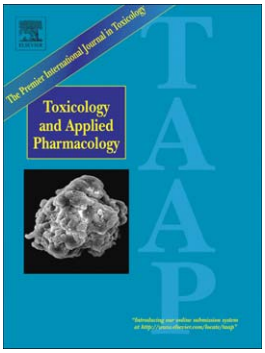

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Nerve cell-mimicking liposomes as biosensor for Botulinum neurotoxin complete physiological activity

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Abstract

Botulinum neurotoxins (BoNT) are the most toxic substances known, and their neurotoxic properties and paralysing effects are exploited for medical treatment of a wide spectrum of disorders. To accurately quantify the potency of a pharmaceutical BoNT preparation, its physiological key activities (binding to membrane receptor, translocation, and proteolytic degradation of SNARE proteins) need to be determined. To date, this was only possible using animal models, or, to a limited extent, cell-based assays. We here report a novel *in vitro* system for BoNT/B analysis, based on nerve-cell mimicking liposomes presenting motoneuronal membrane receptors required for BoNT binding. Following triggered membrane translocation of the toxin's Light Chain, the endopeptidase activity can be quantitatively monitored employing a FRET-based reporter assay within the functionalized liposomes. We were able to detect BoNT/B physiological activity at picomolar concentrations in short time, opening the possibility for future replacement of animal experimentation in pharmaceutical BoNT testing.

Keywords:

in vitro, botulinum neurotoxin, liposome, potency testing, animal testing alternatives

1. Introduction

Botulinum Neurotoxin (BoNT) is a highly toxic bacterial toxin. In its physiologically active form it is known to be a potent inhibitor of cholinergic motoneuron activity. By blocking the secretion of acetylcholine neurotransmitter, the signal transmission is impeded and the target muscle is paralyzed. If administered in minute doses, a constrained paralysing effect can be achieved, which is employed for the treatment of an increasing number of diseases, disorders and in aesthetic surgery (Wheeler and Smith, 2013). While Botox® may be the best known pharmaceutical BoNT-preparation, there is a range of related products, which contain either BoNT type A (BoNT/A) or BoNT/B as active compound (Bigalke, 2013). To gain approval, and alongside production, every batch of pharmaceutical BoNT needs to be tested for its specific overall toxic activity, termed potency (EDQM, 2011). This directly correlates to the different physiological activities of the toxin at the motoneuron, which can be divided into three key steps: 1) binding to specific nerve-cell receptors via the toxin's heavy chain (HC), 2) translocation of the toxin's light chain (LC) across the plasma membrane, induced by a decrease in pH, and 3) proteolytic cleavage of SNARE proteins by the catalytically active LC (Binz, 2013; Rummel, 2013).

Currently, the mouse bioassay is still considered to be the "gold standard" for determination of BoNT potency (e.g. AOAC Official Method 977.26). For this, mice are injected intraperitoneally with dilutions of the respective pharmaceutical preparation, monitored for typical symptoms of botulism for up to 96 hours, and the LD50 determined accordingly. Depending on the exact BoNT type, the test exhibits a detection limit between 5-100 pg (Dorner et al., 2013; Weingart et al., 2010). It was estimated that in the year 2008, batch potency controls of BoNT-pharmaceuticals required approximately 600,000 mice (Bitz, 2010). However, not only the high number of animals, but also the required facilities and personnel result in enormous costs. Above all, due to the severity of the experimental conditions and the resulting distress for the animals, the mouse LD50 test is widely criticized. With respect to *in*

vitro test systems, a few cell-based assays have been reported to monitor the key steps that govern the toxin's physiological activity (Pellett, 2013). Recently, a relatively sensitive testing scheme based on cultured neuroblastoma cells gained FDA-approval for testing of the manufacturer's own BoNT product line (Fernández-Salas et al., 2012). However, the need for expensive lab equipment, specifically trained personnel, and lengthy assay times present considerable shortcomings. Furthermore, most cell-based assays require additional methods to measure the actual toxic effect caused by BoNT, e.g. quantification of intracellularly cleaved SNARE-proteins, thus adding further hands-on time and need for accurate and reproducible protocols (Dong et al., 2004; Fernández-Salas et al., 2012; Shi et al., 2009). Also, in spite on-going research efforts, there is no validated, cell-based method for BoNT/B commercially available to date (Dong et al., 2007; Whitemarsh et al., 2012). In order to completely replace animal testing of BoNT pharmaceuticals, development of alternative and easy-to-use *in vitro* methods is highly desirable (Adler et al., 2010).

Towards this aim, we here describe a novel *in vitro* system to assay the function of BoNT/B. We employed fully assembled and functionalized liposomes as nanometre-sized reaction compartments providing a nerve-cell mimicking system that allows detection of the toxin's three physiological key activities in a single assay format.

2. Material and Methods

2.1. Toxins

The study was performed using an analytical grade, un-nicked 150 kDa BoNT type B1 preparation (strain Okra) with a potency of 1.1 x 10^8 MLD_{50} mg^{-1} from a commercial manufacturer (Metabiologics Inc., Madison, WI, USA). When appropriate, the toxin was diluted in assay buffer (12.5 mM HEPES pH 7.4; AppliChem GmbH, Darmstadt, Germany).

2.2. Test for endopeptidase activity

PL150 peptide substrate (Pharmaleads, Paris, France) was used for the detection of BoNT/B-LC endopeptidase activity. The peptide substrate consists of an oligopeptide with an N-terminally linked Pyrenylalanine fluorophore (Pya), and a C-terminally coupled Para-nitro-phenylalanine quencher molecule (Nop). The oligopeptide contains the specific cleavage site for BoNT/B-LC. Förster resonance energy transfer (FRET) between fluorophore and quencher impedes emission of fluorescence in the intact molecule. When the oligopeptide chain is cleaved, fluorophore and quencher are spatially separated and, if excited, the fluorophore emits a quantifiable fluorescence signal (Anne et al., 2001). Lyophilized PL150 was first dissolved in ultrapure H_2O at 200 µM and diluted for experiments to the desired concentrations in PL150-specific reaction buffer, constituting of 10 mM HEPES pH 7.4, 75 µM $ZnSO_4$, and 2.5 mM TCEP pH 7.0 (Sigma-Aldrich, Buchs, Switzerland). Unless stated otherwise, PL150 was used at a concentration of 10 µM, and the reaction took place in a final volume of 100 µL. Fluorescence was measured in non-binding, black 96 half-well microtitre plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Fluorescent signals were recorded every 30 seconds for 180 minutes with a spectrofluorometer (SpectraMax GeminiXS or M2e; Molecular Devices, Sunnyvale, CA, USA), at excitation and emission wavelengths of 341 and 397 nm, respectively.

Data were further analysed using Prism Version 6.0 (GraphPad Inc., La Jolla, CA, USA). All measurements were performed in duplicates, i.e. two independent experiments were performed concurrently with individual substrate and toxin dilutions. Substrate turnover kinetics, i.e., half-time values, were calculated from the curve progressions using one-phase association non-linear regression models. To calculate SD values for half-times, curves were fitted with the described models onto the measured signal intensities from each replicate measurement. The half-time value derived from each fit was then combined with the half-time from its replicate measurement and standard deviation calculated accordingly.

2.3. Preparation of functionalized liposomes

For production of fully assembled and functionalized liposomes, purified Trisialoganglioside GT1b (Matreya LLC, Pleasant Gap, PA, USA) was used as lipid receptor for BoNT/B. As the toxin's protein receptor, a GST-SytII fusion protein (toxogen GmbH, Hannover, Germany) was used (Rummel et al., 2004). The protein receptor consists of an N-terminal Glutathione-S-Transferase (GST) bound to the luminal and transmembrane domain of rat Synaptotagmin II (SytII; amino acid residues 1 to 90), and was produced in *Escherichia coli*. For purification, the protein receptor was N-terminally coupled to GST without hindering binding to BoNT/B-HC. GST-SytII was purified and stored until use in phosphate-buffered saline (PBS), and 0.5% Triton X-100. Large unilamellar vesicles (LUV) were prepared by hydration of a thin film of dried lipids and ganglioside receptor molecules. Unless otherwise stated, the lipids used for assembly of functionalized liposomes consist of a commercially available, native lipid mixture, i.e. total lipid extract derived from porcine brain (Avanti Polar Lipids Inc., Alabaster, AL, USA). As such, the extract contains numerous components including phospholipids (approximately 41%), e.g. phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, as well as other chloroform soluble compounds such as lipo-proteins, sterols,

cerebrosides, ceramides, sphingolipids, glycolipids, and other neutral lipids (approximately 59%). To match GT1b concentrations found in native neuronal membranes, 0.1 mM GT1b was used, which corresponds to 0.5% of approximately 20 mM total lipid content (Breckenridge et al., 1972; Kozaki et al., 1998). For production of 500 µL LUV, 3.5 mg of dry brain lipid extract, and 109 µg of GT1b were dissolved in 200 µL chloroform and methanol (50:50) in a glass vial by gentle agitation. Subsequently, the solvent was evaporated by applying a vacuum using a Eppendorf concentrator 5301 (Eppendorf, Hamburg, Germany) to create a dry lipid film. Unless stated otherwise, the lipid film was reconstituted in 500 µL PL150-specific reaction buffer supplemented with GST-SytII and PL150 at concentrations of 1.8-3.6 µM and 20 µM, respectively. Shaking at 4°C for 60 minutes resulted in a turbid vesicle emulsion. Triton X-100 content in the vesicle emulsion was below 0.1%. GT1b integration into vesicle membranes takes place via spontaneous incorporation of its ceramide chain during reconstitution of the lipid film (Shen et al., 2013). The apolar transmembrane domain of GST-SytII and associated Triton X-100 molecules facilitate integration of the molecule into the vesicular membranes (Rigaud and Lévy, 2003). It should be noted that due to the production method, GT1b and GST-SytII may possibly be present in an orientation facing either outside or inside from the liposome membrane (Girard et al., 2004). Following lipid film hydration, unilamellar LUV with uniform size distribution were produced by repeated extrusion of the vesicle emulsion through nucleopore track-etched polycarbonate membranes of 400 nm, 200 nm, and finally 100 nm pore size, flanked by a double stack of polyethylene drain discs on each site (Whatman plc, Kent, UK) assembled in a Mini-Extruder (Avanti Polar Lipids Inc., Alabaster, AL, USA). Remaining Triton X-100 from supplementation of protein receptor was removed by incubation of the emulsion with 40 mg of Bio-Beads SM-2 Adsorbent (Bio-Rad Laboratories, Hercules, CA, USA) on an overhead rotator for 90 minutes at 4°C. Then, non-encapsulated substrate molecules and components of the cleavage buffer were removed by a minimum of 12

hours of dialysis at 4°C against 12.5 mM HEPES pH 7.4 using SpectraPor Dialysis Membranes type 7 with a molecular weight cut-off of 25 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Liposomes were used directly after production or stored until use at 4°C. Storage did not have a significant effect on liposome integrity (Fig. S1). Liposomes devoid of receptors were produced in the same manner, without supplementation of neither GT1b nor GST-SytII receptor molecules.

2.4. Liposome characterization

2.4.1. Size exclusion chromatography

Unless stated otherwise, 30 µL of a liposome emulsion was subjected to size exclusion chromatography (SEC) using a microcentrifuge spin column system (Thermo Scientific, Rockford, IL, USA) with a gel bed volume of approximately 600 µL for separation from not-integrated and/or not-encapsulated compounds. A 50%-slurry of Sepharose 4B (Sigma-Aldrich, Buchs, Switzerland) in 12.5 mM HEPES pH 7.4 was used as gel matrix. Separation was enhanced by centrifugation in a table top centrifuge (Eppendorf AG, Hamburg, Germany) at 1,200 rpm for 45 seconds at ambient temperature and the eluted sample fraction was collected. After the first elution, 30 µL of 12.5 mM HEPES pH 7.4 were gently added to the top of the Sepharose gel bed, followed by another centrifuge run with following collection of the eluted fraction. This elution step with HEPES buffer was repeated another 11 times to yield a total of 12 fractions.

2.4.2. Electron microscopy

For Cryo-scanning electron microscopy (Cryo-SEM) imaging, liposomes were produced using 50 µM PL150-substrate, 40 mM brain lipid extract, and GT1b and GST-SytII at concentrations of 50 µM and 1.8 µM, respectively. The liposome emulsion was filled between two 6 mm aluminium carriers (planchettes). The planchette sandwich was frozen in a Bal-Tec HPM100 high-pressure freezer (Leica

Microsystems, Wetzlar, Germany), and stored frozen in liquid nitrogen. Based on the weak cohesion of the samples, the planchettes physically separated at this step. Fresh fracturing was induced with a scalpel before the planchettes were transferred to the freeze-fracturing system (EM BAF060; Leica Microsystems, Wetzlar, Germany). There, freeze drying was performed at -95°C for 3 min, before coating the sample with 2 nm tungsten at 45°, followed by an additional 1.5 nm coating at angles varying from 45 to 90°. Cryo-SEM measurements were performed in a field emission scanning electron microscope (Leo Gemini 1530, Carl Zeiss SMT GmbH, Oberkochen, Germany) equipped with a cold stage to maintain specimen temperature at -120°C (VCT Cryostage; Leica Microsystems, Wetzlar, Germany). Secondary electrons (SE) at an acceleration voltage of 5 kV were used for image acquisition.

2.4.3. Liposome size and relative amounts

Hydrodynamic diameter and polydispersty of liposome emulsions were determined by dynamic light scattering using a BI-200SM particle sizer (Brookhaven Instruments Corporation, Holtsville, NY, USA), at an angle of 90° and 25°C sample temperature. For measurement of the relative concentrations of liposomes, emulsions were measured using a spectrophotometer at 230 nm wavelength (NanoDrop ND-1000; NanoDrop Technologies Inc., Wilmington, DE, USA).

2.5. Immunological analysis

2.5.1. Antibodies

Mouse monoclonal anti-VAMP1/2/3 (Synaptic Systems, Göttingen, Germany), mouse monoclonal anti-GT1b (Millipore Corporation, Billerica, MA, USA), and rabbit polyclonal anti-GST (Bethyl Laboratories Inc., Montgomery, TX, USA) were used as primary antibodies against PL150, GT1b and GST-SytII, respectively. Polyclonal goat anti-rabbit antibody with a horse-radish peroxidase (HRP) label (BioFX Laboratories

Inc., Owings Mills, MD, USA) and HRP-labelled, polyclonal goat anti-mouse antibody (Thermo Scientific, Rockford, IL, USA) were used as secondary, species-specific antibodies.

2.5.2. Enzyme-linked immunosorbent assay

For immunological detection of the liposome components, enzyme-linked immunosorbent assay (ELISA) was employed. For detection of PL150, GT1b or GST-SytII, sample cavities of MaxiSorp 96-well plates (Nunc, Langenselbold, Germany) were coated at 4°C overnight with 50 µL of liposomes diluted either 1/1000, 1/2000 or 1/100 in 100 mM bicarbonate buffer (40 mM sodium carbonate, 60 mM sodium hydrogen carbonate; Sigma-Aldrich, Buchs, Switzerland). After 60 minutes of blocking at 37°C with 200 µL blocking buffer, i.e. 2% bovine serum albumin (BSA; Carl Roth, Karlsruhe, Germany) in PBS, sample cavities were washed three times with 200 µL PBS each. Then, 50 µL of either anti-VAMP1/2/3 (1 µg mL^{-1}), anti-GT1b (37 µg mL^{-1}), or anti-GST antibody (1 µg mL^{-1}) diluted to the indicated concentrations in blocking buffer, were applied and incubated for 60 minutes at 37°C. After another washing step, sample cavities were incubated with 50 µL of blocking buffer with either HRP-labelled goat anti-rabbit (2 µg mL^{-1}), or goat anti-mouse antibody (0.8 µg mL^{-1}) for 45 minutes at 37°C. Following a further washing step, 50 µL of 3,3',5,5'-Tetramethylbenzidine (TMB; Thermo Scientific, Rockford, IL, USA) were added as a chromogenic substrate for HRP to each sample cavity. HRP-catalysed conversion of TMB was stopped by addition of 50 µL of 2 M H_2SO_4 per sample cavity. The average absorbance was then measured at 450 nm, subtracting the reference value at 650 nm, using a multi-mode microplate reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). All measurements were performed in duplicates, i.e. two independent experiments were performed concurrently with the same liposomes.

2.6. Assay procedure using functionalized liposomes

For detection of BoNT/B physiological activity, ten µL of BoNT/B at the desired concentrations were gently mixed with 8 µL of undiluted, fully functionalized liposomes. To increase controlled binding of the toxin to the liposomes, the mixture was incubated for 15 minutes at 4°C. Subsequently, samples were transferred to non-binding, black 96 half-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Unless stated otherwise, low-pH buffer (pH 4.0; 12.5 mM HEPES pH 5.5 and 0.38 mM dimethylglutaric acid; Sigma-Aldrich, Buchs, Switzerland) was added to yield a final volume of 100 µL. Prior to fluorescence read-out, microplates were gently shaken for 60 seconds. Fluorescent signals were recorded every 30 seconds for 180-360 minutes with a spectrofluorometer (SpectraMax GeminiXS or M2e; Molecular Devices, Sunnyvale, CA, USA), at excitation and emission wavelengths of 341 and 397 nm, respectively. To quantitatively release encapsulated PL150-molecules from the assembled liposomes, Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) was added to the mixtures to a final concentration of 0.1%. Data were analysed using Prism Version 6.0 (GraphPad Inc., La Jolla, CA, USA). Half-times were calculated from the curve progressions following the initial lag phase of 60 minutes using one-phase association non-linear regression models. All measurements were performed in duplicate, i.e. two independent experiments were performed concurrently with the same liposomes but individual toxin dilutions. To calculate SD values for half-times, curves were fitted with the described models onto the measured signal intensities from each replicate measurement. The half-time value derived from each fit was then combined with the half-time from its replicate measurement and standard deviation calculated accordingly. To calculate for minimal assay times, the initial lag time was added to the average half-time and defined as “time to reach half-maximum” (time to half-max).

3. Results and Discussion

3.1. Analytical principle of the detection method

A schematic presentation of the principle of the detection method is shown in Fig. 1. Similar to the activity at the presynaptic membrane of motoneurons, BoNT/B binds via the receptor binding domain of its HC to specific nerve cell receptors that are integrated into the liposome membrane. The membrane bilayer of the functionalized liposomes encloses a reaction compartment, which contains a specific reaction buffer and peptide substrate molecules. Upon acidification of the surrounding environment, the HC mediates the translocation of the LC into the liposome lumen. There, the LC can proteolytically cleave the encapsulated peptide substrate. Due to the FRET-reporter system, cleavage of the substrate's peptide chain yields a quantifiable fluorescent signal. The increase in fluorescence intensity correlates to the amount of physiologically active toxin in the assay. Combined testing of the three events provides a quantitative assay for BoNT/B potency in a single assay format.

3.2. Determination of BoNT/B endopeptidase activity

To allow for sensitive measurement of BoNT/B endopeptidase activity, a reporter assay was established using PL150 peptide substrate. Preliminary experiments performed without liposomes in PL150-specific reaction buffer showed that an increase of the fluorescence intensity directly correlated to the amount of toxin added (Fig. 2a-b). With respect to sensitivity, the increase in signal intensity observed for 10 pM BoNT/B was still clearly discernible from background noise. However, to quantify and compare the effect of different BoNT/B-concentrations, half-time values were calculated from the presented curve progressions. The calculated half-times correspond to the rate of substrate conversion, i.e. the kinetics of the substrate cleavage by BoNT/B. In the presence of different concentrations of Botulinum neurotoxin, more SNAPtide substrate molecules are cleaved in the same amount of

time, resulting in different half-time values of the reaction. Figure 2c depicts the half-times, i.e. rate of substrate conversion, for different concentrations of BoNT/B in this unrestricted reaction system, where all substrate molecules are available for cleavage. At high concentrations e.g. at 10 nM BoNT/B, the reaction half-time is only 10.6 +/- 1.3 minutes. For a 2- to 10-fold reduction in toxin concentration the half-time values increase accordingly. At 5 nM BoNT/B for example, the half-time takes 2.1 times longer, i.e. 22.8 +/- 2.1 minutes, and at 1 nM BoNT/B the half-time value increases by 7.9 times. At lower BoNT/B concentrations, the differences between the respective half-times are less pronounced and approach a plateau at 125 minutes. There was no indication that frequency of the measurement did have an effect on fluorophore stability (Fig. S2). Although a previous study reported a minimum detection limit of 0.7 pM for BoNT/B-LC, the work used 1.8 times higher substrate concentrations and incubation times of up to four hours, and the BoNT/B holotoxin was not tested (Anne et al., 2001).

3.3. Characterization of functionalized liposomes

3.3.1. DLS-measurement and SEM-imaging

The hydrodynamic diameter of the tested liposomes ranged between 120 and 140 nm with an average diameter of 129.5 nm and an average polydispersity of 0.099. Both the narrow range of measured hydrodynamic diameters and the low polydispersities indicate a highly homogeneous liposome emulsion. In addition, as described before (Berger et al., 2001; Jesorka and Orwar, 2008), a hydrodynamic diameter below 200 nm primarily yields unilamellar liposomes (Fig. S3), an important requirement for the presented test system. Average liposome diameters and homogeneity of the emulsion were further confirmed by EM images acquired via Cryo-SEM freeze fracture of the intact, fully assembled liposomes (Fig. 3).

3.3.2. Substrate encapsulation and receptor integration

To confirm and validate the presence of both receptors and the PL150 peptide substrate in functionalized liposomes, SEC was used to separate the dialysed liposome emulsion. After SEC separation, the eluted fractions were tested for the presence of either liposomes or other components via light scattering or ELISA, respectively (Fig. 4). Light scattering measurements showed that liposomes eluted predominantly in the first SEC fractions. PL150 peptide substrate showed a corresponding elution pattern. In contrast to undialysed liposomes (Fig. S4), no PL150 was detectable in the non-liposomal fractions and the majority of GT1b and GST-SytII also could be detected in the liposome-containing fractions. Apart from GST-SytII present in the liposome-containing fractions, small amounts of the receptor were also detected in subsequent fractions. This may have been due to either isolated GST-molecules lacking the transmembrane domain of the SytII protein receptor, or to a small fraction of co-purified, non-integrated receptor in the liposome emulsion. Although the exclusion limit of 60 nm of Sepharose 4B beads could also lead to an elution of smaller liposomes in later fractions (Ruysschaert et al., 2005),

neither light scattering nor immunoassays of the other components suggest that liposomes were present there. In conclusion, our data not only show that all necessary components are present in the liposomes, but also that prior dialysis efficiently removes non-encapsulated molecules of the peptide substrate from the emulsion of the functionalized liposomes.

3.3.3. Cleavage of peptide substrate released from liposomes

Preliminary experiments confirmed that BoNT/B-mediated cleavage of free, unencapsulated PL150 substrate is not affected by the presence of liposomes (data not shown). To further demonstrate that the encapsulated substrate remained functional and is not degraded without internalization of the BoNT-LC, liposomes containing PL150 substrate were incubated with BoNT/B in PL150-specific reaction buffer. The liposomes used here did contain PL150 peptide substrate only, but no receptor molecules. As shown in Fig. 5a, exposure of intact liposomes to BoNT/B yielded no increase in signal intensity (t = 0 - 70 minutes), which indicates that no unspecific translocation of BoNT/B or its LC subunit through the liposome membrane occurred. However, upon Triton X-100-induced lysis of the liposomes (t = 70 - 180 minutes), the fluorescent signal increased rapidly in the presence of BoNT/B as all encapsulated substrate molecules are released and may be cleaved by the BoNT/B LC proteolytic activity. These findings are in accordance with the SEC experiments and confirm that no un-encapsulated substrate molecules are present in the liposome emulsion. Furthermore, the results demonstrate that PL150 was unaffected by the encapsulation process, and remains sensitive to enzymatic cleavage by BoNT/B.

3.4. Measuring BoNT/B activity with functionalized liposomes

3.4.1. Detection of BoNT/B physiological activity at 37°C

To test for the toxin's physiological activity, i.e. binding, translocation and endopeptidase activity, under conditions as close as possible to a physiological

environmental mixture of BoNT/B and functionalized liposomes was subjected to a pH-shift at an incubation temperature of 37°C (Fig. 5b). In accordance with previous studies (Brandau et al., 2007; Sun et al., 2011), preliminary experiments showed that acidification of the reaction mixture does not impair binding of BoNT/B to its receptors (Fig. S5a). In the first phase of the assay, following a lag time of approximately 25 minutes, fluorescence measurements showed a steep increase in signal intensity, until maximum values were reached after approximately 60 minutes. As previously discussed (Pirazzini et al., 2013), the lag time can most probably be attributed not to membrane translocation but rather to the time needed to cleave a substantial fraction of the PL150 substrate. Following the signal maximum after 60 minutes, a decrease in signal intensity was observed. This may be because of accumulation of free quencher molecules inside the liposomes, as a result of increasing substrate cleavage. Due to a higher degree of freedom, released quencher molecules can then again cause quenching of fluorophore emmission. Another possible explanation, although less likely, could be that protons of the surrounding low-pH buffer may permeate the liposome membrane, potentially due to minor irregularities caused by translocation of the LC across the membrane. This would lead to a decrease of the pH in the liposome lumen and slow down the reaction, and/or decrease fluorescence due to pH-dependent fluorophore quenching. However, preliminary experiments showed no unspecific permeation of proton through the liposomal membranes using a similar liposome with pyranine encapsulated as a pH-sensitive fluorescent dye (Fig. S6). As liposome lysis by addition of Triton X-100 in the second phase of the experiment resulted in an increase in signal intensity this could also imply that the observed signal decrease after reaching maximum may be due to substrate depletion in liposomes containing translocated LC, while other liposomes still contain abundant cleavable substrate molecules. The signal increase following liposome lysis was clearly discernible from that in the first phase of the experiment, with the former being much slower due to inhibition of the endopeptidase activity of BoNT at pH 4 (Fig.

S5b), in addition to pH-dependent fluorescence quenching. In the third phase, addition of PL150-specific reaction buffer leads to pH-neutralization, which leads to a rapid increase of fluorescence signal. Similar to Fig. 5a, all previously encapsulated substrate molecules are released and may be cleaved not only by the translocated LC domains, but also by holotoxin molecules present in the reaction mixture. Altogether, the signal increases in the different phases of the experiment were clearly distinct, and strongly suggest that in the cleavage and release of FRET reporters in the first phase of the experiments takes place in the lumen of intact liposomes.

3.4.2. Sensitivity of the detection system at ambient temperature

To stabilize the signal after reaching the maximum, and to elucidate how lower incubation temperatures would affect the sensitivity, assays were performed at ambient temperature. Preliminary experiments indicated that proteolytic cleavage of substrate molecules also occurs at such conditions, albeit requiring extended reaction time (Fig. S7). With functionalized liposomes, Fig. 6a shows that the fluorescence signal increased after a lag phase of approximately 60 minutes, following the pH shift to trigger translocation of the LC. Signals increased corresponding to the amount of toxin added. Similar to Fig. 2c, Fig. 6b presents the half-time values calculated from the presented curve progressions to compare the rate of substrate conversion by different concentrations of BoNT/B. After the initial lag time of 60 minutes, the half-time calculated for 666.7 pM BoNT/B was 60.2 +/- 2.3 minutes. The half-time for 6.7 pM BoNT/B was approximately 50 minutes longer, i.e. 109.4 +/- 21.3 minutes. Lower concentrations were no longer distinguishable from the background and the negative controls. To determine minimal assay times, the initial lag phase and average half-time are taken together and defined as "time to reach half-maximum" or time to half-max (Table S1).

Our findings clearly demonstrated that the sequential physiological activities of BoNT/B also took place at ambient temperature, albeit at a slightly slower speed than

at 37°C. This difference may be largely attributed to reduction of endopeptidase activity at lower temperature (Kukreja et al., 2010; Mizanur et al., 2014). While the binding reaction should not be affected, the lower temperature may also reduce fluidity of the liposome membranes, thereby possibly affecting LC translocation (Pirazzini et al., 2013; 2011). However, higher membrane rigidity may also reduce influx of protons from the low pH-buffer environment, or leakage of fluorogenic substrate from the liposome lumen (Mansy, 2010). While the latter may result in extraluminal substrate cleavage and fluorescence increase, the acidic environment outside the liposomes actually inhibits endopeptidase activity, and also leads to pH-dependent quenching of fluorophore signals. Accordingly, despite a slightly longer assay time, a decrease in temperature may offer distinct advantages for the overall performance of the assay. Altogether, the current set-up allowed detection of BoNT/B at picomolar concentrations, and represents a large step forward towards development of a sensitive and rapid in vitro test system.

4. Conclusion

In the past, a variety of biochemical approaches have been employed to measure the potency of BoNT, by determination of the activity of either LC endopeptidase (Bagramyan et al., 2013; Ferracci et al., 2005; Ouimet et al., 2013; Stevens et al., 2013; Wang et al., 2010), HC-binding (Ahn-Yoon et al., 2004; Frisk et al., 2011; Mason et al., 2006), or combinations thereof (Evans et al., 2009; Gregory et al., 2014; Liu et al., 2012). Yet, as such assays only yield information on the distinct activities of the individual subunits of the toxins, they fail to yield comprehensive information on the combined physiological activity. We here describe an assay, which is able to monitor and quantify the entire sequence of binding to nerve cell receptors, translocation of the LC across a membrane, and LC-mediated cleavage of a SNARE reporter peptide. Although cell-based assays also allow quantification of BoNT

physiological activity (Pellett, 2013), they often suffer from lengthy assay times and very high costs. In contrast, the *in vitro* system introduced here requires only little preparation time and minimal instrument requirements. Liposomes can be produced beforehand and stored until needed. As most reagents and consumables are standardized and available in high quality, transfer of the method into controlled environment would be feasible. The assay takes only few hours, including all incubation steps and fluorescence measurement. In conclusion, we demonstrate proof of principle for the first *in vitro* detection system able to measure the three key steps involved in BoNT/B physiological activity at picomolar concentrations, without the need for animal testing or living cells. For a replacement of the animal test, the method needs to be tested with pharmaceutical-grade BoNT-products and validated for its accuracy by evaluation of the exact detection limits, robustness and reliability by measuring inter- and intra-assay variations as well as unspecific effects of other compounds or similar toxins. Compliance with international regulations need to be ensured. Furthermore, the test system's performance needs to be compared to other assays, which measure the toxin complete physiological activity or potency. Testing of other toxin forms, e.g. BoNT devoid of receptor binding domain (Fischer et al., 2008), will also be addressed in further studies. We believe that the nerve-cell mimicking liposome assay not only offers a tool to avoid the use of animal experimentation for toxicity measurements of pharmaceutical BoNT preparations, but that the modular character of the test system also allows an expansion towards detection of further BoNT serotypes and other toxins in the future.

5. References

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Author contributions

O.G.W. conceived the project and its components, performed the experiments and analysed the data. M.J.L. supervised and directed the project. O.G.W. and M.J.L. wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

Figures & figure legends

Fig. 1. Use of functionalized liposomes for determining the physiological activity of BoNT. The membrane bilayer of the fully assembled, functionalized liposomes encloses a reaction compartment that contains the specific reaction buffer and fluorogenic reporter substrate. The oligopeptide substrate molecule harbours a cleavage site for BoNT-LC, and a fluorophore and a quencher molecule attached at opposite ends. The BoNT receiving specific nerve cell receptors are integrated into the liposome membrane. Similar to the events at the motoneuron, BoNT binds via its HC to the nerve cell receptors. Upon acidification of the outer milieu, the HC mediates translocation of the LC into the liposome lumen, where it enzymatically cleaves the encapsulated reporter substrate. As a result, the fluorophore is dequenched and yields a quantifiable fluorescent signal following excitation.

Fig. 2. Determination of BoNT/B endopeptidase activity using a FRET substrate. Endopeptidase activity between (a) 10 pM and 10 nM of BoNT/B, measured using PL150 and PL150-specific reaction buffer at a temperature of 37°C. Measurements between 10 pM and 100 pM BoNT/B are also depicted with a different scale in (b). The baseline was corrected using background signal from buffer and liposomes alone as negative controls. Fluorescence signals were recorded every 30 seconds. (c) Substrate turnover kinetics, i.e. half-time values were calculated from curve progressions of different concentrations of BoNT/B by using one-phase association non-linear regression models. Error bars represent SD.

Fig. 3. Homogeneity of liposomes assembled for BoNT testing. Although the assembled and functionalized liposomes are mostly spherical, the sample preparation procedure required for Cryo-SEM imaging might result in somewhat

altered shapes. The average hydrodynamic diameter of the liposomes in this sample was 140 nm. Scale bar is 200 nm.

Fig. 4. Presence of receptors and FRET-substrate in functionalized liposomes. An emulsion of functionalized liposomes was separated by size exclusion chromatography and analysed via light scattering measurement for (a) presence of liposomes, (b) content of PL150 peptide substrate (via ELISA), (c) presence of GT1b, and (d) of GST-SytII. Error bars represent SD.

Fig. 5. Cleavage of released FRET-substrate and liposomal detection of BoNT/B physiological activity at 37°C. (a) Enzymatic degradation of FRET substrate released from liposomes was measured via fluorescence signal read-out. For this, receptor-free liposomes with encapsulated PL150 peptide substrate incubated with either 100 pM BoNT/B (black line) or HEPES buffer as negative control (grey line) in PL150-specific reaction buffer at 37°C. Liposome lysis was triggered by addition of Triton X-100 after 70 minutes. (b) Detection of BoNT/B physiological activity with functionalized liposomes and subsequent substrate release was measured using functionalized liposomes incubated with either 333.3 pM (dark red line) or 66.7 pM (bright red line) of BoNT/B, respectively, in low pH-buffer (pH 4), at 37°C. First, Triton X-100 for liposome lysis and, subsequently, PL150-specific reaction buffer (30 µL) for pH-neutralization were added to the reaction mixture. The baseline was corrected using low pH-buffer and liposomes as background signal. Fluorescence signals were recorded every 30 seconds.

Fig. 6. Sensitivity of the liposome-based BoNT/B physiological activity assay. Sensitivity of the detection method at 25°C was tested using functionalized liposomes and BoNT/B. (a) Fluorescence signals measured at concentrations of BoNT/B between 3.3 pM and 666.7 pM. For clarity, average values are shown and standard

deviations are not indicated in the graph. The baseline was corrected using low pH-buffer and liposomes as background signal. Fluorescence signals were recorded every 30 seconds. (b) Half-times of the reactions were calculated from the curve progressions following the initial lag phase of 60 minutes, using one-phase association non-linear regression models. Error bars represent SD.

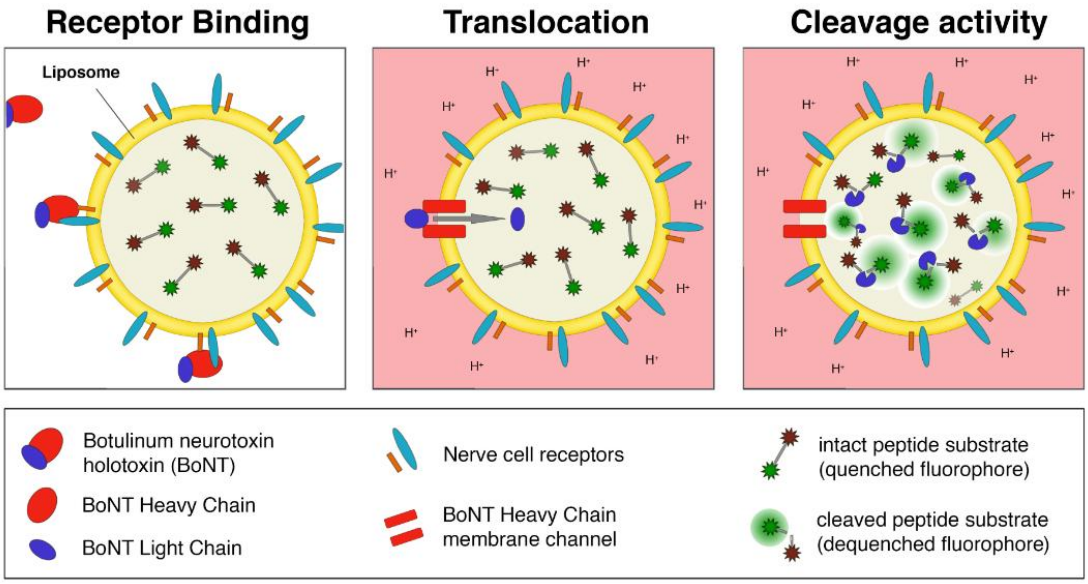

Fig. 1

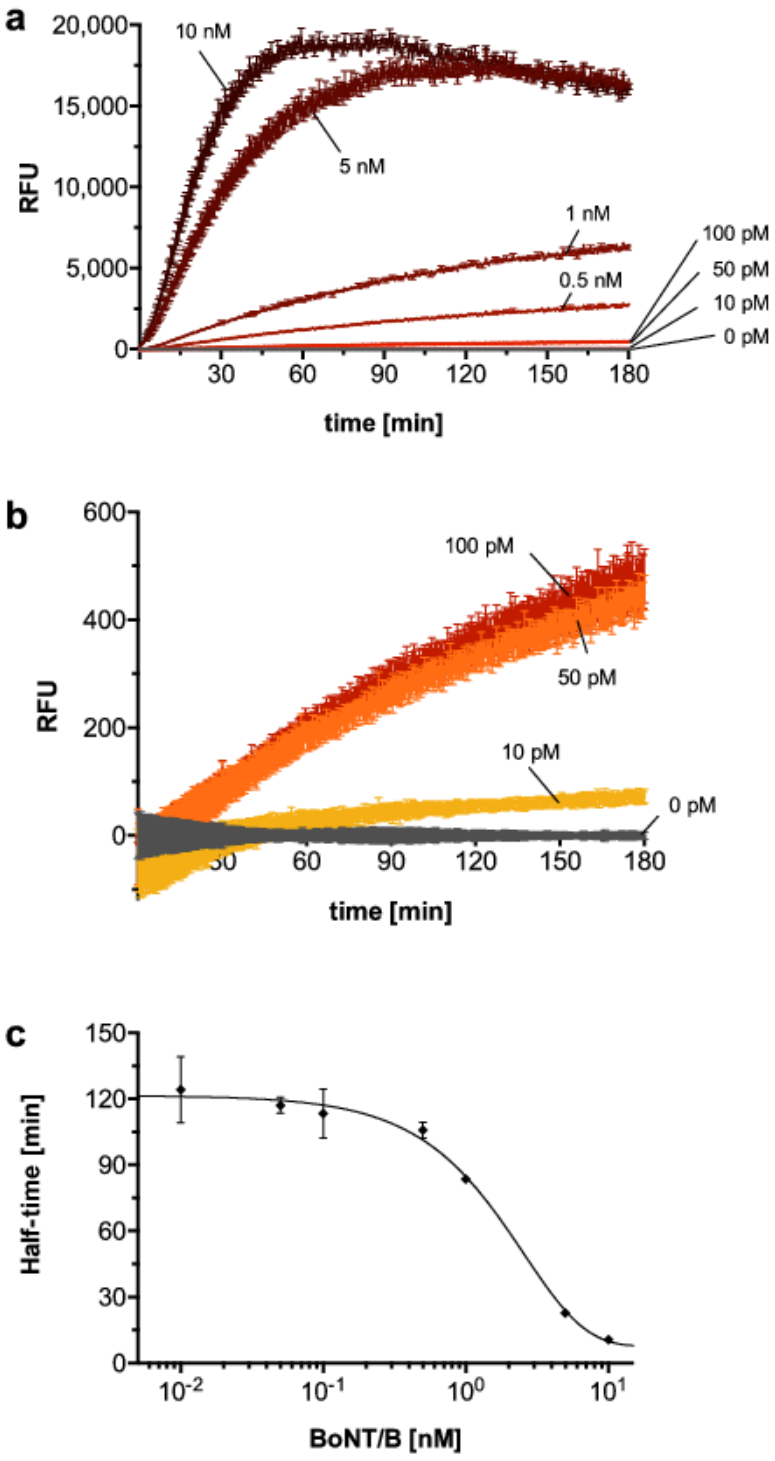

Fig. 2

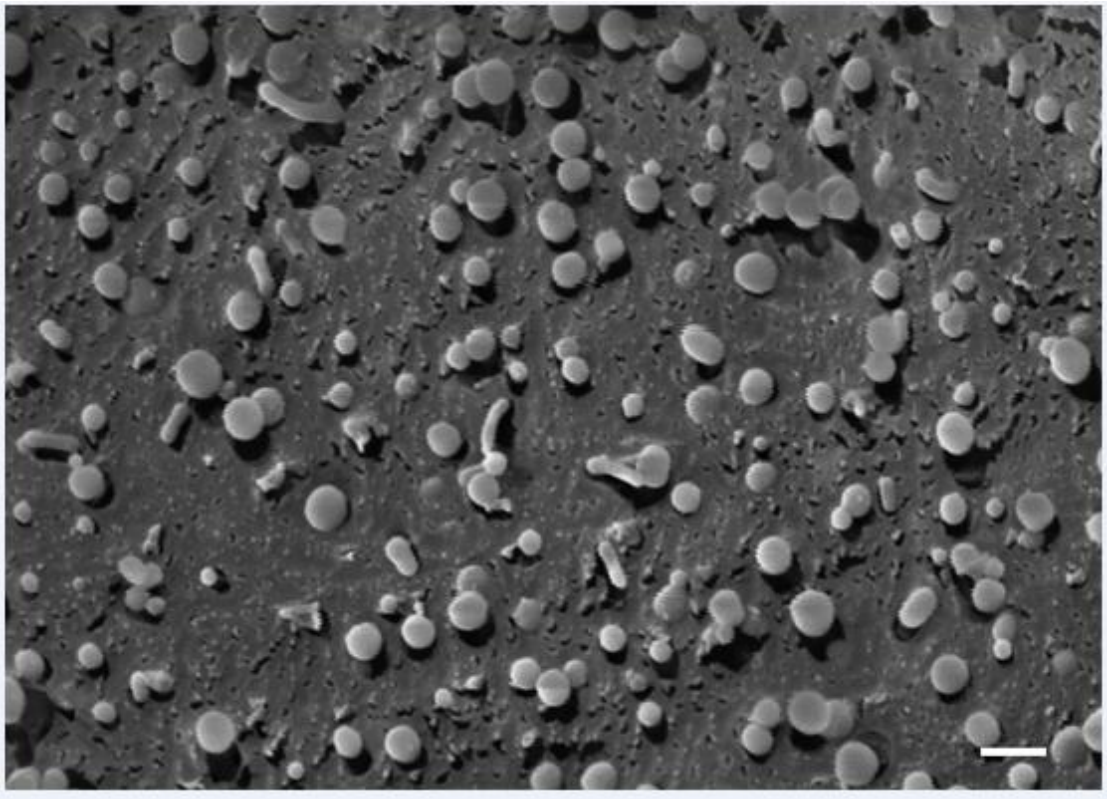

Fig. 3

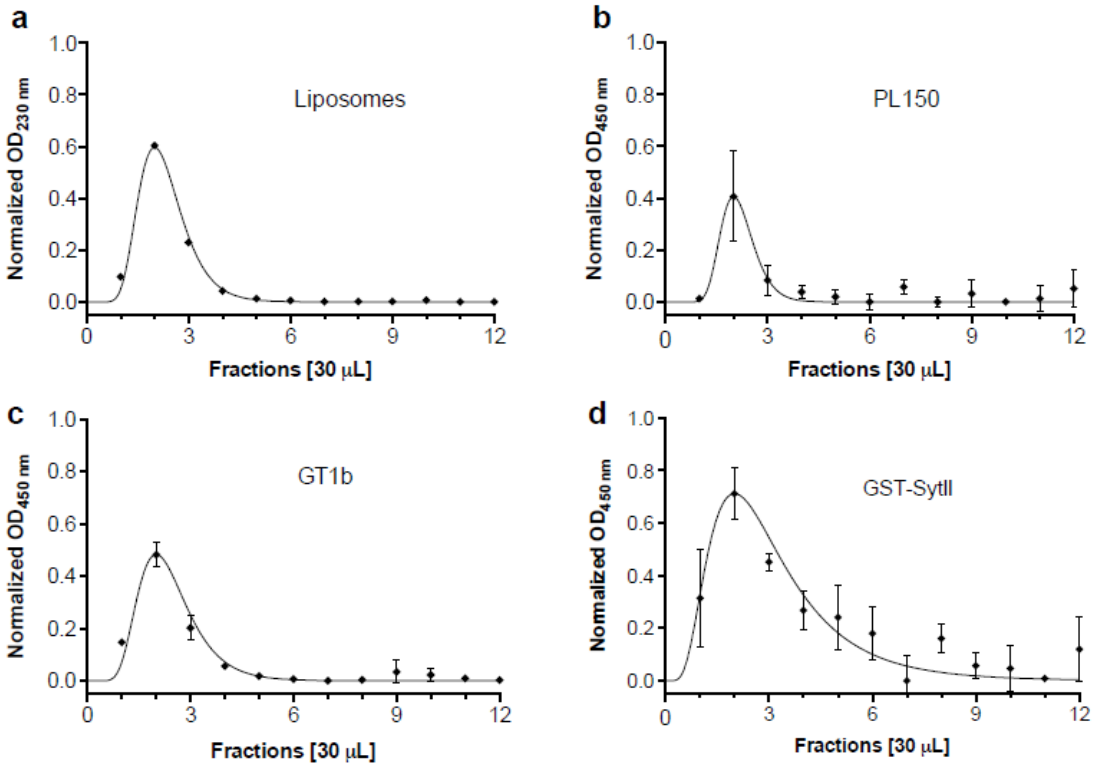

Fig. 4

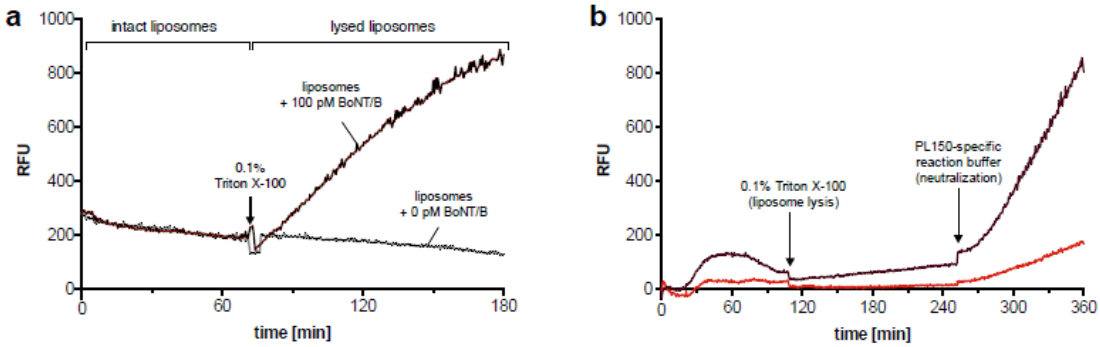

Fig. 5

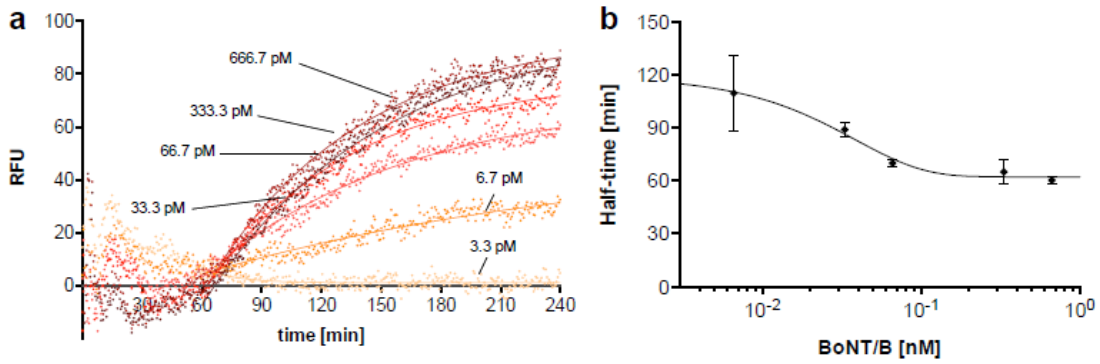

Fig. 6

Highlights

- A cell-free *in vitro* system was used to measure BoNT/B physiological function.
- The system relies on nerve-cell mimicking liposomes as a novel detection system.
- A FRET-based reporter assay is used as final readout of the test system.
- BoNT/B physiological activity was detected at picogram quantities in short time.
- The method opens the possibility to replace animal experimentation in BoNT testing.