Sensors **2007**, *7*, 2808-2824

sensors

ISSN 1424-8220

www.mdpi.org/sensors

Full Research Paper

Antimicrobial Peptides: New Recognition Molecules for Detecting Botulinum Toxins

Nadezhda V. Kulagina, George P. Anderson, Frances S. Ligler, Kara M. Shaffer and Chris Rowe Taitt *

Center for Bio/Molecular Science & Engineering, Code 6900, Naval Research Laboratory, 4555 Overlook Ave. SW, Washington, DC 20375, USA

* Author to whom correspondence should be addressed. E-Mail: chris.taitt@nrl.navy.mil

Received: 24 October 2007 / Accepted: 14 November 2007 / Published: 16 November 2007

Abstract: Many organisms secrete antimicrobial peptides (AMPs) for protection against harmful microbes. The present study describes detection of botulinum neurotoxoids A, B and E using AMPs as recognition elements in an array biosensor. While AMP affinities were similar to those for anti-botulinum antibodies, differences in binding patterns were observed and can potentially be used for identification of toxoid serotype. Furthermore, some AMPs also demonstrated superior detection sensitivity compared to antibodies: toxoid A could be detected at 3.5 LD_{50} of the active toxin in a 75-min assay, whereas toxoids B and E were detected at 14 and 80 LD_{50} for their respective toxins.

Keywords: Antimicrobial peptide, botulinum toxin, detection, array, biosensor

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1. Introduction

Botulism is the clinical term for the neuroparalytic disease caused by one of several protein toxins produced by *Clostridium botulinum*. In spite of its relatively low rate of incidence, foodborne botulism is still considered a public health emergency due to its high mortality rate and the potential for widespread ingestion of contaminated foodstuffs [1]. The mortality rate has decreased to approximately 15% in the last 50 years [2] — primarily due to improvements in supportive and respiratory care and to administration of antitoxin in the early stage of illness [3]. In spite of these

improvements, the high lethality of these toxins has served as an inducement for nefarious activities, as evidenced by state-sponsored programs for their weaponization and two intentional releases of the toxins by the Aum Shinrikyo cult [4].

The neurotoxins produced by *C. botulinum* exist as structurally similar but antigenically distinct serotypes. Each toxin is synthesized as a 150 kDa polypeptide that is activated by proteolysis and selective reduction, yielding a heavy chain (H, 100 kDa) and a light chain (L, 50 kDa) linked by an interchain disulfide. Regions of sequence homology suggest that all serotypes employ similar modes of action in neurotoxicity. The H chains provide cholinergic specificity. The L chains are zinc endopeptidases that cleave the SNARE proteins found in the presynaptic junction of neuronal cells at toxin-specific loci; cleavage of any of these SNARE proteins prevents release of acetylcholine, resulting in blockage of motor nerve terminals and flaccid paralysis. Botulinum neurotoxins are the most potent biological toxins in the world [5, 6]. By extrapolation from primate studies, the lethal amount of neurotoxin A toxin for a 70-kg human would be approximately 0.09-0.15 μg delivered intravenously or intramuscularly, 0.70-0.90 μg delivered by inhalation, and 70 μg taken orally [5].

The only currently approved test for laboratory confirmation of botulism and identification of a source food is the mouse bioassay, which can detect as little as 10 pg of neurotoxin [7, 8]. However, bioassays require a high level of animal use (at least four animals per sample if serotyping is desired), a long time for definitive results (up to 4 days), and shipment of suspect samples to an appropriate testing facility. There are several ELISA-based assays that have been validated for toxin detection in cell cultures with detection limits in the low pg/ml range [9]. Other methods with sensitivities comparable to mouse bioassays have been described, but many are limited by assay complexity, reagent expense, and the requirement for multiple lengthy incubations and/or expensive instrumentation to achieve suitable sensitivities [10-15]. For these reasons, a rapid, sensitive, and easy-to-use test for botulinum toxins requiring only standard laboratory equipment would be highly advantageous for both patient treatment and timely public health response.

With the objective of creating a platform to detect many different organisms *without target-specific reagents*, we have recently evaluated arrays of antimicrobial peptides (AMPs) as alternative recognition molecules; these arrays were capable of detecting and discriminating between multiple bacteria and rickettsiae based on the patterns of binding [16-18]. Many AMPs exert their antimicrobial activity by interacting with invariant components of microbial surfaces and disrupting cell membranes [19-21]. Although current dogma holds that the natural targets of most AMPs are bacteria, fungi, and enveloped viruses, we have determined that some of these peptides also bind to toxins. In this study, we describe an AMP-based assay for inactivated botulinum toxins A, B and E. Several AMPs demonstrated superior detection capabilities when compared to simultaneous, parallel assays on the same instrument using antibodies for target capture. Although binding by AMPs was semi-selective, these assays were able to discriminate between neurotoxoids A and B based on patterns of binding. Kinetic and affinity constants for binding of inactivated botulinum neurotoxins A, B, and E to immobilized AMPs were also determined.

2. Results and Discussion

It is widely held that the natural mechanistic targets for the microbiocidal activity of many AMPs are cellular membranous structures; a large body of work describes the theory and practical aspects of AMP-membrane interactions [19-23]. It was therefore surprising when Garcia and colleagues found that the AMP buforin-I (Table 1) inhibited the protease activity of botulinum neurotoxin B in solution [24]. In large part because of Garcia's original observations and subsequent work [25, 26], we attempted to assess the potential of a number of other AMPs *unrelated to buforins* to detect inactivated botulinum toxins A, B, and E in rapid assays.

Table 1. Amino acid sequences of relevant AMPs and those used in this study.

Polymyxin B	fa-BTBBBFLBBT	fa=fatty acid; B=diaminobutyrate
Polymyxin E	fa-BTBBBLLBBT	fa=fatty acid; B=diaminobutyrate
Polymyxin B nonapeptide	fa-BTBBBFLBBT	B= diaminobutyrate
Buforin-I	AGRGKQGGKVRAKAKTRSSRAGL**QF**PVGRVHRLLRKGNK	
Buforin-II	TRSSRAGL**QF**PVGRVHRLLRK	
Melittin	GIGAVLKVLTTGLPALISWI**KRKR**QQ-$CONH_2$	
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-$CONH_2$	
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL	
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	
Bactenecin	RLC**RI**VVIRVCR	
Magainin-1	GIGKFLHSAGKFGKAFVGEIMKS	
Parasin	KGRGKQGGKVRAKAKTRSS	

Note: amino acid sequences cleaved by peptidase activity of botulinum neurotoxins A, B, and E (QF, KR, RI) are bolded.

As a first step in creating a multiplexed AMP-based screening system, different AMPs (Table 1) were immobilized in arrays on silane-modified microscope slides using direct covalent attachment [17]. Solutions containing inactivated botulinum toxins A and B were incubated with the patterned surfaces and bound toxoids were detected using a fluorophore-labeled antibody directed against toxins A and B (Fig. 1a). For kinetic analyses, toxoids A, B, and E were labeled with fluorescent dye and binding of toxoids to immobilized AMPs was measured directly, in real-time.

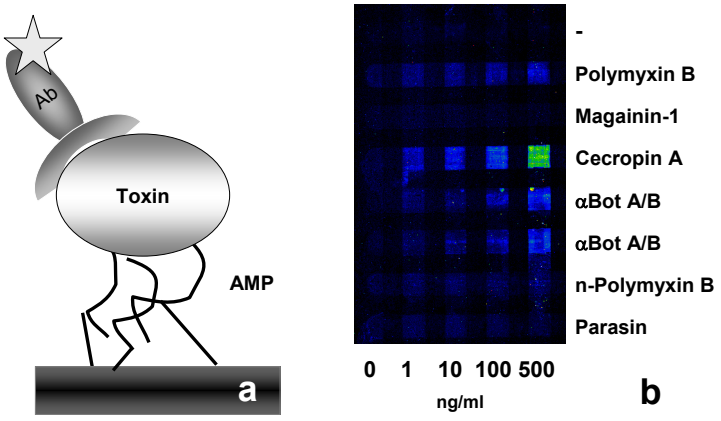

Figure 1. Sandwich assays for botulinum toxoids using immobilized AMPs. Panel a: Schematic of sandwich-format assay for inactivated botulinum toxins. Anti-botulinum toxin antibody (Ab) is shown labeled with a fluorophore (★). Panel b: Representative image of AMP-capture sandwich format assay for inactivated botulinum neurotoxin A. Cecropin A demonstrates high affinity for the toxin with a detection limit below 1 ng/ml; the detection limit for control antibodies (αBot A/B) was 10 ng/ml. No significant binding was observed in the negative control lane (-) or in the buffer blanks.

Fig. 1b shows a representative image of a patterned array of AMPs used to detect botulinum toxoid A in a 75-min "sandwich" format assay using a fluorescent antibody tracer to detect bound toxoid. Dose-response curves were generated for binding of toxoids A and B to the immobilized peptides (Fig. 2). Detection limits were then determined as the lowest concentrations tested with mean net signals ($n \geq 3$ separate array elements) at least 3 standard deviations above the mean of negative controls (buffer blanks) and are shown in Table 2.

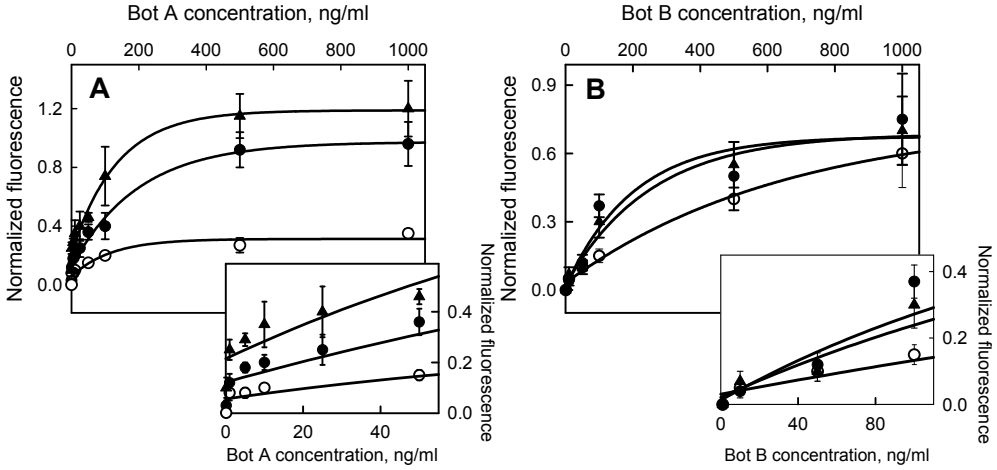

Figure 2. Dose response curves for botulinum toxoids A (a) and B (b) in sandwich format assays. Shown are binding of inactivated toxins to polymyxin B (○), cecropin A (▲), and anti-botulinum toxin A/B (●). Data shown were normalized with respect to chicken positive control signals to account variability between the slides. Error bars represent standard deviations ($n \geq 4$ replicate spots).

Table 2. Detection limits for inactivated toxins in "sandwich" assays.

Immobilized biomolecule	Inactivated botulinum toxin A	Inactivated botulinum toxin A
Anti-botulinum toxin A/B	10 ng/ml	50 ng/ml
Polymyxin B	10 ng/ml	50 ng/ml
Polymyxin B nonapeptide	> 1 μg/ml	n.d.*
Polymyxin E	25 ng/ml	50 ng/ml
Magainin-1	> 1 μg/ml	> 1 μg/ml
Parasin	> 1 μg/ml	> 1 μg/ml
Buforin-II	10 ng/ml	n.d.
Cecropin A	1 ng/ml	50 ng/ml
Cecropin B	5 ng/ml	50 ng/ml
Cecropin P1	> 1 μg/ml	> 1 μg/ml
Melittin	5 ng/ml	10 ng/ml
Bactenecin	5 ng/ml	50 ng/ml

*not determined

In general, detection limits were lower for toxoid A than toxoid B, in large part due to the higher degree of tracer antibody binding to toxoid A (compare antibody lanes for the two serotypes in Fig. 3a). Both toxoids were detectable at LODs that are consistent with previous immunoassay results using the same system [27, 28]. LODs similar to those of the antibody controls were obtained with immobilized polymyxins B and E, which differ by a single amino acid. Interestingly, however, toxoid A did not bind to polymyxin B nonapeptide, which is identical to polymyxin B but lacks the fatty acyl tail. Neither toxoid bound significantly to magainin-1. Sensitivity for botulinum toxoid A detection was significantly enhanced using cecropin A as the immobilized recognition species; the LOD determined for toxoid A binding to cecropin A was 1 ng/ml (3.5 LD_{50}), although samples containing lower concentrations occasionally gave signals above those of negative control ($P < 0.05$) (Fig. 2a). Detection of botulinum toxoid B was most sensitive with immobilized melittin, with an LOD of 10 ng/ml (14 LD_{50}).

Although the buforin-I originally used in Garcia's inhibition studies is no longer commercially available, we were able to test parasin and buforin-II, two peptides analogous to the N- and C-terminal portions of buforin-I, respectively. While buforin-II bound toxoid A, giving a detection limit of 10 ng/ml, parasin failed to bind either of the inactivated toxins at all concentrations tested (up to 1 μg/ml. As previously observed with all other bacteria and viruses tested [16-18], a high surface density was required for optimal binding of both toxoids to immobilized AMPs (Fig. 3a, 3b, additional data not shown), whereas maximal binding to immobilized antibodies was not as greatly affected. The unexpected differences in density effects cannot be not explained by molecular size. Rather, the high surface density of immobilized AMPs may have endowed these surfaces with sufficient avidity to detect bacterial targets at lower concentrations. Furthermore, peptide-peptide interactions have been postulated to be required for strong target binding and microbicidal activity [29-32].

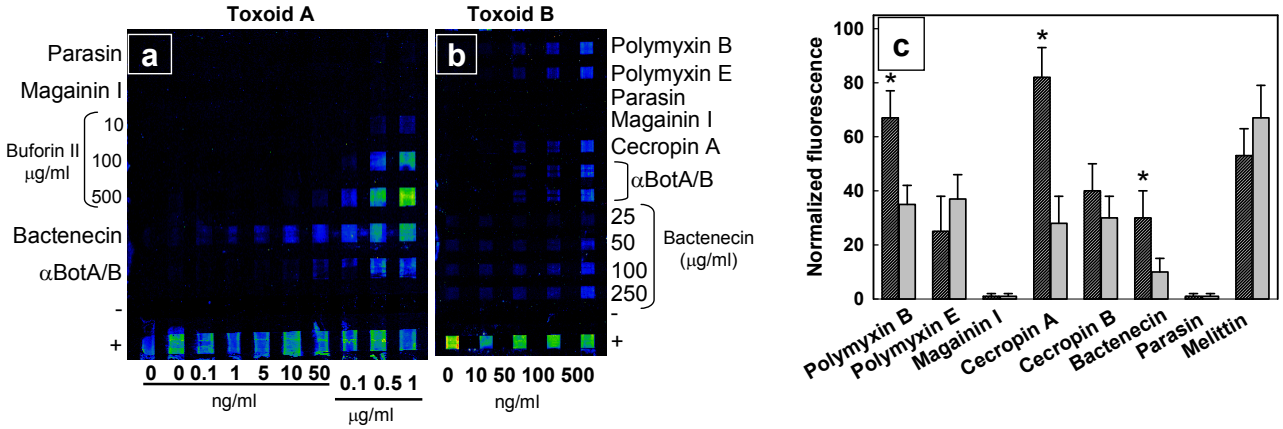

Figure 3. Representative images of AMP-capture sandwich assays for inactivated botulinum toxin A (a) and B (b); note the effect of increasing concentrations of patterned AMPs (buforin II in panel a, bactenecin in panel b). Negative control lanes (-) were patterned with buffer, whereas positive control lanes (+) were patterned with anti-chicken IgY and interrogated with Cy3-chicken IgY. Panel c shows binding patterns of 100 ng/ml inactive botulinum neurotoxins A (striped bars) and B (gray bars) to immobilized AMPs, with fluorescent intensities normalized with respect to chicken positive control signals to account for variability between slides. * indicates a statistical difference ($P < 0.05$). Error bars represent standard deviations ($n \geq 4$ replicate array elements).

In spite of obvious similarities in AMP binding, various AMPs demonstrated some significant differences in binding the inactivated neurotoxins A and B in sandwich assays ($P < 0.05$, analysis of covariance for linear portions of the dose-response curves). For clarity, these differences are shown in Fig. 3c at a single concentration (100 ng/ml) and normalized to the positive control (chicken IgY; * indicates $P < 0.05$, student's t-test). Although binding of target to a single immobilized AMP will not allow unequivocal identification, the patterns of binding to multiple peptides can potentially be used to identify which serotype is present with use of an appropriate pattern recognition algorithm. Although dual-toxin-producing strains have been identified [33, 34], the vast majority of *C. botulinum* strains produce only a single toxin serotype [35], thereby simplifying pattern recognition by minimizing the potential for toxin mixtures.

A series of real-time binding experiments were performed to determine the kinetics of interactions between the inactive toxins and immobilized AMPs. Toxoids A, B and E were labeled with fluorophore and direct format assays were performed (Fig. 4a). Since this format does not require use of a fluorescent tracer antibody, direct monitoring of binding of the labeled toxoids to immobilized AMPs was possible. Furthermore, this format allowed binding of inactive botulinum toxin E to AMPs to be assessed; antibodies with sufficient affinity to this serotype were not available to use as "tracer" antibodies in "sandwich" assays (cf. Fig. 1a). Fig. 4b shows concentration-dependent binding of labeled toxoid E to melittin and cecropins A and B. Direct assay LODs for binding of inactivated botulinum toxin E to melittin, polymyxin B and polymyxin E were 25 ng/ml (75 LD_{50}), and 50 ng/ml (150 LD_{50}) for binding to bactenecin and cecropins A and B. None of the labeled toxoids bound to immobilized magainin-1 or parasin.

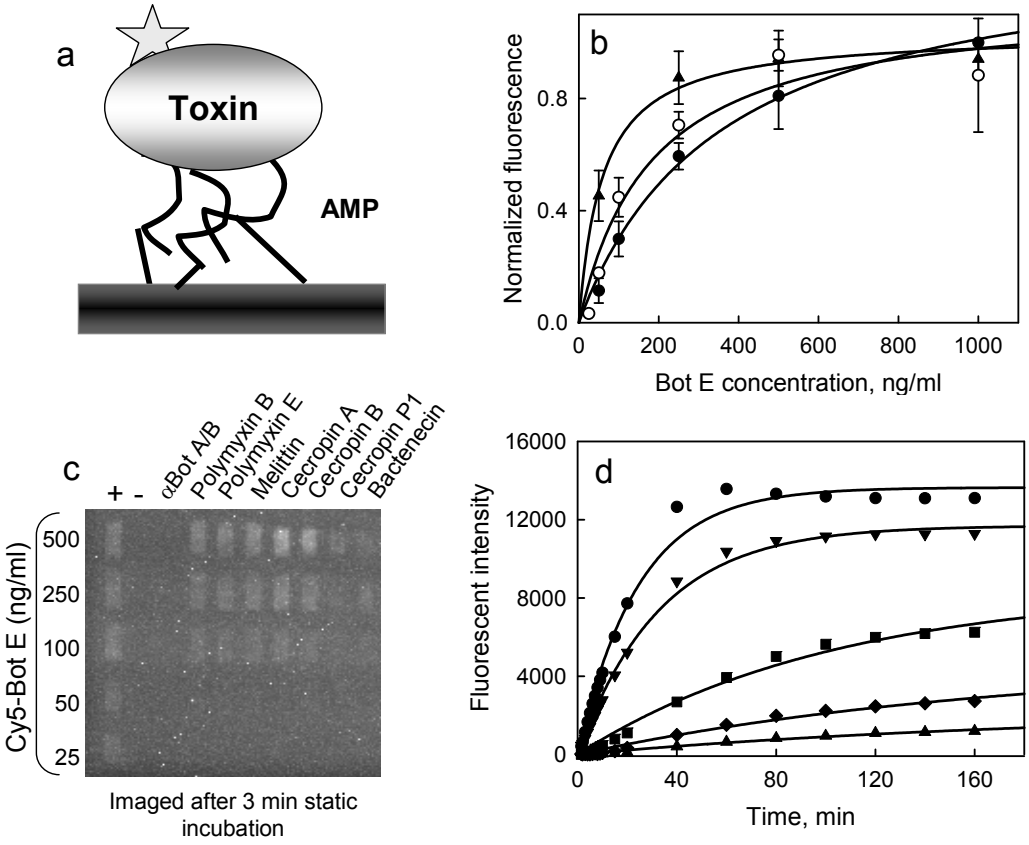

Figure 4. Direct assays for botulinum toxoids using immobilized AMPs. Panel a: Schematic of direct assay used for kinetic determination; the toxin is shown labeled with fluorophore (★). Panel b: Dose-response curve of Cy5-labeled inactivated neurotoxin E binding to immobilized cecropin A (▲), cecropin B (●), and melittin (○). Error bars represent standard deviations ($n \geq 4$ replicate array elements). Panel c: Representative image of labeled inactivated botulinum toxin E binding to an array patterned with multiple AMPs (toxoid concentrations shown to the left of the image). This image was taken 3 minutes after addition of the labeled toxoid and shows that binding of toxin occurs very rapidly. Panel d: Representative timecourse curves of various concentrations of labeled toxoid E binding to cecropin B; data were extracted from images taken at different incubation times (such as that shown in panel c). Shown are 1000 ng / ml (●), 500 ng / ml (▼), 250 ng / ml (■), 100 ng / ml (♦), and 50 ng / ml (▲) toxoid E.

Binding of labeled toxoids A, B, and E to the immobilized AMPs was followed in real-time, a time-dependence curve was generated at each concentration of the toxoids (Figs. 4c, 4d), and the kinetic and dissociation constants determined. The toxoids bound with high affinity to the immobilized AMPs, with nanomolar to sub-nanomolar K_ds (Table 3). In general, differences in kinetic or dissociation constants were not significant ($P > 0.05$). This general trend was primarily due to the large standard deviations observed in both k_{on} and k_{off}, used to calculate the K_ds; coefficients of variation for the kinetic constants ranged from 4 to 68%, due to between-slide variability, but were generally lower than those calculated for the K_ds. The differences observed in sandwich assay results ($P < 0.05$; Figure 3c) presumably reflect variations in the k_{off} rates. The sandwich assays had several wash steps, performed under flow, as well as an additional incubation step; we have previously observed that higher

sensitivities were obtained when samples were incubated static (versus under flow) in both direct and sandwich assay formats (data not shown). This effect has also been observed for other targets binding to AMPs [17].

Table 3. Kinetic and dissociation constants for binding of inactivated botulinum toxins A, B, and E to immobilized AMPs

Capture molecule	Inactivated botulinum toxin A			Inactivated botulinum toxin B			Inactivated botulinum toxin E		
	K_d (nM)	k_{on} ($M^{-1}s^{-1}$) $\times 10^4$	k_{off} (s^{-1}) $\times 10^{-4}$	K_d (nM)	k_{on} ($M^{-1}s^{-1}$) $\times 10^4$	k_{off} (s^{-1}) $\times 10^{-4}$	K_d (nM)	k_{on} ($M^{-1}s^{-1}$) $\times 10^4$	k_{off} (s^{-1}) $\times 10^{-4}$
Antibody	2.6 ± 0.6	8.3 ± 1.6	2.2 ± 0.5	4.8 ± 1.7	2.3 ± 0.6	1.1 ± 0.2	n/a	n/a	n/a
Polymyxin B	7.5 ± 0.4	6.7 ± 0.3	5.0 ± 1.0	3.2 ± 2.0	6.7 ± 3.1	2.2 ± 0.9	12.3 ± 8.1	2.8 ± 1.8	3.5 ± 0.6
Polymyxin E	13.4 ± 6.8	3.1 ± 1.4	4.2 ± 0.5	5.2 ± 3.0	4.1 ± 2.1	2.2 ± 0.6	12.9 ± 8.5	2.8 ± 1.8	3.7 ± 0.7
Cecropin A	nd	nd	nd	7.3 ± 3.4	2.3 ± 1.0	1.7 ± 0.3	0.11 ± 0.02	28.4 ± 5.4	0.32 ± 0.02
Cecropin B	2.5 ± 0.8	11.9 ± 2.3	3.0 ± 0.7	6.5 ± 2.7	3.4 ± 1.0	2.2 ± 0.6	1.9 ± 1.7	6.2 ± 2.1	1.2 ± 0.5
Cecropin P1	6.9 ± 2.5	3.9 ± 1.3	2.7 ± 0.4	16.1 ± 7.6	1.3 ± 0.6	2.2 ± 0.4	18.4 ± 5.0	1.5 ± 0.4	2.7 ± 0.1
Melittin	9.7 ± 5.3	2.8 ± 1.4	2.7 ± 0.5	4.2 ± 1.7	4.4 ± 1.4	1.8 ± 0.5	2.5 ± 1.5	8.0 ± 2.8	2.0 ± 0.9
Bactenecin	3.3 ± 1.2	8.0 ± 2.5	2.7 ± 0.4	5.0 ± 3.4	4.7 ± 2.6	2.3 ± 0.9	6.1 ± 1.6	4.4 ± 1.1	2.7 ± 0.2

n/a - Due to unavailability of antibody against botulinum E toxin, kinetics parameters were not determined for this toxoid.
nd - Cy5-labeled inactivated botulinum A toxin did not bind to immobilized cecropin A.

In the direct assays, several instances of significantly different affinity and kinetic values in the direct assays were noted. A higher k_{on} was observed for toxoid A binding to cecropin B ($P < 0.05$), but this was offset by a slightly higher k_{off}, leading to a K_d similar to those from the other AMPs ($P > 0.05$). A second notable exception was the inability of fluorescently labeled toxoid A to bind to cecropin A, shown to have the highest binding activity in sandwich assays. Since binding to other AMPs did not appear affected, it is possible that the mechanism of this interaction differs from those of the other AMPs. On the other hand, cecropin A had the highest affinity for labeled toxoid E; its K_d (10^{-10} M) was at least ten-fold lower than all others measured and was due to both significantly higher k_{on} and lower k_{off} rates when compared to other AMPs and the other toxins ($P < 0.01$). These K_ds were significantly lower than the IC_{50}s measured for buforin-II and botulinum toxin B in inhibition assays [24, 25], as well as the inhibition or Michaelis constants of other peptide substrate analogs and inhibitors of botulinum neurotoxins [36-40]. However, a number of peptide inhibitors with nanomolar dissociation constants have recently been developed [41].

In their initial 1999 report and subsequent work [24-26], Garcia's group obtained data to support the hypothesis that buforins, previns, and related AMPs inhibit botulinum neurotoxin B by interacting with the proteolytic active site. These interactions were postulated to require an α-helical stretch upstream of the cleavage site, a helix-turn-helix or extended helix motif, and the QF cleavage sequence of its native substrate, VAMP-2 [42]. By analogy, inhibitors of botulinum neurotoxins A and E using a similar inhibitory mechanism would require the appropriate QR/KR and RI cleavage sequences from SNAP-25, respectively [43, 44]. In contrast, the present study demonstrates interaction of inactivated

neurotoxins with a number of AMPs that do not possess the appropriate cleavage sites for the neurotoxins tested (Table 1); cleavage sites are found in only buforin-II (QF), melittin (KR), and bactenecin (RI). More recently, others have demonstrated the importance of a hinge region for binding and inhibition of botulinum neurotoxins by combinatorially-derived peptides [45, 46]. Several of the AMPs that interacted with the toxoids (polymyxins, bactenecin) clearly do not form the requisite extended α-helical or helix-turn-helix motifs. However, the majority of the α-helical AMPs showing high binding to the toxoids possess a helix-breaking proline, while those with minimal binding (magainin-1, parasin) do not.

Exosites have recently been confirmed by crystallographic evidence as sites of substrate interaction remote from the active site [47-49]. These exosites are hypothesized to be responsible for specificity and may require specific chemical and structural motifs (SNARE sequences) for target recognition [50-53]. In spite of the tremendous sequence conservation of SNARE sequences among target proteins, none of the AMPs tested in this study possess the requisite numbers of acidic, hydrophobic and polar residues with the appropriate positioning [50, 53]. Other sites of toxin-ligand interactions have also been described [54]. The preparations used in the current study were derived from neurotoxin complexes, rather than individual purified toxins. The neurotoxin associated proteins (NAPs) within the complexes protect the neurotoxins from proteases, heat, and acidity within the gut [55-57] and may be responsible for mediating interactions with the immobilized AMPs. Although future experiments with purified toxins will indicate whether binding is mediated by the toxin or by associated proteins, it is clear that the naturally secreted, complexed forms interact with AMPs.

While not yet eliminating the need for an immunological component, the AMP-based assays developed here (AMP capture, antibody tracer) demonstrated the potential for simple and rapid (< 75-min) detection of inactivated botulinum neurotoxins; only standard laboratory instruments (peristaltic pump, microarray reader) were required. When using the optimal AMPs for target capture, assays for the toxoids were more sensitive than immunoassays previously developed on the same instrument and those performed in parallel [Table 2; 27, 28].

Although the detection limits were also in the same range as a number of other rapid fluorimetric or colorimetric immunoassays [58-64], they are still not sufficient to detect levels comparable to mouse bioassays (pg/ml levels). The AMP-based assays described in this study were also significantly less sensitive than several recently described assays using hydrolysable peptide substrates; LODs for these latter assays are in the low to sub-pg/ml ranges, with cleavage products detected using capillary electrophoresis, atomic force microscopy, mass spectrometry, or fluorescence [11, 13, 14, 65, 66]. These activity-based assays have the additional advantage of serotype discrimination and determination of toxin functionality (versus immunogenicity). However, a limitation to these substrate-based assays is the potential for peptide degradation by endogenous proteases, which may result in false-positive results if cleavage products are not individually characterized. Although the effect of clinical or food matrices on binding and stability of the immobilized AMPs was not addressed in the present study, temperature, salt, and proteolytic stability of some AMPs has been documented [17, 19, 23, 67].

A number of ELISAs and other antibody-based assays have also been described with detection limits in the low pg/ml range [11, 12, 62, 68-74]. The most sensitive of these combine high affinity antibodies with an extremely sensitive platform, and in many cases, incorporate one or multiple

amplification steps [9, 10, 12, 62, 71, 73, 74]. Several of these also utilize liposomes for signal enhancement, either as scaffolds for fluorophores or as a means of encapsulating signal generating elements [75-78]. A recent report [79] describes immunoPCR using encapsulated PCR templates, with sub-fg/ml detection limits. As the AMPs tested here have similar K_ds to the antibodies used in many of these studies, we anticipate that with suitable modifications, AMP-based assays can be integrated into these alternative platforms, with appropriate improvements in sensitivity. Incorporation of AMPs into tests utilizing liposomes must, however, proceed with great care to avoid interference or disruption of the signal-generating element by the AMP [79, 80].

In conclusion, this is the first demonstration that the same array of semi-selective AMPs used to detect bacterial, fungal, rickettsial, and viral targets can also be used to detect analytes that do not possess membranous structures and are presumably not their natural targets. Similar to the bacterial targets, differences in the patterns of toxoid binding were demonstrated and may potentially be used for target identification. Although the present study required a tracer antibody for sandwich assays, it is the goal of future AMP-based systems to eliminate target-specific elements. If used as a complementary method to immunoassays, AMP-based tests can potentially detect a wider variety of targets, and as demonstrated in the present study, may significantly shorten the time required for results; direct binding was detectable after only 3 min (Fig. 4c); similarly short binding times for toxoids A and B were also observed. Ultimately, studies with native toxins in BL3 facilities will be required to validate the binding interactions observed in this study and to further elucidate the mechanisms involved. If those studies confirm the present work, AMPs show potential for insertion into multi-analyte assay systems as a recognition molecule complementary to antibodies.

3. Experimental Section

Antibodies, antigens and peptides.

The US Naval Research Laboratory is not equipped to handle the highly toxic botulinum toxins and therefore, inactivated *C. botulinum* type A, B, and E complex toxoids were purchased from the US Department of Defense Critical Reagents Program (CRP, Edgewood, MD); these preparations were certified inactive prior to shipment. Specific activities of the toxin complexes used to prepare the toxoids (LD_{50}/ml) were determined by the manufacturer (Metabiologics, Inc., Madison, WI) and ranged from 1.4×10^7 to 3.6×10^7 LD_{50}/mg, depending on the toxoid and lot. Rabbit anti-botulinum toxin A/B antibody was also obtained from CRP and cross-reacts with both the native and the toxoided forms of botulinum toxin; the extreme toxicity of the native toxins precludes their use during immunization, and thus, toxoided forms are the most common immunogens. Polymyxins B and E, polymyxin B nonapeptide, and buforin-II were obtained from Sigma-Aldrich (St. Louis, MO), cecropins A and B from Anaspec (San Jose, CA), and melittin, bactenecin, and parasin from American Peptides (Sunnyvale, CA). Stock solutions of peptides were prepared in phosphate-buffered saline (PBS), pH 7.4, and kept refrigerated until use.

"Tracer" anti-botulinum toxin A/B antibodies were labeled with Cy3 bis-reactive N-hydroxysuccinimidyl ester (GE Healthcare Life Sciences, Piscataway, NJ) as described in detail elsewhere [17]. The dye-to-protein ratios (mole/mole) ranged from 2 to 4.

Preparation of fluorescently-labeled inactivated toxins for kinetic experiments

Inactivated botulinum toxins A, B, and E (500 µL, 1 mg/ml) were incubated for 30 min in 50 mM sodium borate, pH 8.5, with Cy5 bisfunctional N-hydroxysuccinimidyl ester (GE Healthcare) dissolved in 25 µL anhydrous DMSO immediately before use. Following separation from unincorporated dye by gel filtration, labeled toxoids were concentrated using Centricon® centrifugal filter units (Millipore, Billerica, MA) and subsequently incubated overnight with 1 packet of Cy5-maleimide (GE Healthcare). Labeled targets were then separated from free dye as above. Sequential labeling of both amino and thiol moieties allowed dye-to-protein ratios of 1.5 to 2 to be achieved. Labeled toxoids were stored in the dark at 4°C until use.

Preparation and patterning of sensor substrates

Conventional glass microscope slides (Daigger, Wheeling, IL) were patterned with immobilized AMPs and control antibodies using direct covalent coupling chemistry and poly(dimethy)siloxane (PDMS) patterning templates as described previously [17]; patterned slides were blocked with 10 mg/ml gelatin, dried, and stored at 4°C for up to 2 weeks. Negative controls consisted of lanes patterned with buffer only, whereas positive controls were patterned with anti-botulinum toxin A/B antibodies (10 µg/ml). This method results in an array of stripes oriented along the short (25 mm) axis of the slide, with a different recognition molecule immobilized within each striped area. After patterning, slides were blocked with a 1 mg/ml gelatin solution in PBS for at least 40 min and were stored dry at 4°C until use.

Sandwich assays

Binding assays were performed essentially as described for detection of bacterial targets using immobilized AMPs [17]. Briefly, patterned slides were incubated with 0.1 mL toxoid solution for 1 hour, rinsed in PBS containing 1 mg/ml BSA and 0.05% Tween-20 (PBSTB), and then incubated, under flow, with Cy3-labeled tracer antibody (0.4 mL of 10 µg/ml Cy3-antibody in PBSTB, flow rate of 0.3 ml/min). After a final rinse, slides were then imaged with a Packard ScanArray Lite confocal microarray scanner (Packard Biochip Technologies, Billerica, MA). The assays therefore took approximately 75 min and required no specialized equipment except a standard microarray scanner and a multi-channel peristaltic pump.

Fluorescence intensities were extracted from the images using QuantArray microarray analysis software Program (Packard). Concentration-dependence curves were fitted to a 3-parameter exponential rise to a maximum function (Sigma Plot software, Version 8.0, Chicago, IL). Detection limits (LODs) were calculated as lowest concentrations tested with mean net signals ($n \geq 3$ separate array elements) at least 3 standard deviations (SD) above the mean of negative control values.

Binding kinetics assays

Kinetic measurements were made using the NRL Array Biosensor essentially as previously described [81], with the assay unit mounted above an imaging system to measure surface binding events in real time. After an initial wash of each lane with PBS, different concentrations of Cy5-

labeled toxoid (diluted in PBSTB) were injected into each channel and incubated without flow ("static"). Binding of labeled toxoids to AMPs was monitored for 2 h. Digital images (in Flexible Image Transport System format) were collected every min for the first 10 min, at 15 min and 20 min, and finally at 20-min increments for an additional 140 min. Fluorescent intensities were extracted from the images using a custom analysis program written in LabWindows and both specific and non-specific (background) signals were determined [81].

Kinetic constants were determined by fitting the intensity data to the following curve:

$$y = a(1-e^{-(Kapp)x}), \quad (1)$$

where y indicates the intensity measured at time t, and x indicates the time allowed for interaction. For each interaction, the equation was solved for K_{app}, the apparent rate constant. The rate constants were then determined from a plot of K_{app} versus the toxoid concentration (C), where

$$K_{app} = k_{off} + k_{on}C, \quad (2)$$

and the dissociation constant, K_d, was then determined as the ratio of k_{off}/k_{on}.

Acknowledgements

N.V.K. is recipient of a National Research Council fellowship. The authors thank the National Institutes of Health (R01 EB00680) and JSTO-CBD for their financial support. The views expressed herein are those of the authors and do not represent those of the US Navy, the US Department of Defense, or the US government.

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