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Short Communication Open Access

# Stabilization of *Clostridium botulinum* Toxin Complex Using Chemical Cross-linkers

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## Abstract

*Clostridium botulinum* produces seven antigenically-distinct botulinum neurotoxins (BoNTs) which cause severe neuroparalytic illness botulism in humans and animals. In bacterial cultures, secreted BoNTs are associated with several neurotoxin-associated proteins (NAPs) forming toxin complexes (TCs). NAPs play an important role in the oral toxicity of BoNTs by protecting toxin from degradation and digestion by gastric acid and proteolytic enzymes. TCs are pH sensitive and can easily dissociate to BoNT and NAPs under neutral to basic pH condition. The stabilization of TCs is critically important for studying interaction domains (NAPs and BoNT), and also to develop better antibodies for the use in BoNT detection methods. In this study, cross-linking of TC was performed with three maleimide base sulfhydryl cross-linking reagents (e.g. 1, 4-bis (maleimido) butane (BMB); 1, 11-bismaleimidotriethyl glycol BM $(PEG)_3$, and Tris (2-maleimidoethyl) amine TMEA) to stabilize TC. Based on cross-linking data of TC, BM $(PEG)_3$ was found to be most suitable amongst three tested cross-linking reagents.

**Keywords:** Botulism; Neurotoxin complex; Neurotoxin associated proteins; Chemical cross-linkers

## Abbreviations:

BoNT: Botulinum Neurotoxin; TC: Toxin Complex; NAPs: Neurotoxin Associated Proteins; NTNH: Nontoxic Non-Hemagglutinin; HAs: Hemagglutinins

## Short Communication

Botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* are responsible for the neuroparalytic disease botulism in humans and animals. Regarded as the most potent of all biological toxins, BoNTs are classified as Category A agents by the Centers for Disease Control and Prevention (CDC), and categorized into seven serologically-distinct types A-G. Neurotoxins (NTs) from serotypes A-D, G, and recently discovered new serotype FA mosaic/H are encoded by two gene clusters located in close proximity to each other. Genetic variations in the sequences of these toxin genes are used to characterize *C. botulinum* strains and distinguish serotypes and subtypes [1-4]. Serotype-specific arrangement of BoNTs and neurotoxin associated proteins (NAPs) are known as botulinum progenitor neurotoxin gene clusters that encode for 3-7 proteins which then aggregate to form the toxin complexes (TCs) [4]. All TCs contain a 150 kDa neurotoxin (NT), and a ~ 130 kDa non-toxic non-hemagglutinin (NTNH). Some TCs contain three hemagglutinin (HA) proteins (HA-17, HA-33, and HA-70); other TCs lack these HAs. The production of botulinum TC is known to vary with different serotypes, strains [5]. Size of HA containing TCs varies from ~300 kDa (M-TC), ~500 kDa (L-TC) and ~900 kDa (LL-TC) [6-9]. There is some evidence that higher level of BoNT antibodies are produced by rabbits injected with TC rather than BoNT alone [10]. Antibodies derived from TC are therefore crucial for developing sensitive BoNT detection methods.

Although the functions of NAPs are not fully understood, it is thought these NAPs assist in stabilizing BoNT and protecting it from proteolytic cleavage in the acidic environment of the stomach. NAPs assist in the absorption of BoNT in the stomach and also in the transport of BoNT from the intestinal area into the bloodstream [8,11,12]. Under neutral to basic pH conditions, the TCs can easily dissociate to release BoNT, and NAPs. However, the factors and conditions which influence the dissociation of the TCs and release of BoNTs during *C. botulinum* infections have not yet been adequately described. Previously, a report described the influence of pH changes, protein concentrations, and presence of salts on the dissociation of TCs [13]. TCs have been shown to be 10 to 100- times more toxic than BoNT alone through the oral route [8,11].

Chemical cross-linking of reactive groups is a promising method for understanding the formation, stability, and dissociation of TCs. This method of cross-linking based on reactive groups in native proteins and protein complexes has been in use for more than a decade and, a large number of chemical cross-linking reagents have been developed [14,15]. Broadly, these cross-linkers may be classified in several categories according to their reactivity (e.g. amine-, or thiol-reactive, homo and heterobifunctional) or incorporation of additional functional groups such as cleavable sites, and affinity tags. The selection of appropriate cross-linkers and cross-linking protocol are critical to achieve an optimal cross-linked protein complex.

We used our recently characterized TC from PS-5 strain for chemical cross-linking [16] using three maleimide cross-linkers [e.g. 1, 4-bis (maleimido) butane (BMB); 1,11-bismaleimidotriethyl glycol BM $(PEG)_3$, and Tris (2-maleimidoethyl) amine TMEA] from Thermo Scientific Inc. (Rockford, IL). This project was a part of the

collaborative effort between the Center for Food Safety and Applied Nutrition (US FDA) and the US Department of Homeland Security to generate stable TC as immunological reagents for the evaluation and development of BoNT detection assays.

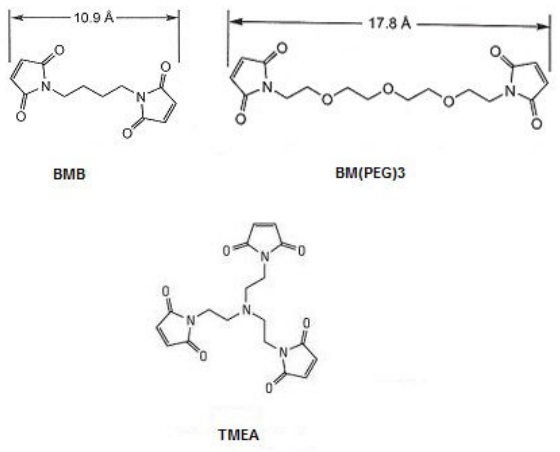


**Figure 1**: Chemical Structure of cross-linkers. 1, 4-bis (maleimido) butane [BMB, M.Wt. 248.23], 1, 11-bis (maleimido) triethyl glycol [BM $(PEG)_3$, M. Wt. 352.13], Tris (2-maleimidoethyl) amine [TMEA, M. Wt. 386.36].

To optimize the cross-linking of TC, we took into consideration several parameters, such as solubility, length and types of spacer arms, reaction conditions, and concentrations of cross-linker and protein complex. Three maleimide base sulfhydryl cross-linkers with bifunctional (e.g. BMB, and BM $(PEG)_3$) and trifunctional (e.g. TMEA) arms varying in length of spacer arm were selected for cross-linking of TC as shown in Figure 1.

The cross-linking reaction was first carried out with BMB and BM $(PEG)_3$ for 2 h and 4 h at room temperature using cross-linker and TC ratio of 2:1 according to the standard protocol supplied by the manufacturer. The cross-linked TC was analyzed on 4-15% SDS-PAGE under denaturing condition. It is clearly evidenced from the SDS-PAGE shown in Figure 2 that TC was comprised of at least 7 bands and the size of smallest band was >15 kDa. There was no change in protein banding pattern of TC with and without cross-linkers (Figure 2A (2 h), 2B (4 h); lane 4, TC with BMB; lane 5, TC with BM $(PEG)_3$).

The cross-linking protocol is designed for cross-linking smaller polypeptides. Given these results, we decided to optimize cross-linking reactions of TC with BMB and BM $(PEG)_3$ and extended the reaction incubation time and changed the reaction temperature from room temperature to 4°C for 16 h. We also increased the ratio of cross linker and TC to 4:1. One large sized band (>250 kDa) is clearly visible as indicated by the arrow with only BM $(PEG)_3$ cross-linked TC as progression of the cross-linking reaction (Figure 2C; lane 5).

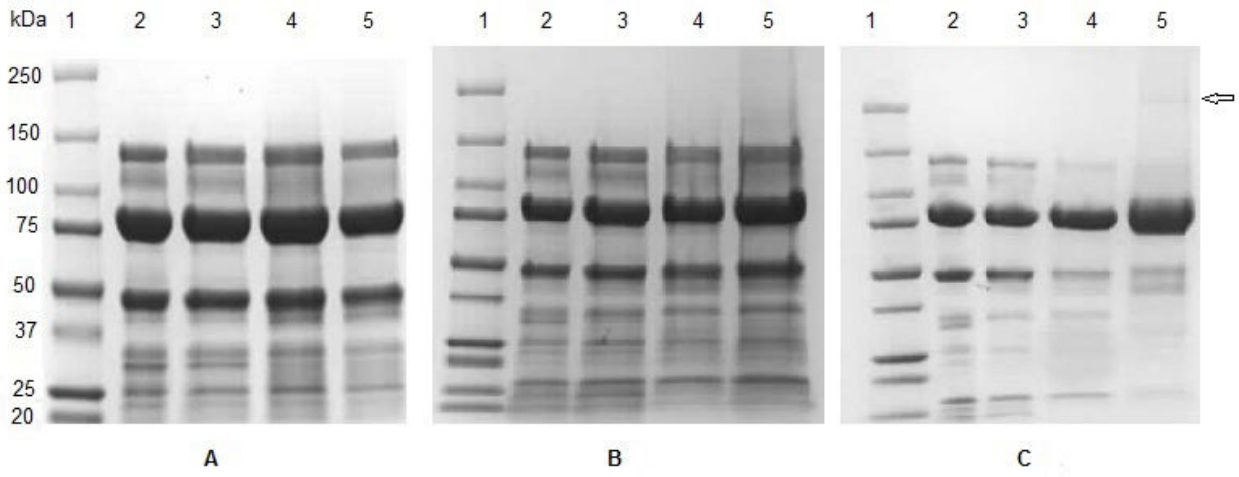


**Figure 2:** 4-15% PAGE analysis of chemically cross-linked toxin complex under denaturing condition. The cross-linking reaction was performed for 2 h (A) 4 h (B), at RT with 2:1 ratio and 16 h (C) at 4°C with 4:1 ratio of either BMB or BM $(PEG)_3$ with toxin complex. Toxin complex proteins were visualized by staining with Coomassie Blue R-250 stain. Standard protein marker 10 (lane 1), unreduced toxin complex (lane 2), reduced toxin complex (lane 3), reduced toxin complex + BMB (lane 4); reduced toxin complex + BM $(PEG)_3$ (lane 5).

Based on the BM $(PEG)_3$ cross-linked TC data, we decided to further increase the cross-linker and TC ratio to 10:1 and performed cross-linking reaction with only BM $(PEG)_3$ for 16 h, 32 h, and 40 h at 4°C. This resulted in a significant change in banding pattern and noticed appearance of >150 kDa protein bands as indicated by arrow in Figure 3A. We also noticed a shift in band patterns of proteins with sizes ranging from 25 -75 kDa (Figure 3A; TC with BM $(PEG)_3$, lane 4. 16 h; lane 5. 32 h; lane 6. 40 h).

The cross-linked TC sample (Figure 3A; lane. 6) with BM $(PEG)_3$ was also checked on a native gel to verify the presence of larger bands. The majority of the cross-linked toxin complex did not migrate from the well because of high molecular weight (Figure 4; lane 5).

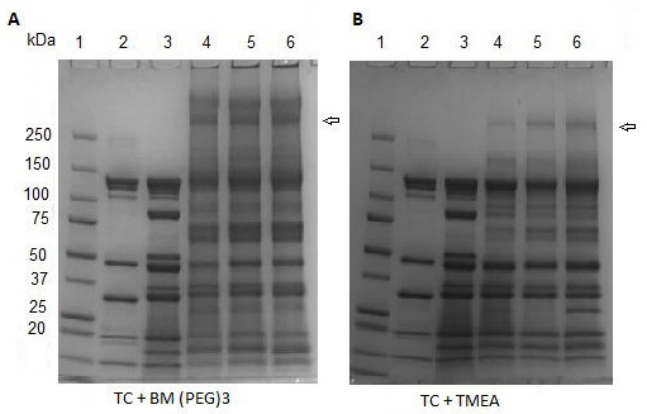


**Figure 3:** 4-15% SDS-PAGE analysis of chemically cross-linked toxin complex under denaturing condition (A & B). The cross-linking reaction was performed at 4°C with 10:1 ratio of cross-linkers and TC. (A) Standard protein marker (lane 1), reduced toxin complex from Hall strain (lane 2), reduced toxin complex from PS5 strain (lane 3), reduced toxin complex from PS5 strain + BM (PEG)3 for 16 h (lane 4), 32 h (lane 5), and 40 h (lane 6). (B) Standard protein marker (lane 1), reduced toxin complex from Hall strain (lane 2), reduced toxin complex from PS5 strain (lane 3), reduced toxin complex from PS5 strain + TMEA for 16 h (lane 4), 32 h (lane 5), and 40 h (lane 6)

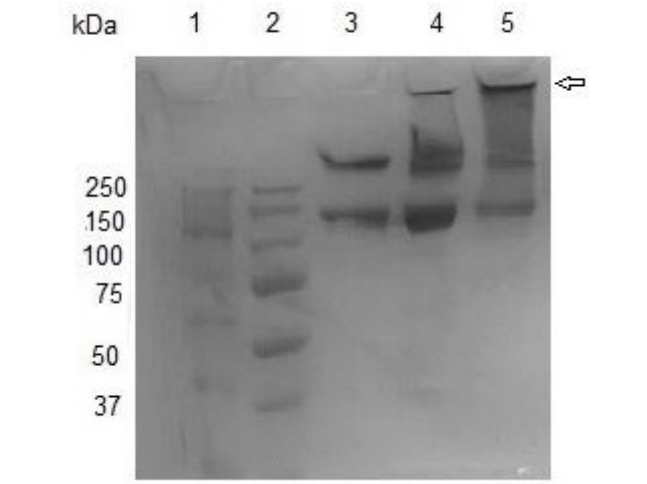


**Figure 4:** 4-15% SDS-PAGE analysis of TC + BM (PEG)3 (Figure 3A, lane 6) under native condition. Toxin complex proteins were visualized by staining with Coomassie Blue R-250 stain. High molecular standard protein marker (lane 1), low molecular standard protein marker (lane 2), reduced toxin complex from Hall strain (lane 3); reduced toxin complex from PS 5 strain (lane 4) reduced toxin complex from PS 5 strain + BM (PEG)3 40 h (panel A; lane 6) was loaded in lane 5.

Attempts to use the same cross-linking conditions and procedures for TC with a trifunctional cross-linker TMEA, and this cross-linker was not promising (Figure 3B; lane 4. 16 h; lane 5. 32 h; lane 6. 40 h) as BM $(PEG)_3$. Structure of cross-linkers were taken into consideration to better understand the differences in cross-linking pattern of TC with BM $(PEG)_3$ and TMEA. The main structural differences between these two cross-linkers are in the length and number of spacer arms (e.g. TMEA (10.3 Å), and BM $(PEG)_3$ (17.8 Å). It is possible that the lower level of cross-linking with TMEA is due to the short distances between the three spacer arms, which might not allow sufficient contact between the cross-linker and the amino acid residues of complex proteins to form covalent bonds. Earlier research has established that the chain length of the spacer arm determines many essential properties of the cross-linking reagents [15]. The longer the spacer arm has greater ability to accommodate two cross-linkable reactive groups within the distance range of the reagent [15]. Based on the above TC cross-linking data, we have developed stable TC which can be utilized for structure-function analysis and antibody production.

In conclusion, the stabilization of TCs is important to study interaction domains of BoNT and NAPs and also to develop better antibodies to use for BoNT detection method. TC was cross-linked using three maleimide based chemical compounds in this study. Amongst three tested maleimide cross-linkers, BM $(PEG)_3$ found only effective with TC, and work is in progress to raise antibodies against BM $(PEG)_3$ cross-linked TC of PS-5 strain of *C. botulinum*.

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