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PAPER IN FOREFRONT

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Ganglioside–liposome immunoassay for the detection of botulinum toxin

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Abstract A rapid and highly sensitive receptor immunoassay for botulinum toxin (BT) has been developed using ganglioside-incorporated liposomes. Botulism outbreaks are relatively rare, but their results can be very severe, usually leading to death from respiratory failure. To exert their toxicity, the biological toxins must first bind to receptors on the cell surface, and the trisialoganglioside GT1b has been identified as the cell receptor for BT. Therefore, in this study, GT1b was used to prepare the ganglioside–liposomes by spontaneous insertion into the phospholipid bilayer. In a sandwich-based, hybrid receptor immunoassay, BT is detected as a colored band on a nitrocellulose membrane strip, where BT bound to the GT1b-liposomes are captured by anti-BT antibodies immobilized in a band across the strip. The intensity of the colored band can be visually estimated, or measured by densitometry using computer software. The limit of detection (LOD) for BT in the lateral-flow assay system was 15 pg mL^{-1}, which is comparable to the limits of detection achieved with the most sensitive assays previously reported. However, this rapid assay can be completed in less than 20 min. These results demonstrate that the sandwich assay using GT1b-liposomes for detection of BT is rapid and very sensitive, suggesting the possibility for detecting BT in field screening, simply and reliably, without the need for complex instrumentation.

Keywords Botulinum toxin · Ganglioside · Liposomes · Ganglioside–liposomes · Immunoliposomes · Receptor immunoassay

Introduction

Biological toxins, viruses, and hormones must first bind to cell surface receptors in order to act inside the cells. After binding to the receptors, these biologically active molecules penetrate through the cell membrane, usually via endocytosis, and then exert their activity inside the cell. Carbohydrates, existing as glycolipids or glycoproteins on the cell surface, have long been implicated as major receptors for biological toxins [1] and as receptors for hormones and other small molecules. Gangliosides, sialic acid-containing glycosphingolipids, are present in the plasma membranes of most vertebrate cells. The various functions of gangliosides have been studied, including their use as receptors for biological toxins [2, 3]. Since van Heyningen et al. reported that brain gangliosides bound and deactivated cholera toxin [4], the toxin deactivation effect of gangliosides has been studied with other toxins such as botulinum toxin [5] and tetanus toxin [6], which also suggested the function of gangliosides as toxin receptors. Gangliosides contain both hydrophilic and hydrophobic regions and carry a negative charge. The hydrophobic portion, ceramide, consists of a long-chain fatty acid linked to the amino alcohol sphingosine through an amide bond. The hydrophilic carbohydrate moiety is composed of hexoses, *N*-acetylated hexosamines, and at least one sialic acid molecule. In the membrane, the ceramide portion is imbedded in the lipid bilayer, while the hydrophilic oligosaccharide chain is exposed to the outer environment [7]. This structure makes gangliosides well suited as a surface receptor for toxins. The structure of trisialoganglioside GT1b is shown in Fig. 1. As toxin receptors, gangliosides have been used in model membrane systems where the gangliosides were incorporated into liposome bilayers or lipid monolayers. Since gangliosides are natural cell membrane receptors, these ganglioside-incorporated liposomes can be a useful biomimetic model system to study the interaction between biological toxins and cell-surface gangliosides. Improved techniques to prepare ganglioside–liposomes have also been applied to the development of toxin detection assays that take advantage of the strong and specific interactions between toxins and gangliosides [8, 9, 10].

Liposomes, spherical vesicles composed of a phospholipid bilayer surrounding an aqueous cavity, were originally

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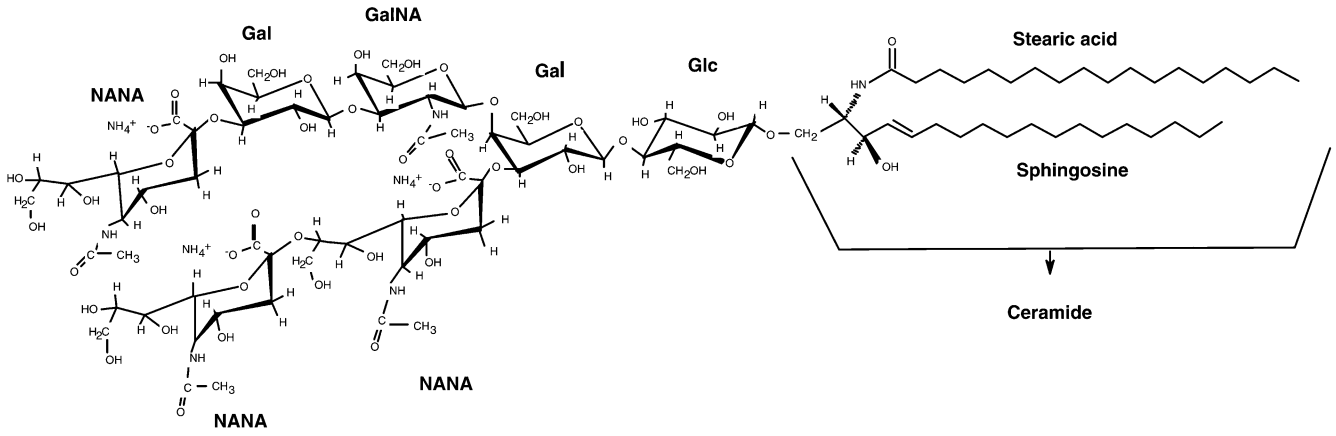

Fig. 1 Structure of the trisialoganglioside GT1b, which is one of the natural receptors for botulinum neurotoxin. Abbreviations: Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NANA, *N*-acetylneuraminic acid (sialic acid)

developed to study cell membranes. However, because of their ability to carry various water-soluble agents in their aqueous cavity, liposomes have been used in clinical diagnostics, drug delivery, and even in the cosmetics and food industries [11]. The use of liposomes in diagnostics has several advantages over enzyme-linked assays. Liposomes have the sites for ligands exposed on their surface and relatively large volumes for containing dye or other markers in their cavity, thus providing greatly enhanced signals. Liposomes utilized in sandwich assay detection systems mostly exist as immunoliposomes with antibodies on their surface, or as nucleic acid-tagged liposomes. Despite having specificity and strong affinity for biological toxins comparable to those of antibodies, gangliosides have not been widely used as receptors in liposome-based assays until recently. Ganglioside-incorporated liposomes have advantages over immunoliposomes because of the amphiphilicity of the gangliosides. Gangliosides contain the hydrophobic ceramide, which can be spontaneously incorporated into a lipid bilayer structure, while antibodies need several chemical steps for covalent conjugation to the liposome structure.

Botulinum neurotoxin (BT) produced by *Clostridium botulinum* is the most toxic substance known: as little as 0.05–0.1 μg is a lethal dose in humans. Patients with botulism show neurological symptoms of flaccid muscular paralysis, with death resulting from respiratory failure if left untreated. In addition, the high probability of bioterrorists using biological toxins as agents of mass destruction, makes these toxins of even more serious concern [12]. Therefore, the development of rapid and sensitive detection methods for BT is urgently needed. At present, the mouse bioassay is the commonly accepted "gold-standard" method for the detection of BT [13]. Although it is highly sensitive, with a detection limit of 10–20 pg mL^{-1}, the mouse bioassay is costly, time-consuming, and requires the use of animals. To date, several detection methods have been developed for BT as alternatives to the mouse bioassay: immunoassays, enzyme activity-based assays, and polymerase chain reaction (PCR)-based assays [14, 15, 16, 17]. In this study, BT was detected using ganglioside–liposomes containing the intensely red sulforhodamine B (SRB) dye as the visual marker, and the trisialoganglioside GT1b receptor for BT was used for the preparation of the liposomes. Anti-BT antibodies were immobilized in narrow zones on plastic-backed nitrocellulose (NC) membrane sheets, which were then cut into test strips. In this sandwich assay system, BT was first bound to the GT1b on the liposomes and these were then captured by the antibodies in the analytical zone during capillary migration through the test strip. The presence of BT was observed as a colored band in the analytical zone on the strip. The intensity of the dye color in the band was measured either by visual estimation or by densitometry utilizing a computer scanner. As described below, in addition to its speed and specificity, the method has very high sensitivity, comparable to ELISA and the mouse bioassay, thereby providing a promising alternative detection approach.

Materials and methods

Materials

Dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylethanolamine (DPPE), and dipalmitoyl phosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). *N*-(κ-maleimidoundecanoyloxy)sulfosuccinimide ester (sulfo-KMUS), *N*-succinimidyl-*S*-acetylthiopropionate (SATA), hydroxylamine hydrochloride, and *N*-ethylmaleimide were purchased from Pierce (Rockford, IL). Trisialoganglioside (GT1b), sulforhodamine B (SRB), cholesterol, *N*-acetylneuraminic acid (NANA), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). To avoid biological hazards, commercialized toxin subunits or toxoids (formaldehyde-inactivated toxins) were used, if available, in this study. Botulinum neurotoxin type A heavy chain, tetanus toxoid, diphtheria toxoid, and *Escherichia coli* heat-stable toxin (STa) were obtained from List Biological Laboratories, Inc. (Campbell, CA). Cholera toxin B subunit was purchased from Sigma Chemical Co. Affinity-purified rabbit polyclonal antibodies to botulinum toxin subtype A were purchased from Biogenesis (Poole, England). Nitrocellulose (NC) membranes with plastic backing (10-μm pore size) were obtained from Millipore (Bedford, MA). Polycarbonate (PC) filter membranes of 0.2-μm pore size came from Whatman International Ltd. (Maidstone, England). The STa used in this study is an intact toxin, so it requires handling precautions. Appropriate laboratory attire should be worn, including a lab coat, gloves, and safety glasses. In case of exposure, the area of the

body that comes into contact with STa should be washed thoroughly. STa can be inactivated by 0.04 mM dithiothreitol or 0.1 M β-mercaptoethanol. STa-contaminated materials can be inactivated by autoclaving at 121°C and 15 psi.

Preparation of GT1b-liposomes

GT1b-liposomes were prepared by the extrusion method, after repetitive freeze–thaw cycles [18], from a mixture of DPPC, DPPG, cholesterol, and GT1b in a molar ratio of 40.3:4.2:40.9:1.3. An 86.7-μmol aliquot of the lipid mixture was completely dissolved in a 100-mL round-bottom flask by swirling in 7 mL of a chloroform/methanol mixture (6:1, v/v). The dissolved lipid mixture was dried by evaporation under vacuum on a rotary evaporator to form a thin lipid film on the flask wall. Four mL of a 150 mM aqueous SRB solution, in 20 mM HEPES buffer (pH 7.5) containing 0.01% sodium azide, were added to the dry lipid mixture. After gentle swirling, 5 cycles of freezing and thawing were performed, by alternating placement of the flask in a dry ice/acetone bath and a 50°C water bath. The hydrated liposomes were extruded through a 0.2-μm pore size PC filter membrane using a mini-extruder (Avanti Polar Lipids, Inc.). The resulting liposomes were gel-filtered through a 1.5×25 cm Sephadex G-50 column to remove unencapsulated dye.

The phospholipid concentration in the resulting liposomes was determined by quantitation of phosphorus using Bartlett's phosphorus assay [19]. The mean diameter of the liposomes was measured by laser-diffraction particle-size analysis with an LS particle-size analyzer (Coulter Scientific Instruments, Hialeah, FL). The ganglioside concentration in the liposomes was quantified by the method of Hikita et al. [20] using NANA as the standard. Liposome concentration, receptor concentration and the number of SRB molecules per liposome were determined as described previously [21].

Preparation of immunoliposomes

Preparation of DPPE-ATA

For conjugation of antibodies to liposomes, DPPE-ATA was prepared from DPPE and a thiolating reagent, SATA (21). DPPE (7.2 μmol) was dissolved in 1 mL of 0.7% (v/v) triethylamine in chloroform. SATA (14.3 μmol) was added to the DPPE solution and sonicated for 1 min under nitrogen gas. The flask containing the mixture was capped and stirred for 20 min at room temperature. Addition of 3 mL of chloroform followed by evaporation was repeated until all traces of triethylamine were completely removed. The final product was dissolved in 1 mL of chloroform.

Preparation of liposomes

Liposomes were prepared by the extrusion method described above. For the immunoliposomes, DPPE-ATA (3.6 μmol) was used in place of GT1b. Hydroxylamine solution (0.5 M hydroxylamine, 25 mM EDTA in 0.1 M HEPES buffer, pH 7.5) was added to the final liposome solution (1:10, v/v) and the mixture was incubated in the dark for 2 h at room temperature.

Modification of antibodies for conjugation

Polyclonal antibodies to botulinum subtype A were dialyzed overnight against PBS (pH 7.4), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.01% sodium azide. Sulfo-KMUS was added to the dialyzed antibodies at a molar ratio of 15:1 (sulfo-KMUS:antibody), and the mixture was incubated with shaking for 3 h at room temperature in the dark. The reaction was stopped by adding 0.5 M Tris (pH 7.8) at 20 times the molar ratio of the sulfo-KMUS used. The reaction mixture was incubated for an additional 15 min at room temperature and then dialyzed in a DispoDialyzer (molecular weight cut-off 15,000, Spectrum Lab. Inc., Rancho Dominguez, CA) against Tris-buffered saline (TBS, pH 7.4) containing 0.01% sodium azide.

Conjugation of maleimide-derivatized antibodies to liposomes

Conjugation was achieved by incubating derivatized antibodies with thiolated liposomes for 3.5 h at room temperature and then overnight at 4°C. The reaction was carried out in the dark under nitrogen gas. After the incubation, the immunoliposomes were treated with 100 mM *N*-ethylmaleimide in PBS (pH 7.4) for 30 min at room temperature to quench the remaining thiol groups and then filtered through a 1.5×17 cm Sepharose CL-4B column, equilibrated with TBS containing 0.01% sodium azide, to remove unconjugated antibodies.

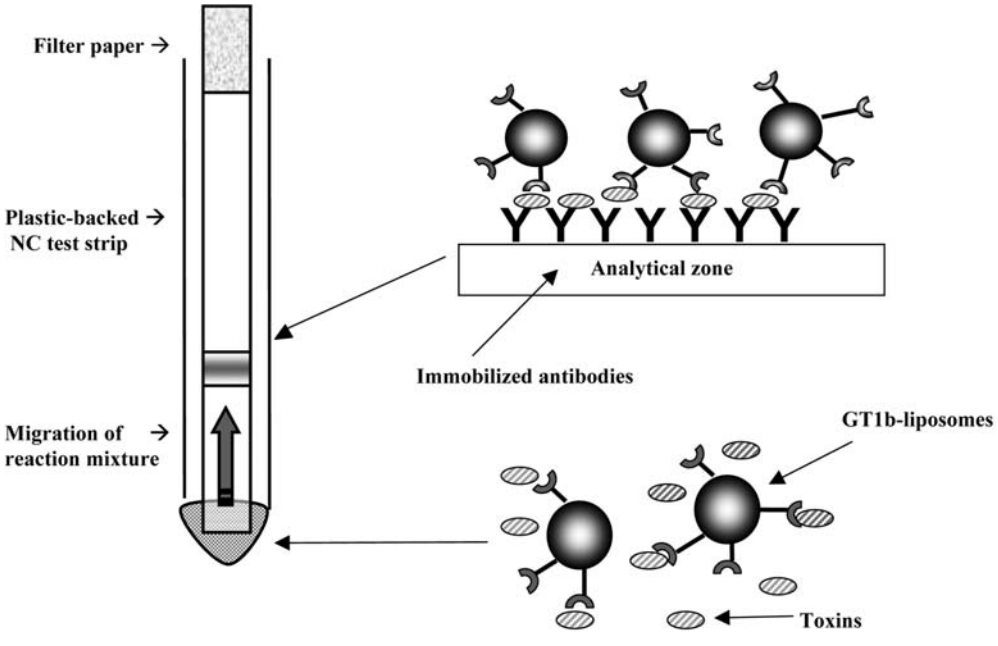

Fig. 2 The test strip assay format. The BT in the reaction mixture binds to the gangliosides on the liposome surface. The BT–GT1b-liposome complex migrates through the nitrocellulose test strip by capillary action until it reaches the analytical zone, where toxins in the complexes are captured by immobilized antibodies. This binding is shown as a *dark band* on the test strip

Preparation of test strips

Test strips were prepared as reported previously [21], with modifications. Plastic-backed NC membrane sheet was cut to the desired size (20×5 cm or 20×8 cm), pre-wetted with 10% (v/v) methanol in phosphate-buffered saline (PBS, pH 7.4) containing 0.01% sodium azide, and dried under vacuum at room temperature. Antibodies to botulinum neurotoxin subtype A (concentration of 1 mg mL^{-1}) in PBS were applied to the analytical zone (approximately 2 cm from one end of the membrane) of the NC membranes using a Linomat IV TLC Sampler (Camag Scientific, Wrightsville Beach, NC). The antibody-immobilized membranes were dried for 1.5 h at room temperature, then incubated in the blocking solution containing 2% polyvinylpyrrolidone (PVP), 0.01% gelatin, 0.002% Tween 20 in PBS, for 1 h with constant shaking, and dried overnight under vacuum at room temperature. After drying, the membranes were cut into test strips (5×50 mm or 5×80 mm) and a filter paper pad was attached to the top of the test strip to provide additional absorbency for the migration process.

Assay formats

The assay was performed by adding 60 µL of GT1b-liposome stock solution, diluted with TBS, to 40 µL of the sample in glass test tubes (10×75 mm). The total volume of the reaction mixture was 100 µL. After the contents were mixed briefly by swirling, the test strip was inserted into the mixture and left in the tube until all of the mixture solution was drawn from the bottom of the test tube. This capillary migration process took approximately 15–20 min. The assay format is depicted in Fig. 2.

Detection and quantitation

The signal color on the test strips can be detected visually. For quantitation of the signal intensity, grayscale densitometry can be used. The test strips were scanned using an Expresstion 636 color image scanner (Epson, Torrance, CA), and the scanned images were converted into grayscale readings. The intensity of each signal was quantified with Scan Analysis densitometry software (Biosoft, Ferguson, MO)

Results and discussion

Preparation and characterization of GT1b-liposomes

Following repetitive freeze–thaw cycles, the GT1b-incorporated liposomes, encapsulating SRB, were prepared by the extrusion method. To decide the optimal amount of GT1b, several batches of liposomes were prepared with different amounts of GT1b in the lipid mixture, and the binding of BT to those liposomes was compared. The highest binding was observed when GT1b was approximately 1–2 mol% of the total lipids in the lipid mixture (data not shown). Higher concentrations of GT1b made the liposomes unstable because of the large carbohydrate moiety. Therefore, to achieve the highest stability, the concentration of GT1b was maintained at less than 2 mol% of total lipids in the lipid mixture. The DMB assay showed that about 40% of the GT1b in the lipid mixture was incorporated into the resulting liposomes.

The characteristics of the liposomes used in these studies are shown in Table 1. The mean diameter of GT1b-liposomes was measured by a particle-size analyzer to be 197 nm. This result seems reasonable because the liposomes were extruded through 0.2-µm pore size PC membrane filter and, without any sonication during preparation, the size of liposomes are only affected by the pore size of the membrane filter used during the extrusion [22]. With the assumption that the thickness of the bilayer is 4 nm [23], the internal volume of a single liposome can be calculated from the diameter. From the diameter of a liposome, the lipid concentration, and the concentration of encapsulated SRB, all other characteristics could be calculated as described previously [21]. The mean diameter, volume, and SRB content of a single GT1b-liposome molecule were comparable to those of liposomes prepared for previous studies of liposomal test strip assay [24]. The number of ganglioside molecules on the liposome surface, 2.1×10^4, was similar to the number of 1.9×10^4, reported by Singh et al. [9]. The stability of the liposomes was determined by measuring the fluorescence of SRB that leaked out of the liposomes during storage, and no significant changes in liposome stability were observed over 9 months of storage at 4°C in the dark. All buffers used in the liposome preparation and the assay were adjusted to have the same osmolarity as the encapsulated SRB, in order to prevent osmotic pressure-related swelling or crenation.

Table 1 Characteristics of GT1b-liposomes

Parameter	Value
Mean diameter (nm)	197±33
Volume (µL)	4.0×10^{-12}
Liposome concentration (liposomes mL^{-1})	5.3×10^{11}
SRB concentration (mM)	150
Number of SRB per liposome (molecules $liposome^{-1}$)	3.2×10^5
Number of GT1b per liposome (molecules $liposome^{-1}$)	2.1×10^4

Development of the test strip assay for BT detection

The test strip assay format used in this study is depicted in Fig. 2. The assay is based on the strong binding between the sample BT and GT1b on the liposome surface, capillary migration on a nitrocellulose strip, and detection of the captured BT–liposome complex in an analytical zone. To minimize the nonspecific binding of GT1b-liposomes to the test strip, the NC membrane was treated with a blocking solution containing PVP. Of the blocking agents tested, PVP, bovine serum albumin (BSA), and gelatin showed consistently low backgrounds (data not shown). However, in the test strips blocked with BSA and gelatin, the reaction mixture appeared to migrate more slowly. Since the objective of this study was to develop a rapid detection method, PVP was chosen as the blocking reagent, with only a small amount (0.01%) of gelatin added. Tween-20 was also added to the blocking solution for uniform migration of liposomes, but its concentration was only 0.002% to avoid the lysis of the liposomes.

In the assay, BT in the sample binds strongly and specifically to GT1b on the liposomes to form BT–GT1b-liposome complexes that can migrate through the NC test

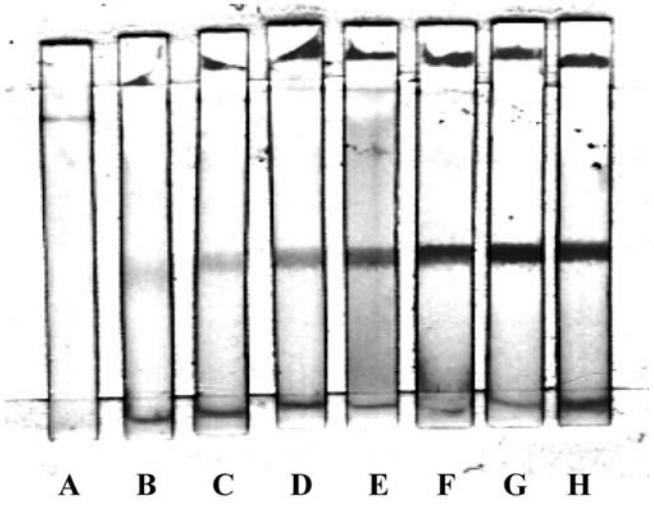

Fig. 3 Scanned images of representative nitrocellulose test strips. Strips were run at room temperature, as described in "Materials and methods". Each strip was inserted into the test tube containing 100 µL of the reaction mixture of GT1b-liposomes and BT at the indicated concentrations: *A* negative control, *B* 1×10^{-5} µg mL^{-1}, *C* 1×10^{-4} µg mL^{-1}, *D* 1×10^{-3} µg mL^{-1}, *E* 1×10^{-2} µg mL^{-1}, *F* 1×10^{-1} µg mL^{-1}, *G* 1 µg mL^{-1}, *H* 10 µg mL^{-1}

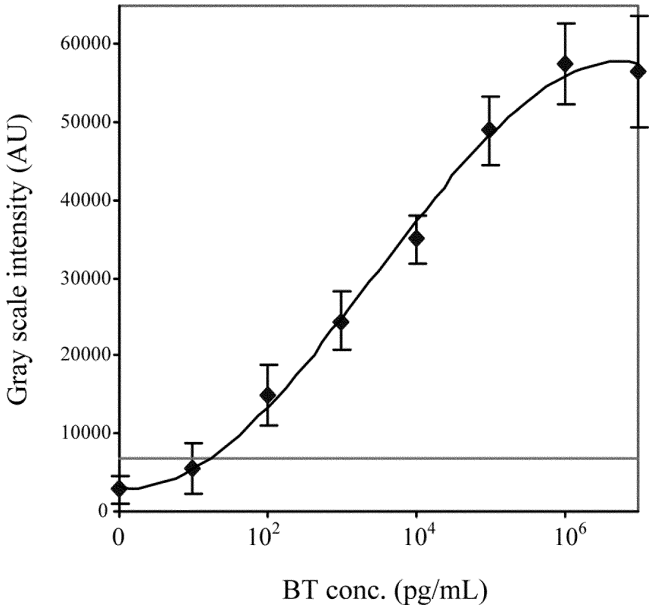

Fig. 4 Dose–response curve for botulinum neurotoxin, generated from test strip assays using GT1b-liposomes. The *solid line* represents the third-order polynomial curve fit, with an R^2 value of 0.996. The *straight horizontal line* indicates the limit of detection, defined as the color intensity 3 times higher than the standard deviation of the background (negative control) signal. Each *point* represents four replicates of grayscale values in the analytical zone

strip by capillary action. These complexes are then captured by immobilized anti-BT antibodies in the analytical zone of the strips and can be observed as a colored band in this zone due to the SRB encapsulated inside the transparent liposomes. The appearance of the actual test strips showing the colored bands due to different concentrations of BT is illustrated in Fig. 3. With various concentrations of BT (0–10 µg mL^{-1}) in buffer, the intensity of the SRB signal in the analytical zone can be visually detected. The intensity of the band is proportional to the amount of toxin in the sample, and the visual detection limit for BT is approximately 100 pg mL^{-1}.

The analytical sensitivity and detection limit of the test strip assay for BT detection was determined from a dose–response curve (Fig. 4). Dose–response data were obtained by scanning densitometry of the test strips, which were run in various concentrations of BT. The limit of detection (LOD) is defined as the lowest concentration of toxin producing a signal intensity 3 times higher than the standard deviation of the intensity of the sample without toxin (i.e., the negative control). By this definition, the instrumental (densitometry) LOD of the current assay for BT is estimated to be 15 pg mL^{-1}, which is about 3 mLD_{50} (mouse 50% lethal dose). The visual detection limit is about a factor of 10 higher. In the dose–response curve for BT, the intensity of the binding signal increases with increasing concentration of BT in the sample, providing a dynamic analytical range between approximately 10^1 and 10^6 pg mL^{-1}, or about 5 orders of magnitude. Several research groups reported that their detection assays for biological toxins, including BT, utilizing ganglioside–liposomes showed high sensitivity [8, 9, 25]. However, these assays detected the color change due to conformational change in the liposomes [8] or the signal from dye-markers or enzymes on the liposome surface [9, 25]. In this study, we used dye-encapsulating liposomes, which, because of the much larger number of dye molecules contained in each liposome, produced a much higher signal intensity, thereby resulting in higher sensitivity. The LOD of 3 mLD_{50} is comparable to the most sensitive BT detection assays previously reported [15, 16, 26, 27] for which the detection limits reach the level of the mouse bioassay.

Table 2 Sensitivity of various detection assays for botulinum neurotoxin[a]

Method	LOD of toxin type A (mLD_{50})[b]	Assay time	Reference
Mouse bioassay	1–2	3–4 days	[32]
RPHA	1.3–1.6	5–6 h	[33]
ELISA		1–5 h	
	2		[34]
	9		[35]
	1–2		[15]
ELCA	<1	>18 h[c]	[27]
Enzymatic assay[d]	0.5	5–6 h	[16]
This study	3	20 min	

[a]Abbreviations: mLD_{50}, mouse 50% lethal dose; RPHA, reversed passive hemagglutination; ELISA, enzyme-linked immunosorbent assay, ELCA, enzyme-linked coagulation assay
[b]1 mLD_{50} for chromatographically purified type A botulinum neurotoxin has been calculated as approximately 6 pg mL^{-1} [32]. At 1 mLD_{50}, 50% of mice injected with 1 mL will die
[c]Including the immunobinding phase
[d]Performed with botulinum neurotoxin type B

These previously reported assays, based on ELISA or the enzymatic activity of BT, are time-consuming and need to be performed by well-trained personnel. In contrast, the assay developed in this study can be completed within 20 min and is very easy to perform. Therefore, this result suggests a BT detection assay using GT1b-liposomes can replace existing methods. In Table 2, the GT1b-liposome assay is compared to other previously reported BT detection assays.

Comparison of the sensitivity of GT1b-liposomes to immunoliposomes for BT detection

The sensitivity of the BT detection assay using GT1b-liposomes was compared to immunoliposomes with antibodies to BT on the liposome surface (Fig. 5). The LOD for BT in the immunoliposome assay system was estimated from the dose–response curve to be 40 pg mL^{-1}. At concentrations equal to or higher than 100 pg mL^{-1}, the signal could be visually detected. This result suggests that the binding of the ganglioside to the toxin is as strong and specific as the binding of antibodies to the toxin. The sensitivity of the immunoliposome assay system is slightly lower, the detection limit a little higher (LOD=40 pg mL^{-1} versus 15 pg mL^{-1}), and the signal intensity decreased more at higher concentrations of toxin as compared to the GT1b-liposome assay system. This poorer performance of the immunoliposome assay can possibly be explained by the format used in this study. In the sandwich assay format of the immunoliposome assay, the same antibodies were used for coating the analytical zone on test strips and for conjugation onto the liposome surface. This would result in these antibodies competing for the same epitopes on the toxin, which could ultimately lead to the lower sensitivity of the assay. This problem could possibly be mitigated by using antibodies to two different BT epitopes, one for use on the test strip analytical zone and the other for conjugation to liposomes. However, considering the complex and time-consuming (at least 2 days) process required to prepare immunoliposomes, ganglioside–liposomes have a distinct advantage over immunoliposomes in their ease of preparation.

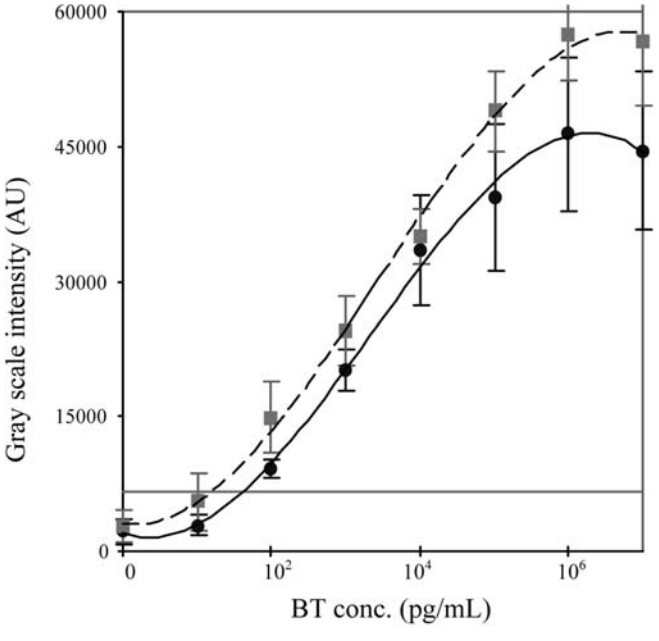

Fig. 5 Comparison of the dose–response curves for immunoliposomes (●) with GT1b-liposomes (■) for botulinum neurotoxin detection. The *solid line* represents the curve fit for immunoliposomes, and the *dashed line* represents the curve fit for GT1b-liposomes (as shown in Fig. 4), with R^2 values of 0.997 and 0.996, respectively. The *straight horizontal line* indicates the limit of detection for the immunoliposomes, defined as the color intensity 3 times higher than the standard deviation of the background (negative control) signal. Each *point* represents four replicates of grayscale values in the analytical zone

Specificity of a capillary migration test strip assay for BT detection

To evaluate the specificity of the test strip assay for BT, the assay was performed substituting various other ganglioside-binding toxins for BT. For this purpose, cholera toxin (CT) from *Vibrio cholerae*, diphtheria toxin (DT) from *Corynebacterium diphtheriae*, *E. coli* heat-stable toxin (STa), and tetanus toxin (TT) from *Clostridium tetani* were used. For safety purposes, commercially available toxoids or subunits of toxin were used in this study, except for STa. Each toxin was added to the assay system at high concentration (10 µg mL^{-1}), and the signal intensity was measured, as described in "Materials and methods". As shown in Fig. 6, the BT detection assay using GT1b-liposomes showed a high signal intensity for BT over the

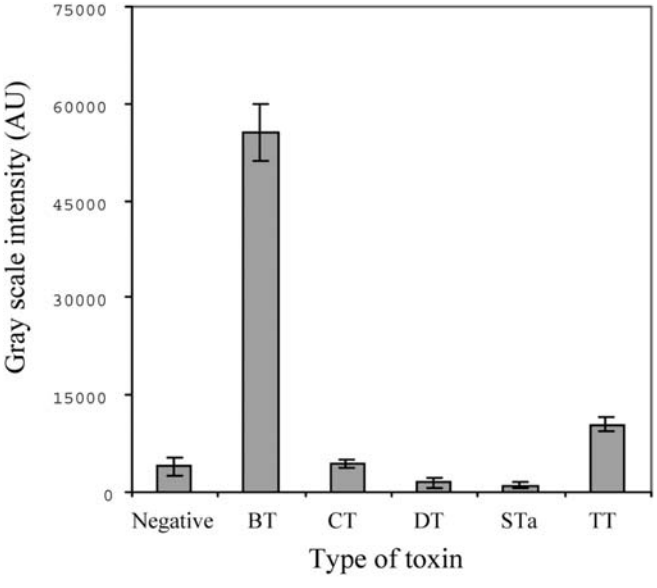

Fig. 6 Specificity of the BT detection assay using GT1b-liposomes. A 10 µg mL^{-1} aliquot of each toxin was dissolved in TBS and used in the assay. The data shown are an average of 3 replicates. Abbreviations: BT, botulinum toxin; CT, cholera toxin; DT, diphtheria toxin; STa, *E. coli* heat-stable toxin; TT, tetanus toxin

other toxins, which suggests specificity of the assay for BT detection. In this experiment, TT showed a little higher binding signal than the other toxins and this could be the result of the similarity of TT and BT in terms of structure and amino acid sequence [28, 29]. In addition, it has been reported that tetanus toxin binds to gangliosides, specifically to the disialoganglioside GD1b and GT1b, as membrane receptors [30, 31]. Despite the fact that TT in the sample can also bind to GT1b on the liposomes, the result from this study suggests that the antibodies to BT, immobilized on the test strip, can provide enough specificity to distinguish BT from TT. The high specificity of the detection assay, which requires binding to two separate and distinct receptors, provides an advantage over other immunological detection methods, which show the problems of false-positive signals from cross-reactivity, especially in sandwich-type assays.

Conclusions

In this study, GT1b-liposomes were used in a sandwich test strip assay for botulinum neurotoxin detection. We demonstrated that GT1b-liposomes can interact with BT at least as strongly as immunoliposomes. The assay developed in this study provides detection levels comparable to the mouse bioassay and most of the other previously reported assays, but has the added advantages of simplicity and rapidity. These results show that a capillary migration test strip assay can be an alternative assay system for BT detection, which can also be applied to the field screening of food or environmental samples. This assay system could be applied to the detection of other biological toxins that use gangliosides as their cell receptors. However, for the application of this assay to field screening, the effect of food matrices will be evaluated. Preliminary studies on a variety of vegetable and seafood samples have demonstrated only a moderate loss in sensitivity with the exception of certain fish, such as salmon, that are very high in fatty acids that appear to interfere with liposome integrity. Also, the effect of other organisms present in the sample on the sensitivity of this assay will be studied.

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