

# High Sensitivity Detection of Active Botulinum Neurotoxin by Glyco-Quantitative Polymerase Chain-Reaction

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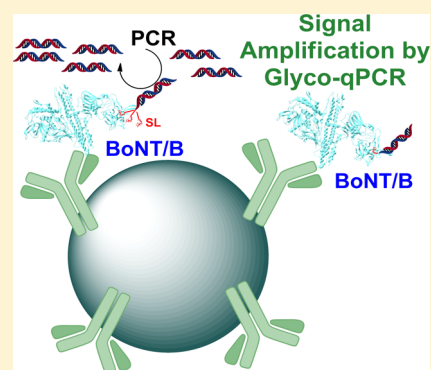
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## S Supporting Information

**ABSTRACT:** The sensitive detection of highly toxic botulinum neurotoxin (BoNT) from *Clostridium botulinum* is of critical importance because it causes human illnesses if foodborne or introduced in wounds and as an iatrogenic substance. Moreover, it has been recently considered a possible biological warfare agent. Over the past decade, significant progress has been made in BoNT detection technologies, including mouse lethality assays, enzyme-linked immunosorbent assays, and endopeptidase assays and by mass spectrometry. Critical assay requirements, including rapid assay, active toxin detection, sensitive and accurate detection, still remain challenging. Here, we present a novel method to detect active BoNTs using a Glyco-quantitative polymerase chain-reaction (qPCR) approach. Sialyllactose, which interacts with the binding-domain of BoNTs, is incorporated into a sialyllactose-DNA conjugate as a binding-probe for active BoNT and recovered through BoNT-immunoprecipitation. Glyco-qPCR analysis of the bound sialyllactose-DNA is then used to detect low attomolar concentrations of BoNT and attomolar to femtomolar concentrations of BoNT in honey, the most common foodborne source of infant botulism.



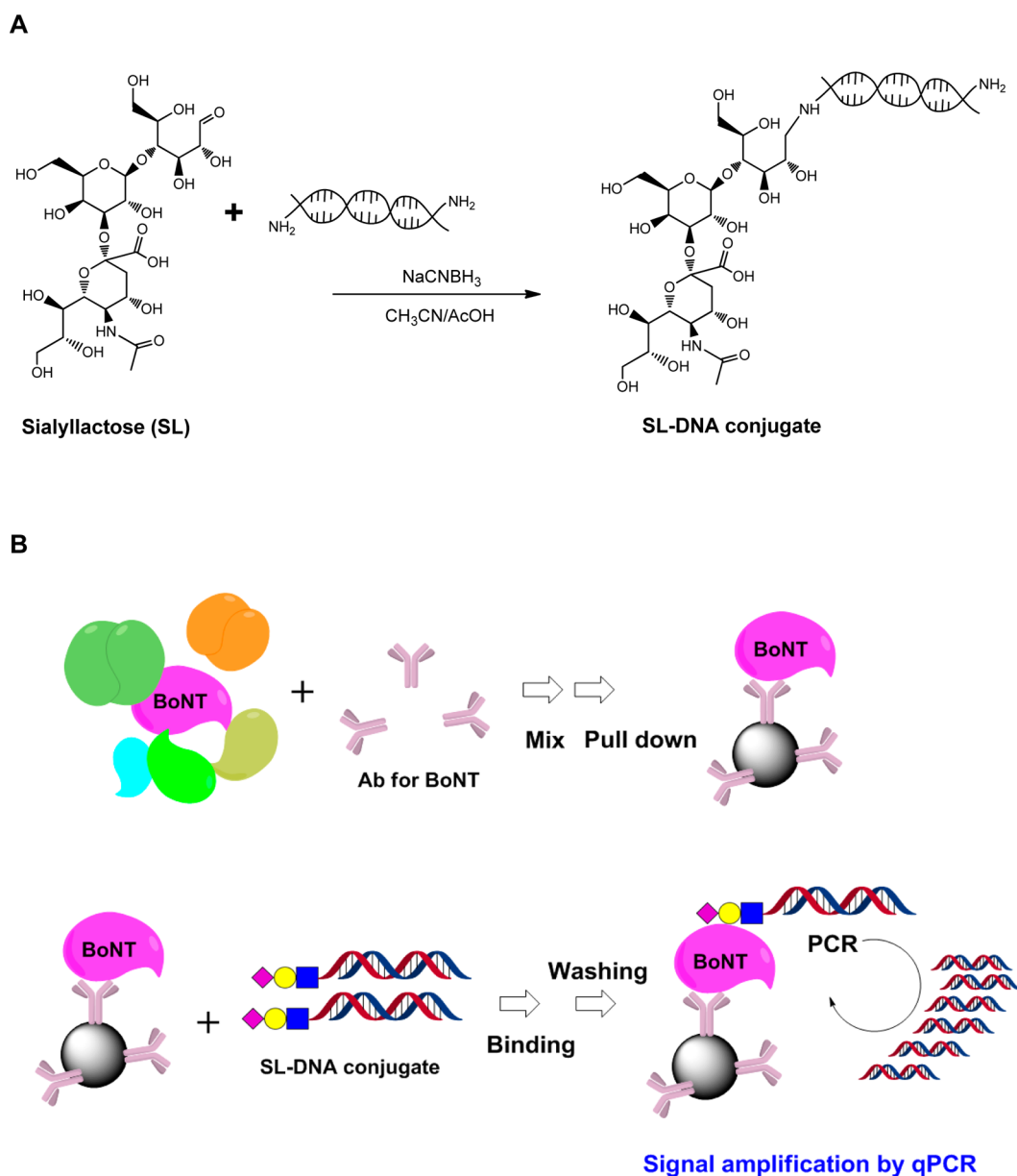
The botulinum neurotoxins (BoNTs), which are the most poisonous toxins known, are produced by the strictly anaerobic gram-positive bacillus, *C. botulinum*, as single inactive polypeptide chains of ~150 kDa. These polypeptides are then cleaved by tissue proteinases into two chains: a heavy (H) chain of ~100 kDa and a light (L) chain of ~50 kDa linked by a single disulfide bond. This active form of these toxins has proteinase and lectin activities and causes botulism, a fatal disease of the nervous system of humans and other mammals.<sup>1</sup> The classic food-borne botulism occurs after ingestion of food containing preformed neurotoxin, produced by *C. botulinum* that contaminate inadequately processed food such as home-canned foodstuffs processed at temperatures below 120 °C.<sup>2</sup> Food-borne botulism in the United States is now uncommon as most of canned foods consumed at home are commercially prepared. Infant botulism has become the most common form of botulism reported in the United States. Between 1976 and 1996, 1442 cases of infant botulism were reported to the Center for Disease Control and Prevention. Infant botulism occurs because infants produce reduced amounts of bile acids, which inhibit the germination of *C. botulinum* spores, within the gastrointestinal tract when compared to the bile acids formed in an adult, and infant intestinal flora is immature.<sup>3</sup> Honey consumption has been associated with a number of cases of infant botulism since 15–25% of honey products harbor botulinum spores (especially type B).<sup>4</sup> Recently, the new

serotype of the toxin-type H, from an infant botulism patient, was by the Arnon group<sup>5,6</sup> and added to the other seven known serotypes, BoNT/A, B, C1, C2, D, E, F, and G. Although this new toxin's DNA sequence will be added to a public database,<sup>7</sup> there is still no effective treatment.<sup>5</sup> BoNTs are also considered high-priority biological agents that are easily produced and could be used in terrorist attacks against food supplies.<sup>8</sup> BoNTs are highly toxic, with estimated inhalation and oral lethal doses in humans of approximately 10 ng/kg and 1 µg/kg body weight, respectively.<sup>9</sup> Ironically, highly toxic BoNTs are used in many human therapeutic and cosmetic applications,<sup>10</sup> and there has been concerns that patients might be treated with higher than recommended doses of unsuitable BoNT preparations by unlicensed practitioners.<sup>11</sup> Thus, development of highly sensitive, simple, and rapid methods to detect active BoNT, not only in foods, but also patient specimens are required for protecting consumers of BoNT-containing foodstuffs, patients from BoNT-containing therapeutics, and civilians and military personnel from bioterrorism. *C. botulinum* cells or spores can be sensitively detected using polymerase chain reaction (PCR), but the detection of BoNT proteins is considerably less

Received: November 15, 2013

Accepted: February 8, 2014

Published: February 8, 2014



**Figure 1.** Scheme for high-sensitivity detection of BoNT with Glyco-qPCR. (A) Coupling of 5'-amine-terminated DNA marker to the reducing end of a 3'-sialyllactose (SL) in the presence of  $\text{NaCNBH}_3$ . (B) Analysis of tiny amounts of BoNT by sequential immunoprecipitation of BoNT, binding of SL-DNA conjugate, washing, and signal amplification by PCR.

sensitive because of the lack of similar molecular amplification tools. The current standard for measuring active BoNT is a live-mouse bioassay, which can detect as little as 5–30 pg of active BoNT.<sup>12</sup> However, the mouse bioassay is expensive to perform, requires many animals, and can take several days to obtain results. Assays that detect the intrinsic endopeptidase activity of BoNT have also been developed for detecting active BoNT.<sup>13</sup> Recently, the Kalkum group developed an assay with a large immunosorbent surface area (ALISSA) that could concentrate the zinc-dependent metalloprotease protease located in 50 kDa L-chain of BoNT and monitor the conversion of fluorogenic peptide substrates by the intrinsic endopeptidase activity of bead-captured BoNT.<sup>14</sup> Using the ALLISSA, attomolar detection of BoNT-A was possible.<sup>14,15</sup> DNA represents an outstanding marker because it is easily amplified using either normal or real time-PCR amplification<sup>16</sup> and offers a potentially

highly sensitive alternative to the use of expensive fluorogenic peptide substrates for signal amplification.

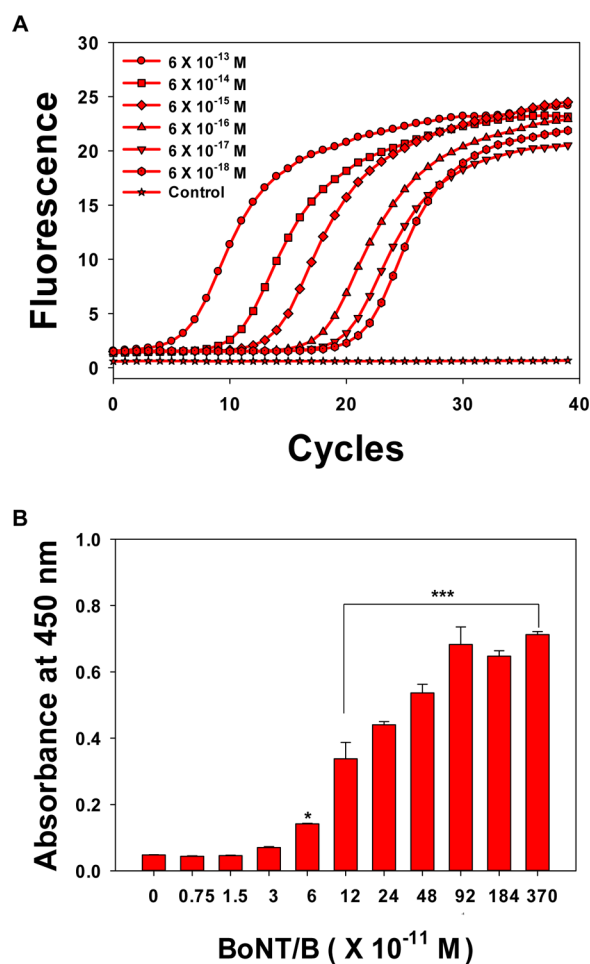
Previously, we reported the use of Glyco-qPCR to detect zmol amounts of carbohydrates.<sup>17</sup> This method can also use carbohydrate conjugates with DNA markers to quantitatively analyze protein–carbohydrate interactions. Here, we demonstrate a novel highly sensitive method for detecting active BoNT/B spiked into honey by combining immunoprecipitation with Glyco-qPCR.

## RESULTS AND DISCUSSION

**Strategy for Detecting Small Amounts of BoNT in Test Samples.** BoNTs are composed of three domains including the L-chain, the translocation domain ( $H_N$ ), and the binding domain ( $H_C$ ). The Zn-protease in L-chain (~50 kDa) specifically cleaves the soluble NSF attachment protein receptor (SNARE) proteins and inhibits acetylcholine release at

the neuromolecular junctions.<sup>18</sup>  $H_N$  is responsible for transport of the L-chain over the endosomal membrane.  $H_C$  binds to receptors on nerve terminals.<sup>19</sup> BoNTs achieve their high affinity and specificity for neurons by binding to disialogangliosides and trisialogangliosides (i.e., GD1a, GT1b, and GD1b). Gangliosides bind in ganglioside-binding site (SXWY motif in  $H_C$ ) conserved in all serotypes except for BoNT/C and D.<sup>20,21</sup> The crystal structure of BoNT/B in complex with SL, a partial mimic of a ganglioside, has been used to model the interactions between the sugar moieties of the ganglioside and its BoNT binding domain.<sup>21</sup> We used the SL-BoNT interaction for detecting active BoNT because SL binds to active BoNT but not to inactive BoNT. In addition, DNA has been used as sensitive barcodes due to the tremendous amplification power of PCR.<sup>16</sup> Thus, we synthesized SL-DNA conjugate as a BoNT detection probe (Figure 1A). The reducing end of the glucose residue in SL was conjugated with 5'-amine-terminated DNA marker using reductive amination,<sup>17</sup> after preparing the 5'-amine-terminated DNA marker with PCR amplification and obtaining a qPCR standard curve for quantifying the amounts of DNA (Supporting Information Figure S1). The conjugates of SL-DNA marker could be used as a detection probe for binding BoNT, and the modification of the glucose residue of SL does not influence the affinity for BoNT.<sup>21–24</sup> The overall schematic for detecting small amounts of BoNT is illustrated in Figure 1B. First, the BoNTs spiked into test sample mixture are recovered using the antibodies against the heavy H-chain,  $H_N$  and  $H_C$  (~100 kDa) of BoNT. The BoNT H domain is captured by antibody since SL interacts with  $H_C$  domain not the L-chain (~50 kDa) of BoNT. Second, the SL-DNA conjugate is bound to the BoNT immobilized on beads in the form of a BoNT-SL-DNA complex. After washing the immobilized BoNT beads 5-times with PBS to remove unbound SL-DNA conjugates as well as residual unreacted DNA (Figure 1B), the washed beads are then used as a template for qPCR. Finally, the DNA marker conjugated with SL was amplified, resulting in increased fluorescence based on the SL-BoNT interaction. This assay approach offers several advantages, including heightened detection sensitivity, the potential for online monitoring, no need for postreaction analyses, and a reduced risk of contamination.

**Detection Limit of BoNT/B.** Deionized water solutions, containing different amounts of BoNT/B (from 600 fM to 6 aM) and a negative control (600 fM of denatured BoNT/B), were prepared using serial dilutions to determine the detection limit for BoNT/B. The samples were bound with excess amounts of BoNT/B specific antibody (BTBH-N1, 10 pM), which interacts with a heavy chain of BoNT/B.<sup>25</sup> At this time, we also performed an SL-inhibition study using ELISA to demonstrate that different epitopes are bound to the BoNT/B antibody interacting with SL (Supporting Information Figure S2). Thus, the binding of antibody (BTBH-N1) did not influence the affinity of SL binding to BoNT/B, which was consistent with our previous results.<sup>25</sup> The BoNT/B-antibody complex was then pulled down using protein A-Sepharose beads. The SL binding site of recovered BoNT allowed SL-DNA conjugates to form BoNT-SL-DNA complex. The complex was then used as a template for qPCR. Glyco-qPCR analysis detected as little as 6 aM concentrations of BoNT ( $6 \times 10^{-18}$  M, Figure 2A). The average cycle threshold (Ct) values obtained by qPCR was determined as a function of the log of the number of BoNT/B molecules per plate well for three replicate Glyco-qPCR measurements on BoNT/B in deionized

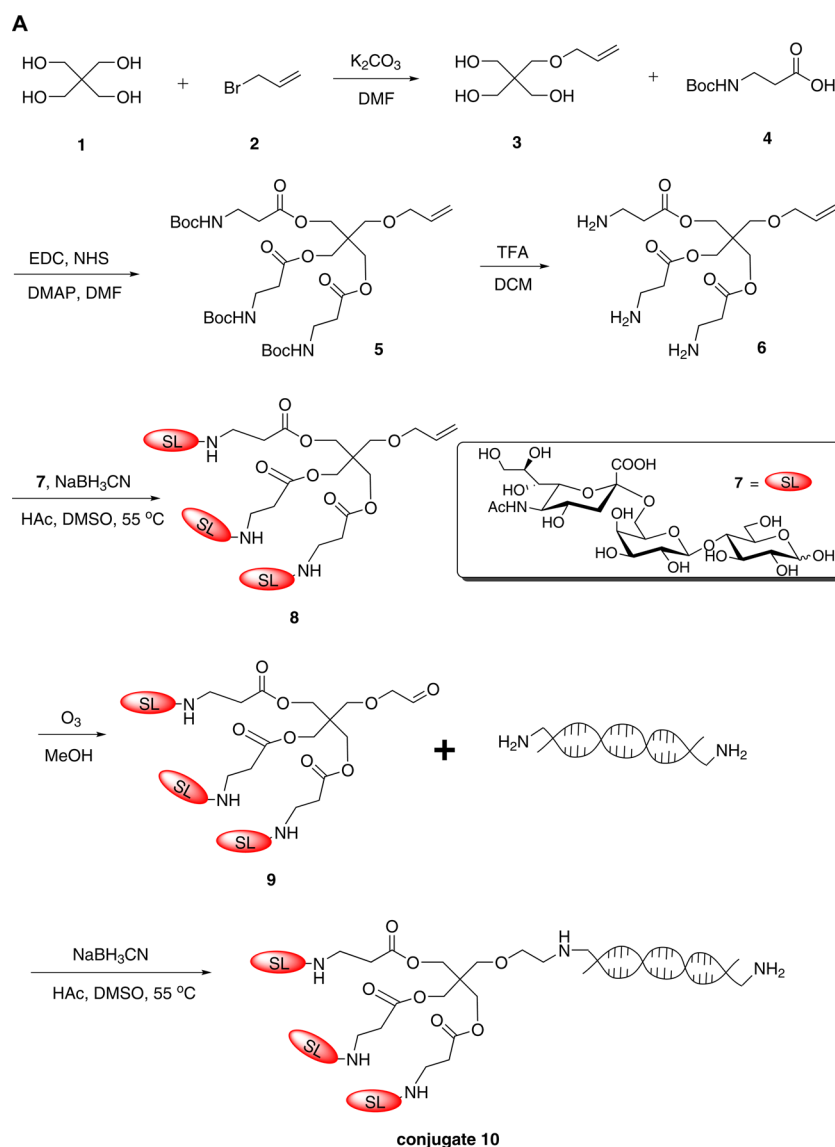


**Figure 2.** Detection limit of BoNT/B using Glyco-qPCR and ELISA assay: (A) Glyco-qPCR amplification plots of BoNT/B-SL-DNA complex. Different amounts of BoNT/B and denatured BoNT/B as a negative control were used to determine detection limitation. All samples and controls were amplified in triplicate. (B) Immunoassay for BoNT/B with sequential binding of BoNT/B-specific monoclonal antibody (BTBH-N1) and HRP-conjugated goat antimouse IgG1. Statistical analysis was performed, and the results were indicated as \* for  $P < 0.05$  and \*\*\* for  $P < 0.001$ . No indication means  $P > 0.05$ . All samples and controls were amplified in triplicate.

water (Supporting Information Figure S3). This is  $10^7$ -fold more sensitive than the 60 pM ( $6 \times 10^{-11}$  M) detection limit of ELISA (Figure 2B). Exceptionally sensitive detection was achieved despite the relatively weak binding affinity between SL and BoNT/B ( $K_D = \sim 0.1 \mu\text{M}$ ).<sup>26</sup> This unexpectedly high sensitivity can be explained based on the following reasons. First, this two-phase assay concentrates BoNT/B ( $\sim 6$  amol) on the surface of beads. We estimate the local concentration of BoNT/B using eq 1.

$$\begin{aligned} & \text{local concentration of BoNT/B} \\ &= \text{no. of beads} \\ & \times \frac{\text{no. of BoNT (6 amol)}}{\text{volume of BoNT bead complex} - \text{volume of bead}} \end{aligned} \quad (1)$$

The volume of BoNT bead complex was estimated from the length of an antibody ( $\sim 8$  nm),<sup>27</sup> the length of BoNT/B-antibody complex ( $\sim 15$  nm),<sup>28</sup> and the average diameter of



**Figure 3.** Detection of different concentrations of BoNT/B in honey. (A) Chemical synthesis of multivalent SL-DNA conjugates. (B) Glyco-qPCR amplification plots of BoNT/B-multivalent SL-DNA complex.

beads ( $50 \mu\text{m}$ ). An elevated local concentration of BoNT/B ( $\sim 0.3 \text{ nM}$ ) results from the colocalized volume ( $\sim 0.1 \text{ nL}$ /bead) and number of beads used ( $\sim 200$ ) in spite of the small amount of BoNT present. Similarly, elevated local concentrations ( $\sim 0.6 \text{ mM}$ ) of both SL-DNA conjugates and antibody

results from their colocalization in this two-phase assay, which promotes the effective binding of small amounts of BoNT/B. However, this elevated local concentration ( $\sim 0.3 \text{ nM}$ ) of BoNT/B is still insufficient to ensure SL binding to BoNT/B, assuming a binding affinity of  $\sim 0.1 \mu\text{M}$ .<sup>26</sup> Thus, we suggest a

second reason for the enhanced binding affinity of SL-DNA conjugate, relative to SL for BoNT/B, resulting from an ionic interaction between DNA and BoNT/B (Supporting Information Figure S4). Numerous positively charged residues (K1225, K1226, K1260, K1264, K1267, R1268, K1269, K1274, P1283, K1284) flank the SL-binding site of BoNT/B. The high negative charge of the DNA in the SL-DNA conjugate contributes to this interaction, enhancing binding affinity. Previous reports<sup>29</sup> show that during the binding of BoNT/B to ganglioside on the surface of presynaptic membrane, this positive charged loop on BoNT/B is closely oriented toward negative charged membrane, supporting this hypothesis. Thus, we suggest that both high local concentration in this two-phase assay and charge interaction between BoNT/B and SL-DNA synergistically contribute to elevate binding affinity, resulting in ultrasensitive detection of BoNT/B. Glyco-qPCR also shows comparable sensitivity to an ultrasensitive liposome-PCR immunoassay for detecting biotoxins that relies on liposomes with encapsulated DNA reporters, and ganglioside receptors embedded in the bilayer, as a detection reagent. In this ultrasensitive liposome-PCR immunoassay, after capturing the target biotoxin by an antibody and cobinding the ganglioside-embedded liposomes, the liposomes are ruptured to release the reporter DNA, which are then quantified by real-time PCR.<sup>24</sup> However, the preparation of ganglioside-embedded liposomes is a very expensive process and a high background signal can occur due to the nonspecific binding of liposomes.<sup>30</sup> In contrast to liposome-PCR immunoassay, Glyco-PCR represents a simpler method for the ultrasensitive detection of BoNT/B.

**Detection of BoNT/B in Honey.** Encouraged by the highly sensitive detection of BoNT/B, we examined the detection of BoNT subtypes (60 fM of BoNT/B) in food using Glyco-PCR. We selected to detect BoNT/B in honey because it represents the major source of infant botulism, resulting from the absorption of heat-labile neurotoxin produced *in situ* by *C. botulinum* colonizing the intestines of infants younger than 1-year of age.<sup>4,31</sup> The critical values (Ct) of qPCR were dependent on the BoNT/B concentration in honey (Supporting Information Figure S5A). The detection sensitivity of 60 fM of BoNT/B (Ct = 17.5) in honey was ~10-fold lower than that (6 fM of BoNT/B; Ct = 17.9) observed in deionized water, based on the Ct plot from deionized water (Supporting Information Figure S3). The average Ct values from multiple replicate Glyco-qPCR measurements of BoNT/B in honey are shown in Supporting Information Figure S5B. These results indicate that 60 fM levels of BoNT could be determined in honey, but that these data were statistical unreliable at 6 fM. In addition, a false positive signal was observed in the negative control (denatured BoNTs). The reduced sensitivity might result from inefficient immunoprecipitation and/or competitive inhibition by the sugars present at high concentrations in honey.

We next synthesized multivalent SL-DNA conjugates (10) starting from pentaerythritol (1) (Figure 3A), since polyvalent interactions can be collectively much stronger than corresponding monovalent interactions.<sup>32</sup> Detailed experimental procedures are described in the Supporting Information and Figures S6–S9. After immunoprecipitating different concentrations of BoNT/B in honey, multivalent SL-DNA conjugates were bound to BoNT/B-antibody complex on beads and washed 5-times for removing the unbound SL-DNA conjugates. The BoNT/B bound multivalent SL-DNA complex was then analyzed by Glyco-qPCR (Figure 3B). Compared to monovalent SL-DNA conjugates, multivalent interaction of SL

improved the affinity for BoNT/B, avoiding false positive signals and increasing the detection sensitivity to 600 aM BoNT/B in honey (Figure 3B).

## CONCLUSION

We demonstrate a simple and inexpensive method for high specificity and attomolar sensitivity detection of active BoNT/B in both PBS and honey through the dual binding of antibodies and monovalent or multivalent SL-DNA conjugates to toxins. Conjugation of specific glycans with DNA marker can be used as detection probes for determining various toxic proteins, including ones with potential use in biological warfare agents. Glyco-qPCR is a particularly general detection method because many toxins, viruses, and pathogens interact with the specific glycans on the surface of cells.<sup>23</sup> In addition, since glycans only interact with active toxic proteins (detection targets), Glyco-qPCR with multivalent SL-DNA conjugates can also reduce false positive signals resulting from denatured target proteins, which are commonly detected by immuno-PCR methods.<sup>33</sup>

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We acknowledge funding from the U.S. National Institutes of Health in the form of Grants GM38060, HL62244, and HL096972 to R.J.L. and ES020903 to J.S.D.

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