in the range of 1000 to 2000 m/z (B). Sequence coverage was improved by utilizing the ZipTip fractionation procedure (C) with detection of rBoNTE(H_c) peptides in the range of 1000 to 2000 m/z . Dominant peptide m/z values assigned to rBoNTE(H_c) are given as well as internal mass standards, which are in italics.

Figure 2. Chemical modification of rBoNTE(Hc) peptide E20. The intact m/z of E6 (LYINGNLIDQK) of 1290.7 (*top panel*) was detected by LysC peptide mapping. A shift of +3.1 m/z was observed during stability testing, which indicates the deamidation of the peptide at three residues (*bottom panel*).

Residue	4 °C	30 °C
1		
2		
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Figure 3.

Sites of rBoNTE(H_c) chemical degradation detected during long-term storage of rBoNTE(H_c) only formulations. rBoNTE(H_c) residues that are susceptible to degradation during long-term storage 4 °C and 30°C are shown in the primary sequence of the protein. Modifications detected at 4 °C are shown in bold and underlined whereas modifications that are detected at 30 °C are indicated with gray shading. In many cases, the same modifications were found at both incubation temperatures. Note that the N-terminal Met residue has been removed.

Figure 4. Chemical modification of rBoNTA(H_C) peptide A11. The intact m/z of A11 (YVDVNNVGIRGYMYLK) of 1904.0 (*top panel*) was detected by LysC peptide mapping. A shift of +16.0 m/z was observed during stability testing, suggestion oxidation (*bottom panel*).

Figure 5. Chemical modification of rBoNTA(H_C) peptide A5. The intact m/z of A5 (NAIVYNSMYENFSTSFWIRIPK) of 2721.3 (*top panel*) was detected by LysC peptide mapping. A shift of +16.0 m/z was observed during stability testing, suggestion oxidation (*bottom panel*).

Figure 6. Chemical modification of rBoNTA(H_C) peptide A6. The intact m/z of A6 (NAIVYNSMYENFSTSF~~W~~IRIPK) of 2680.3 (*top panel*) was detected by LysC peptide mapping. A shift of +16.1 m/z was observed during stability testing, suggestion oxidation (*bottom panel*).

Figure 7. Chemical modification of rBoNTB(Hc) peptide B20. The intact m/z of B20 (LGCNWQFIPK) of 1205.6 (*top panel*) was detected by LysC peptide mapping. A shift of +16.1 m/z was observed during stability testing, suggestion oxidation (*bottom panel*).

Figure 8. Chemical modification of rBoNTE(Hc) peptide E7. The intact m/z of E7 (ELDETEIQTLYSNEPNTNLIK) of 2465.2 (*top panel*) was detected by LysC peptide mapping. A shift of +16.1 m/z was observed during stability testing, suggesting that Tyr oxidation was responsible (*bottom panel*).

Residue	4°C	30°C	Notes
Met			Removed
Asp			
Asn			
Glu			
Gln			
His			
Ile			
Leu			
Lys			
Phe			
Pro			
Ser			
Thr			
Val			

Figure 9.

Sites of rBoNTA(H_c) chemical degradation detected during long-term storage of trivalent rBoNT(H_c) formulations. rBoNTA(H_c) residues that are susceptible to degradation during long-term storage 4°C and 30°C are shown in the primary sequence of the protein.

Modifications detected at 4°C are shown in bold and underlined whereas modifications that are detected at 30°C are indicated with gray shading. In many cases, the same modifications were found at both incubation temperatures. Note that the N-terminal Met residue has been removed.

1	11	21	31	41
ANKYNSEIL	NNIILNLRYK	DNNLIDLSGY	GAKVEVYDGV	ELNDKNQFKL
51	61	71	81	91
TSSANSKIRV	TQNQNIIFNS	VFLDFSVSEW	IRIPKYKNDG	IQNYIHNEYT
101	111	121	131	141
IINCMKNNSG	WKISIRGNRI	IWTLIDINGK	TKSVFFEYNI	REDISEYINR
151	161	171	181	191
WFFVTITNNL	NNAKIYINGK	LESNTDIKDI	REVIANGEII	FKLDGDIDRT
201	211	221	231	241
QFI <u>WM</u> KYFSI	FNTELSQSNI	EERYKIQSYS	EYLKDFWGNP	LMYNKE <u>YYMF</u>
251	261	271	281	291
NAGNKNSYIK	LKKDSPVGEI	LTRSKYNQNS	KYINYRDLYI	GEKFIIRRKS
301	311	321	331	341
NSQSINDDIV	RKED <u>YIYL</u> DF	<u>FNLNQEW</u> RVY	<u>TY</u> KYFKKEE	KLFLAPISDS
351	361	371	381	391
DELYNTIQIK	EYDEQPTYSC	QLLFKKDEES	TDEIGLIGIH	RFYESGIVFE
401	411	421	431	
EYKDYFCISK	WYLKEVKRKP	YNLKLGC <u>NW</u>	FIPKDEGWTE	

Figure 10.

Sites of rBoNTB(H_c) chemical degradation detected during long-term storage of trivalent rBoNT(H_c) formulations. rBoNTB(H_c) residues that are susceptible to degradation during long-term storage 4°C and 30°C are shown in the primary sequence of the protein. Modifications detected at 4°C are shown in bold and underlined whereas modifications that are detected at 30°C are indicated with gray shading. In many cases, the same modifications were found at both incubation temperatures. Note that the N-terminal Met residue has been removed.

Figure 11.

Sites of rBoNTE(H_c) chemical degradation detected during long-term storage of trivalent rBoNT(H_c) formulations. rBoNTE(H_c) residues that are susceptible to degradation during long-term storage 4°C and 30°C are shown in the primary sequence of the protein. Modifications detected at 4°C are shown in bold and underlined whereas modifications that are detected at 30°C are indicated with gray shading. In many cases, the same modifications were found at both incubation temperatures. Note that the N-terminal Met residue has been removed.

TABLE I

LysC Peptide Map of rBoNTA(H_C)

Peptide ID	Theoretical [M+H] ⁺	Observed [M+H] ⁺	Position	Primary Sequence
A1	4237.1	(4238.7) ^a	399-434	LVASNWYNRQIERSSRTLGCSEWIFVDDGWGERPL
A2	3417.7	3419.7	139-166	YSQMINISDYINRWIFVTITNNRLNNSK
A3	3047.6	3049.2	10-35	NIINTSILNLRYESNHLIDLSRYASK
A4	2854.3	2856.0	90-113	YFNSISLNNEYTIINCMENNSGWK
A5	2721.3	2724.4	375-398	MNLQDNNGNDIGFIGFHQFNIAK
A6	2680.3	2680.6	68-89	NAIVYNSMYENFSTSFWRIPK
A7	2415.2	2415.2	276-297	GPRGSVMTTNIYLNSSLYRGTK
A8	2250.2	2250.2	114-132	VSLNYGEIHWTLQDTQEIK
A9	2125.2	2125.2	343-362	ILSALEIPDVGNLSQVVVMK
A10	2030.1	2030.1	309-325	DNIVRNNDRVYINVVVK
A11	1904.0	1904.0	260-275	YVDVNNVGIRGYMYLK
A12	1870.0	1871.0	178-194	PISNLGNIHASNNIMFK
A13	1775.9	1775.9	195-208	LDGCRDTHRYIWIK
A14	1636.8	1636.8	328-342	EYRLATNASQAGVEK
A15	1530.7	1530.7	248-259	PYYMLNLYDPNK
A16	1466.7	1466.8	224-236	DLYDNQNSGILK
A17	1449.6	1449.7	237-247	DFWGDYLYQYDK
A18	1420.7	1420.8	50-61	NQIQLFNLESSK
A19	1332.8	1333.8	167-177	IYINGRLIDQK
A20	988.5	nd ^b	2-9	STFTEYIK
A21	947.5	nd ^b	42-49	VNFDPIDK
A22	946.5	946.4	209-215	YFNLFDK
A23	889.4	(889.5)	365-372	NDQGITNK
A24	776.5	776.4	133-138	QRVVFK
A25	714.5	714.2	62-67	IEVILK
A26	639.3	nd	303-308	YASGNK
A27	632.3	nd	216-220	ELNEK
A28	631.4	(631.4)	36-41	INIGSK
A29	520.3	(520.3)	298-301	FIK

Theoretical and observed LysC digestion peptides of rBoNTA(H_C) protein. Samples were eluted from C₁₈ ZipTip with 90% ACN. Peptide masses with *m/z* values less than 500 are not considered due to matrix interference in the mass spectrum. Singly charged species [M+H]⁺ are assumed. The theoretical values represent average masses. Note that the N-terminal Met residue has been removed.

^a *m/z* values in italics and parenthesis represent peptides that were not detected when the one-step 90% ACN elution was used but were detected by the fractionation procedure.

^b “nd” indicates that the peptide was not detected in the mass spectrum when either the one-step 90% ACN elution or fractionation was used.

TABLE II

LysC Peptide Map of rBoNTB(H_C)

Peptide ID	Theoretical [M+H] ⁺	Observed [M+H] ⁺	Position	Sequence
B1	3957.0	3960.7	133-164	SVFFRYNIREDISEYINRWFFVTITNNLNAK
B2	3381.8	(3381.8) ^b	58-85	IRVTQNQNIIFNSVFLDFSWSWIRPK
B3	3175.5	3175.4	377-403	DEESTDEIGLIGHRFESGIVFEEYK
B4	2819.3	2819.4	313-333	EDYIYLDFFNLNQEWRVYTYK
B5	2368.1	2368.1	207-225	YFSIFNTELSQSNIERYK
B6	2283.1	2283.1	88-106	NDGIQNYIHNEYTIINCMK
B7	2180.1	2180.2	342-360	LFLAPISDSELYNTIQIK
B8	2082.2	2082.2	113-130	ISIRGNRIIWTIDUNGK
B9	1980.1	1980.1	5-20	YNESILNNIILNRYK
B10	1863.8	1863.9	361-375	EYDEQPTYSCQLLFK
B11	1737.9	1737.9	193-206	LDGDIFRTQFIWMK
B12	1616.9	1616.9	179-192	DIREVIANGEIIFK
B13	1546.8	1546.8	282-293	YINYRDLYIGEK
B14	1475.7	(1475.7)	300-312	SNSQSINDDIVRK
B15	1384.6	1384.6	235-245	DFWGNPLYNK
B16	1379.7	1379.7	21-33	DNNLIDLSGYGAK
B17	1379.7	1379.7	34-45	VEVYDGVELNDK
B18	1301.7	1301.7	264-275	DSPVGEILTRSK
B19	1236.5	1236.5	246-255	EYYMFNAGNK
B20	1205.6	1205.6	425-434	LGCNWQFIPK
B21	1130.6	1130.6	226-234	IQSYSEYLK
B22	919.5	919.0	171-178	LESNTDIK
B23	875.4	875.3	404-410	DYFCISK
B24	832.6	832.5	294-299	FIIRRK
B25	807.4	nd ^b	50-57	LTSSANSK
B26	753.4	(753.4)	276-281	YNQNSK
B27	736.3	(736.3)	435-440	DEGWTE
B28	707.4	nd	165-170	IYINGK
B29	705.3	nd	107-112	NNSGWK
B30	634.4	(634.4)	420-424	PYNLK
B31	624.3	nd	256-260	NSYIK
B32	609.3	609.2	411-414	WYLK
B33	536.3	nd	46-49	NQFK
B34	534.2	nd	338-341	EEEE

Theoretical and observed LysC digestion peptides of rBoNTB(H_C) protein. Samples were eluted from C₁₈ ZipTip with 90% ACN. Peptide masses with *m/z* values less than 500 are not considered due to matrix interference in the mass spectrum. Singly charged species [M+H]⁺ are assumed. The theoretical values represent average masses. Note that the N-terminal Met residue has been removed.

^a *m/z* values in italics and parenthesis represent peptides that were not detected when the one-step 90% ACN elution was used but were detected by the fractionation procedure.

^b “nd” indicates that the peptide was not detected in the mass spectrum when either the one-step 90% ACN elution or fractionation was used.

TABLE III

LysC Peptide Map of rBoNTE(H_C)

Peptide ID	Theoretical [M+H] ⁺	Observed [M+H] ⁺	Position	Sequence
E1	4446.9	4449.5	413-449	ADTVVASTWYYTHMRDHTNSNGCFWNFISEEHGWQEK
E2	2847.3	2847.4	374-399	ISSSGNRFNQVVVMNSVGNCTMNFK
E3	2662.5	2663.5	300-323	DSTLSINNIRSTILLANRLYSGIK
E4	2597.2	2597.2	127-148	IVNVNNEYTHINCMRDNNSGWK
E5	2485.3	2487.0	230-249	IVNCSYTRYIGIRYFNIFDK
E6	2464.2	2465.2 ^a	250-270	ELDETEIQTLYSNEPNTNILK
E7	2452.2	n/d	2-23	GESQQELNSMVTDTLNN SIPFK
E8	2407.2	2407.3	149-169	VSLNHNEIHWTLQDNAGINQK
E9	2137.0	2137.9	59-77	YVDTSGYDSNININGDVYK
E10	2028.0	2028.0	92-108	LSEVNISQNDYIYDNK
E11	2000.0	2000.0	111-126	NFSISFWVRIPNYDNK
E12	1897.1	1897.0	213-229	SILNLGNIHVSDNILFK
E13	1873.9	(1873.9) ^b	170-186	LAFNYGNANGISDYINK
E14	1858.0	(1858.0)	326-341	IQRVNNSSTNDNLVRK
E15	1764.9	1765.0	187-201	WIFVTITNDRLGDSK
E16	1692.9	1692.9	354-368	THLFPLYADTATTNK
E17	1433.7	1433.7	271-281	DFWGNLYLLYDK
E18	1397.7	1397.7	342-353	NDQVYINFVASK
E19	1374.7	(1374.7)	400-412	NNNGNNIGLLGFK
E20	1290.7	1290.7	202-212	LYINGNLIDQK
E21	1184.6	1184.6	46-55	SSSVLNMRYK
E22	1159.6	1159.7	291-299	PNNFIDRRK
E23	1154.6	1155.6	282-290	EYYLLNVLK
E24	1098.5	1098.5	83-91	NQFGIYN DK
E25	997.6	997.3	32-39	ILISYFNK
E26	928.4	(928.4)	24-31	LSSYTDDK
E27	622.3	(622.3)	78-82	YPTNK

Theoretical and observed LysC digestion peptides of rBoNTE(H_C) protein. Samples were eluted from C18 ZipTip with 90% ACN. Peptide masses with m/z values less than 500 are not considered due to matrix interference in the mass spectrum. Singly charged species [M+H]⁺ are assumed. The theoretical values represent average masses. Note that the N-terminal Met residue has been removed.

^a BoNTE peptide m/z overlaps with a calibration mass standard.

^b m/z values in italics and parenthesis represent peptides that were not detected when the one-step 90% ACN was used but were detected by the fractionation procedure.

Table IV

rBoNTE(H_c) Chemical Modifications Detected During Incubation at 4 °C

Peptide ID	Intact m/z^a	Modified m/z^b	$\Delta m/z^c$	Peptide Primary Sequence ^d	First Detected
<i>Solution Control</i> (Buffer only; 10 mM succinate, 10 mM sodium phosphate, pH 4.0)					
E2	2847.4	2864.3	33.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	9 wk
E2	2847.4	2864.3	17.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	9 wk
E2	2847.4	2864.3	1.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	9 wk
E4	2597.2	2646.3	49.1	IVNVNNEYTTINCMRDNNNSGWK	9 wk
E4 [*]	2597.2	2623.2	26.0	IVNVNNEYTTINCMRDNNNSGWK	9 wk
E4 [*]	2597.2	2621.6	24.4	IVNVNNEYTTINCMRDNNNSGWK	9 wk
E4	2597.2	2614.2	17.0	IVNVNNEYTTINCMRDNNNSGWK	9 wk
E11	2000.0	2016.0	16.0	NFSISFWVRIPNYDNK	9 wk
E11 [*]	2000.0	2012.9	12.9	NFSISFWVRIPNYDNK	9 wk
E17	1433.7	1451.7	18.0	DFWGNLYLLYDK	9 wk
E22	1159.7	1160.5	0.8	PNNFIDRRK	9 wk ^e
<i>Formulation 1</i> (Buffer with 0.2% Alhydrogel TM)					
E11	2000.0	2016.0	16.0	NFSISFWVRIPNYDNK	9 wk ^e
E20	1290.7	1293.8	3.1	LYINGNLIDQK	2 wk
E22	1159.7	1160.5	0.8	PNNFIDRRK	9 wk ^e
<i>Formulation 2</i> (Buffer with 7.5% trehalose and 0.2% Alhydrogel TM)					
E11 [*]	2000.1	2012.9	12.8	NFSISFWVRIPNYDNK	2 wk
E15	1764.9	1781.0	16.1	WIFVTITNDRGLGDSK	9 wk ^e
E19	1374.7	1379.8	5.1	NNNGNNIGLLGFK	2 wk
E22	1159.7	1160.5	0.8	PNNFIDRRK	9 wk ^e
<i>Formulation 3</i> (Buffer with 7.5% trehalose, 0.01% polysorbate 20, and 0.2% Alhydrogel TM)					
E2	2847.4	2864.3	33.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	2 wk
E2	2847.4	2864.3	17.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	2 wk
E2	2847.4	2864.3	1.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	2 wk
E4	2597.2	2646.3	49.1	IVNVNNEYTTINCMRDNNNSGWK	2 wk
E4 [*]	2597.2	2621.6	24.4	IVNVNNEYTTINCMRDNNNSGWK	2 wk

Peptide ID	Intact m/z^a	Modified m/z^b	$\Delta m/z^c$	Peptide Primary Sequence ^d	First Detected
E4	2597.2	2614.2	17.0	IVNVNNEYTTIINC <u>MRD</u> NNSGWK	2 wk
E8*	2407.3	2415.3	8.0	VSLNHNEIHWTLQDNAGINQK	2 wk
E11	2000.0	2016.0	16.0	NFSISFWVRIPNYD <u>NK</u>	2 wk
E11*	2000.0	2012.9	12.9	NFSISFWVRIPNYD <u>NK</u>	2 wk
E17	1433.7	1451.7	18.0	D <u>FWG</u> NYLLYDK	2 wk
E22	1159.7	1160.5	0.8	PNNFIDRRK	9 wk ^e

^a Intact m/z is given as the average mass of the singly charged rBoNTE(H₂) peptide, [M+H]⁺.

^b The m/z is of the modified peptide, [M+H]⁺.

^c The change in m/z is given as the difference between the intact and modified m/z .

^d Possible sites of modification are bolded and underlined in the primary sequence.

^e Indicates that peptides were detected only in the “extreme desorb” (ED) conditions at the 9 week time point.

* Indicates a new m/z that suggests chemical modification of the peptide shown, though the exact chemical change is not known.

Table V

rBoNTE(H_c) Chemical Modifications Detected During Incubation at 30 °C

Peptide ID	Intact m/z^a	Modified m/z^b	$\Delta m/z^c$	Peptide Primary Sequence ^d	First Detected
<i>Solution Control</i> (Buffer only; 10 mM succinate, 10 mM sodium phosphate, pH 4.0)					
E2*	2847.4	2889.6	42.2	ISSSGNRFNQVVVMNSVGNNCTMNEK	9 wk
E2	2847.4	2864.3	33.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	9 wk
E2	2847.4	2864.3	17.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	9 wk
E2	2847.4	2864.3	1.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	9 wk
E4	2597.2	2646.3	49.1	IVNVNNEYTIINC MRDNNNSGWK	9 wk
E4	2597.2	2623.2	26.0	IVNVNNEYTIINC MRDNNNSGWK	9 wk
E4	2597.2	2621.6	24.4	IVNVNNEYTIINC MRDNNNSGWK	9 wk
E4	2597.2	2614.2	17.0	IVNVNNEYTIINC MRDNNNSGWK	9 wk
E11	2000.0	2016.0	16.0	NFSISFWVRIPNYDNK	9 wk
E17	1433.7	1451.7	18.0	DFWGNLYLYDK	9 wk
E22	1159.7	1160.5	0.8	PNNFIDRRK	9 wk ^e
<i>Formulation 1</i> (Buffer with 0.2% Alhydrogel TM)					
E22	1159.7	1160.5	0.8	PNNFIDRRK	9 wk ^e
<i>Formulation 2</i> (Buffer with 7.5% trehalose and 0.2% Alhydrogel TM)					
E11	2000.0	2016.0	16.0	NFSISFWVRIPNYDNK	9 wk
E11*	2000.0	2012.9	12.9	NFSISFWVRIPNYDNK	2 wk
E22	1159.7	1160.5	0.8	PNNFIDRRK	9 wk ^e
<i>Formulation 3</i> (Buffer with 7.5% trehalose, 0.01% polysorbate 20, and 0.2% Alhydrogel TM)					
E2*	2847.4	2889.6	42.2	ISSSGNRFNQVVVMNSVGNNCTMNEK	9 wk
E2	2847.4	2864.3	33.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	2 wk
E2	2847.4	2864.3	17.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	2 wk
E2	2847.4	2864.3	1.6	ISSSGNRFNQVVVMNSVGNNCTMNEK	2 wk
E4	2597.2	2646.3	49.1	IVNVNNEYTIINC MRDNNNSGWK	2 wk
E11	2000.0	2016.0	16.0	NFSISFWVRIPNYDNK	2 wk
E11*	2000.0	2012.9	12.9	NFSISFWVRIPNYDNK	2 wk
E15	1765.0	1780.9	15.9	WIFVITITNDRLGDSK	2 wk

Peptide ID	Intact m/z^a	Modified m/z^b	$\Delta m/z^c$	Peptide Primary Sequence ^d	First Detected
E17	1433.7	1451.7	18.0	DFW <u>G</u> NYLLYDK	2 wk
E20*	1290.7	1304.7	14.0	LYINGNLIDQK	2 wk
E20	1290.7	1293.8	3.1	LYINGNLIDQK	2 wk
E22	1159.7	1160.5	0.8	PNNFIDRRK	9 wk ^e

^a Intact m/z is given as the average mass of the singly charged rBoNTE(H_C) peptide, [M+H]⁺.

^b The m/z is of the modified peptide, [M+H]⁺.

^c The change in m/z is given as the difference between the intact and modified m/z .

^d Possible sites of modification are bolded and underlined in the primary sequence.

^e Indicates that peptides were detected only in the "extreme desorb" (ED) conditions at the 9 week time point.

* Indicates a new m/z that suggests chemical modification of the peptide shown, though the exact chemical change is not known.

Table VI
rBoNT(H_C) Chemical Modifications in Trivalent Samples Detected During Incubation at 4 °C

Peptide ID	Intact m/z _a	Modified m/z _b	$\Delta m/z$ _c	Peptide Primary Sequence ^d	First Detected
<i>Solution Control</i> (Buffer only: 25 mM succinate, 15 mM sodium phosphate, pH 4)					
B4*	2819.3	2824.3	5.0	EDYIYLDFFNLNQEWVRVYTK	9 wk
A11*	1904.0	1932.1	28.1	YVDVNNVGIRGYMYLK	2 wk
A11	1904.0	1920.0	16.0	YVDVNNVGIRGYMYLK	9 wk
B11	1737.9	1753.9	16.0	LDGDIERTQFIWMK	9 wk
A17	1449.7	1451.7	2.0	DFWGDYLYQYDK	2 wk
B18*	1301.7	1304.7	3.0	DSPVGEILTRSK	2 wk
B19	1236.5	1252.5	16.0	EYMFNAGNK	9 wk
B20	1205.6	1221.6	16.0	LGCNWWQFIPK	2 wk
E26*	928.4	932.4	4.0	LSSYTDDK	2 wk
<i>Formulation 1</i> (Buffer with 0.2% Alhydrogel™)					
A6	2680.6	2696.4	15.8	NAIVYNSMYENESTSFWRIPK	4 wk
E6*	2465.2	2471.4	6.2	ELDETEIQTLYSNEPTNLIK	2 wk
A7*	2415.2	2427.1	11.9	GPRGSVMTTNIYLNSSLYRGTK	2 wk
A9	2125.2	2141.2	16.0	ILSALEIPDVGNLSQVVVMK	9 wk
E18	1397.7	1414.8	17.1	NDQVYINFAASK	2 wk
B18*	1301.7	1317.5	15.8	DSPVGEILTRSK	2 wk
B20	1205.6	1221.6	16.0	LGCNWWQFIPK	2 wk
B26*	753.4	759.4	6.0	YNQNSK	2 wk
<i>Formulation 2</i> (Buffer with 7.5% trehalose and 0.2% Alhydrogel™)					
B4	2819.3	2851.3	32.0	EDYIYLDFFNLNQEWVRVYTK	4 wk
B4	2819.3	2822.4	3.1	EDYIYLDFFNLNQEWVRVYTK	2 wk
A5	2721.4	2744.2	22.8	MNLQDNNGNDIGFHFQFNIAK	4 wk
A5	2721.4	2737.3	15.9	MNLQDNNGNDIGFHFQFNIAK	4 wk
A6	2680.2	2696.4	16.2	NAIVYNSMYENESTSFWRIPK	4 wk
E3	2662.5	2678.5	16.0	DSTLSINNIRSTILLANRLYSGIK	2 wk
A7	2415.2	2431.2	16.0	GPRGSVMTTNIYLNSSLYRGTK	4 wk

Peptide ID	Intact m/z^a	Modified m/z^b	$\Delta m/z^c$	Peptide Primary Sequence ^d	First Detected
A10*	2030.1	2036.1	6.0	DNIVRNNDRVYINVVVK	4 wk
E10*	2028.0	2036.1	8.1	LSEVNISQNDYIYDNK	4 wk
A13	1775.9	1807.9	32.0	LDGCRDTHRYIWIK	2 wk
A17	1449.7	1451.7	2.0	DFWGDYLYQYDK	2 wk
E18	1397.7	1406.6	8.9	NDQVYINFFVASK	4 wk
B18*	1301.7	1304.7	3.0	DSPVGELTRSK	2 wk
B20	1205.6	1221.6	16.0	LGCNWWQFIPK	2 wk
<i>Formulation 3</i> (Buffer with 7.5% trehalose, 0.01% polysorbate 20, and 0.2% Alhydrogel™)					
A5	2721.4	2737.3	15.9	MNLQDNNGNDIGFHFQFNIAK	2 wk
A6	2680.2	2696.4	16.2	NAIVYNSMYENFSTFWIRPK	4 wk
E6*	2465.2	2481.3	16.1	ELDETEIQTLYSNEPTNLIK	2 wk
A7	2415.2	2431.2	16.0	GPRGSVMTTNIXLNSSLYRGTK	2 wk
E16*	1692.9	1731.0	34.1	THLFPLYADTATTNK	2 wk
E16*	1692.9	1701.9	9.0	THLFPLYADTATTNK	2 wk
A14*	1636.8	1666.8	30.0	EYRLATNASQAGVEK	2 wk
A17*	1449.7	1451.7	2.0	DFWGDYLYQYDK	2 wk
E18	1397.7	1414.8	17.1	NDQVYINFFVASK	2 wk
B18*	1301.7	1317.5	15.8	DSPVGELTRSK	2 wk
B18*	1301.7	1304.7	3.0	DSPVGELTRSK	2 wk
B20	1205.6	1221.6	16.0	LGCNWWQFIPK	2 wk
B26*	753.4	759.4	6.0	YNQNSK	2 wk

^aIntact m/z is given as the average mass of the singly charged rBoNTE(H₂C) peptide, [M+H]⁺.

^bThe m/z is of the modified peptide, [M+H]⁺.

^cThe change in m/z is given as the difference between the intact and modified m/z .

^dPossible sites of modification are bolded and underlined in the primary sequence.

* Indicates a new m/z that suggests chemical modification of the peptide shown, though the exact chemical change is not known. Modifications in italics may be attributed to either peptide as indicated.

rBoNT(H_C) Chemical Modifications in Trivalent Samples Detected During Incubation at 30 °C

Table VII

Peptide ID	Intact m/z _a	Modified m/z _b	$\Delta m/z$ _c	Peptide Primary Sequence ^d	First Detected
<i>Solution Control</i> (Buffer only: 25 mM succinate, 15 mM sodium phosphate, pH 4)					
A5	2721.4	2737.3	15.9	NAIVYNSMYENESTSF ^W IRIPK	9 wk
A7	2415.2	2431.3	16.1	GPRGSVM ^T TNIYLNSSL ^Y RGTK	2 wk
A11	1904.0	1920.0	16.0	YVDVNNVGIRGYMYLK	2 wk
B20	1205.6	1221.6	16.0	LGCN ^W QFIPK	2 wk
B19	1236.5	1248.5	12.0	EYYMFNAGNK	9 wk
E26 [*]	928.4	932.4	4.0	LSSYTDDK	2 wk
<i>Formulation 1</i> (Buffer with 0.2% Alhydrogel TM)					
A6	2680.6	2696.4	15.8	NAIVYNSMYENESTSF ^W IRIPK	2 wk
E6	2465.2	2481.3	16.1	ELDETEIQTLXSNPNTN ^L IK	2 wk
A9	2125.2	2141.2	16.0	ILSALEIPDVGNLSQVV ^V MK	4 wk
A14 [*]	1636.8	1666.8	30.0	EYRLATNASQAGVEK	2 wk
E18	1397.7	1414.8	17.1	NDQVY ^N IFVASK	2 wk
B19	1236.5	1252.5	16.0	EYYMFNAGNK	2 wk
B20	1205.6	1221.6	16.0	LGCN ^W QFIPK	2 wk
B26	753.4	759.4	6.0	YNQNSK	2 wk
<i>Formulation 2</i> (Buffer with 7.5% trehalose and 0.2% Alhydrogel TM)					
A3 [*]	3049.2	3063.9	14.7	NIINTSILNRLRYESNHLIDLSRYASK	2 wk
A5	2721.4	2737.3	15.9	MNLQDNNNGNDIGFHQFN ^N IAK	4 wk
A6	2680.6	2696.4	15.8	NAIVYNSMYENESTSF ^W IRIPK	2 wk
A7	2415.2	2431.2	16.0	GPRGSVM ^T TNIYLNSSL ^Y RGTK	2 wk
E16 [*]	1692.9	1731.0	38.1	THLFPLYADTATTNK	2 wk
B20	1205.6	1221.6	16.0	LGCN ^W QFIPK	2 wk
B26 [*]	753.4	759.4	6.0	YNQNSK	2 wk
<i>Formulation 3</i> (Buffer with 7.5% trehalose, 0.01% polysorbate 20, and 0.2% Alhydrogel TM)					
B4	2819.4	2822.4	3.0	EDYTYLDFFN ^L NQEWRYVYTK	2 wk
A5	2721.4	2737.3	15.9	MNLQDNNNGNDIGFHQFN ^N IAK	2 wk

Peptide ID	Intact m/z^a	Modified m/z^b	$\Delta m/z^c$	Peptide Primary Sequence ^d	First Detected
A6	2680.6	2696.4	15.8	NAIVXNSMYENFSTSF W IRIPK	4 wk
E6	2465.2	2481.3	16.1	ELDETEIQTLXSNEPNTNLIK	2 wk
A7	2415.2	2431.2	16.0	GPRGSVM T TNINSLNSSL Y RGTK	2 wk
E16*	1692.9	1731.0	38.1	THLFPLYADTATTNK	2 wk
E16*	1692.9	1701.9	9.0	THLFPLYADTATTNK	2 wk
A14*	1636.8	1666.8	30.0	EYRLATNASQAGVEK	2 wk
E18	1397.7	1414.8	17.1	NDQVY INNFVASK	2 wk
B18*	1301.7	1304.7	3.0	DSPVGEILTRSK	2 wk
B20	1205.6	1221.6	16.0	LGCN W QFIPK	2 wk
B26*	753.4	759.4	6.0	YNQNSK	2 wk

^aIntact m/z is given as the average mass of the singly charged rBoNTE(H₂C) peptide, [M+H]⁺.

^bThe m/z is of the modified peptide, [M+H]⁺.

^cThe change in m/z is given as the difference between the intact and modified m/z .

^dPossible sites of modification are bolded and underlined in the primary sequence.

* Indicates a new m/z that suggests chemical modification of the peptide shown, though the exact chemical change is not known.