A single-chain variable fragment selected against a conformational epitope of a recombinantly produced snake toxin using phage display

Rimbault, Charlotte; Knudsen, Pelle D; Damsbo, Anna; Boddum, Kim; Ali, Hanif; Hackney, Celeste M; Ellgaard, Lars; Bohn, Markus-Frederik; Laustsen, Andreas H

Published in:
New Biotechnology

DOI:

Publication date:
2023

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
A single-chain variable fragment selected against a conformational epitope of a recombinantly produced snake toxin using phage display

Charlotte Rimbault, Pelle D. Knudsen, Anna Damsbo, Kim Boddum, Hanif Ali, Celeste M. Hackney, Lars Ellgaard, Markus-Frederik Bohn, Andreas H. Laustsen

A single-chain variable fragment selected against a conformational epitope of a recombinantly produced snake toxin using phage display

Phage display technology is a powerful tool for selecting monoclonal antibodies against a diverse set of antigens. Within toxinology, however, it remains challenging to generate monoclonal antibodies against many animal toxins, as they are difficult to obtain from venom. Recombinant toxins have been proposed as a solution to overcome this challenge, but so far, few have been used as antigens to generate neutralizing antibodies. Here, we describe the recombinant expression of α-cobratoxin in E. coli and its successful application as an antigen in a phage display selection campaign. From this campaign, an scFv (single-chain variable fragment) was isolated with similar binding affinity to a control scFv generated against the native toxin. The selected scFv recognizes a structural epitope, enabling it to inhibit the interaction between the acetylcholine receptor and the native toxin in vitro. This approach represents the first entirely in vitro antibody selection strategy for generating neutralizing monoclonal antibodies against a snake toxin.

Introduction

Every year, snakebite envenoming causes a large number of deaths and amputations when victims do not receive timely administration of antivenom [1]. Existing antivenoms on the market consist of antibodies or antibody fragments derived from the plasma of immunized animals [2]. However, new treatment modalities are being investigated, including the use of monoclonal antibodies obtained using in vitro display technologies such as phage display, which can potentially deliver therapeutic antibodies that are highly specific, possess high neutralizing capacities, and benefit from improved safety profiles [3,4]. A number of such monoclonal antibodies and single domain nanobodies that can neutralize snake toxins in vivo have already been reported [5-9]. However, all these efforts have relied on toxins from native sources. Of the estimated 19–25,000 snake toxins predicted to exist [10], only very few are commercially available, thereby imposing a major bottleneck on the development of monoclonal antibodies against most snake toxins. To circumvent this challenge, the use of recombinant toxins may seem obvious. However, while several studies involving immunization using recombinant snake toxins have been reported [11-14], there are so far no reports on the use of recombinant snake toxins as antigens for the discovery of monoclonal antibodies using in vitro display technologies and naïve libraries. This observation could reflect that the structural integrity of snake toxins produced recombinantly thus far is potentially inadequate for such toxins to be used as antigens, i.e., incorrectly folded toxins will only select for suboptimal binders that do not sufficiently recognize the toxins in their native conformation. Therefore, new systems for recombinant protein expression could help unlock access to the myriads of snake toxins that are currently unavailable to researchers. Finally, the ability to manipulate and carefully alter toxins using recombinant DNA technology may also open up further applications, as it may allow easier study of toxin biochemistry and venom evolution via the creation of toxin mutants, as well as facilitating the development of new molecular tools, such as toxoids, tagged toxins, or fusion proteins [15].

Abbreviations: BLI, Biolayer interferometry; scFv, Single-chain variable fragment; MALDI-TOF, Matrix-Assisted Laser Desorption Ionization - Time of Flight; HPLC, High-performance liquid chromatography; TEV, Tobacco etch virus; nAChR, Muscle-type nicotinic acetylcholine receptor; ACh, Acetylcholine; DELFIA, Dissociation-Enhanced Lanthanide Fluorescence Immunoassay; CDR, Complementarity-Determining Region.

E-mail address: ahola@bio.dtu.dk (A.H. Laustsen).

Received 26 October 2022; Received in revised form 29 March 2023; Accepted 7 April 2023
Available online 8 April 2023
1871-6784/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
To address the above challenges and opportunities, we used the csCyDisCo expression system (cytoplasmic disulfide bond formation in *E. coli*) [16] for the generation of a model toxin (α-cobratoxin) from the monocled cobra (*Naja kaouthia*), which was then further employed as an antigen in a phage display-based antibody selection campaign. The CyDisCo system is based on co-expression of one catalyst of disulfide bond formation, the mitochondrial oxidase Erv1p from *Saccharomyces cerevisiae*, and another catalyst of disulfide bond isomerization, hPDI (human protein-disulfide isomerase). Erv1p provides the oxidizing equivalents to generate disulfide bonds *de novo*, and the hPDI isomerizes non-native disulfide bonds. The CyDisCo co-expression system has been shown to accommodate highly complex disulfide-bonded proteins, including Fab antibody fragments [17] (five disulfide bonds), the vtPA [18] (a tissue plasminogen activator fragment with nine intra-molecular disulfide bonds), Resistin [18] (five intra-molecular disulfide bonds and an inter-molecular disulfide bond), and a SARS-CoV-2 spike protein receptor binding domain [19] (five disulfide bonds). In the present study, the modified csCyDisCo system was used, which includes an additional protein-disulfide isomerase expressed in the venom gland of the cone snail species *Conus geographus* and has been successfully used to produce conotoxins with up to five disulfide bonds [20]. It is shown that a toxin recombinantly expressed in the csCyDisCo system can be used to select binders to the native toxins *in vitro*, and that the antigen-antibody interactions rely on structural epitopes. Thereby, a fully *in vitro* pipeline is presented for the selection of monoclonal antibodies against snake toxins, which could find utility for the development of recombinant antivenoms even against snake venoms which cannot be procured, as long as toxin sequence information is available. With the relatively recent rise of snake genomics [21], such entirely *in vitro* methodologies and pipelines may become increasingly important in the field of toxinoLOGY.

**Material and methods**

**Toxin preparation**

α-cobratoxin and *N. nivea* venom were obtained in lyophilized form from Latoxan SAS (Portes-lès-Valence, France), and prepared as previously described [7]. The α-cobratoxin was reconstituted in...
Phosphate-buffered saline (PBS, 10 mM Na$_2$HPO$_4$, 1.8 mM NaH$_2$PO$_4$, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and biotinylated using a 1:1 (toxin:biotinyl reagent) molar ratio as previously described [6]. Following biotinylating, Amicon Ultra-4 Centrifugal Filter Units (Millipore, Burlington, USA) with a 3 kDa membrane cut-off were used for purification of the biotinylated toxin. The protein concentration was measured by the absorbance at 280 nm using a NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted using the molar extinction coefficient predicted by ProtParam (web.expasy.org). The degree of biotinylation was analyzed using MALDI-TOF in an Ultraflex II TOF/TOF spectrometer (Bruker Daltonics, Billerica, USA). N. nivea venom was fractionated by reverse-phase HPLC as previously described [22].

**Plasmid construction**

To produce the recombinant α-cobratoxin, a modified version of the pET39 Ub19 vector [23] was used that allowed the production of toxins with an N-terminal ubiquitin (Ub) solubility tag with an internal His$_{16}$-loop (Ub-His$_{20}$), followed by a Tobacco Etch Virus (TEV) protease cleavage site. The gene encoding the α-cobratoxin was inserted between the KpnI and HindIII restriction sites into pLE879 [16] (Fig. 1A). The toxin sequence, codon-optimized for expression in *E. coli*, was synthesized by Eurofins Genomics GmbH (Ebersberg, Germany) and encoded a C-terminal biotin acceptor peptide (AviTag) preceded by a flexible linker (-GSGGS-). The construction of the helper plasmid, pCSyDisCo (pLE577), containing EnvYp, HPDI, and cspD(pGLU.GH) [24], had previously been described [16]. For the expression of the mCherry-tagged *E. coli* biotin ligase (BirA), the sequence encoding mCherry-BirA was amplified from the pACYC-mCh-BirA (kindly donated by Dr Matthieu Sainlos, Bordeaux University, France) [25] and inserted into pLE879 between *NdeI* and *Xhol*, thus creating an mCherry-BirA construct, fused to a C-terminal His$_{6}$-tag. A plasmid encoding the TEV protease was designed by fusing a codon-optimized mCherry gene with a codon-optimized version of the SuperTEV protease [26]. The mCherry-SuperTEV gene was synthesized by Eurofins Genomics GmbH (Ebersberg, Germany) and PCR amplified. Like the mCherry-BirA construct, the mCherry-SuperTEV was inserted between *NdeI* and *Xhol* in pLE879, hence fusing a C-terminal His$_{6}$-tag to the mCherry-SuperTEV construct.

**Circular dichroism (CD)**

α-Cobratoxin (20 μg/mL) was dialyzed against 10 mM potassium phosphate buffer (pH 7.0). The Far-UV CD was recorded using a JASCO J-1500 spectrophotometer (Easton, MD, USA) using a 0.1 mm quartz cuvette. The spectrum was acquired by conducting 10 measurements between 250 nm and 190 nm with a bandwidth of 1 nm and intervals of 1 nm, with a scan speed of 50 nm/sec. The measurements were carried out at a temperature of 15 °C, and the spectra were processed and smoothened using SpectraManager software (JASCO) and GraphPad Prism (GraphPad Software).

**Expression of recombinant α-cobratoxin, TEV cleavage, biotinylation, and purification**

Recombinant α-cobratoxin (Rs-cobratoxin) was generated as follows. Chemically competent *E. coli* BL21(DE3) cells were co-transformed with pET39 Ub19-α-cobratoxin and pCSyDisCo and plated on an LB agar plate containing kanamycin (50 μg/mL) and chloramphenicol (30 μg/mL). The toxin was then expressed as described above for the SuperTEV endoprotease. Following dialysis into PBS, the protein was concentrated to 40 μM using centrifugal filtration units. The SuperTEV endoprotease was added at a molar ratio of 1:20 (SuperTEV: Rs-cobratoxin), and cleavage was carried out at 30 °C for 1 h, whereafter it was moved to 4 °C overnight. TEV cleavage efficiency was checked by running samples before and after TEV cleavage on an SDS-PAGE gel and stained with coomassie blue. Quantiﬁcation of the intensity of coomassie-stained bands was performed with ImageLab Software. For in vitro biotinylatation, BirA was added at a molar ratio of 1:100 (BirA:Rs-cobratoxin) along with 5 mM ATP and 300 μM biotin. The solution was left at room temperature for 1 h, after which a new batch of 300 μM biotin and 5 mM ATP was added for another hour of incubation at 30 °C. Biotinylatation of the toxin was analyzed by a Streptavidin Gel-Shift assay evaluated by SDS-PAGE analysis [28]. After confirmation of tag cleavage and biotinylation status, the Rs-cobratoxin was filtered through a 0.22-μm filter and purified further on a size exclusion chromatography column (HiLoad 16/600 Superdex 75 pg, Cytiva, Marlborough, USA) using a BioRad NG Quest 10 Plus chromatography system and 1 x PBS as running buffer.

**Phosphatase and 4-nitrophenylphosphate substrate**

Buffer (TA buffer with 400 mM imidazole). *E. coli* extracts and IMAC purification steps samples were run on an sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) gel, stained with coomassie blue. The purified protein was dialyzed three times against 1 x PBS at 4 °C. Following dialysis, the protein was concentrated using centrifugal filtration units and flash-frozen with liquid nitrogen for conservation at − 80 °C.

**Circular dichroism (CD)**

Rs-cobratoxin (20 μg/mL) was dialyzed against 10 mM potassium phosphate buffer (pH 7.0). The Far-UV CD was recorded using a JASCO J-1500 spectrophotometer (Easton, MD, USA) using a 0.1 mm quartz cuvette. The spectrum was acquired by conducting 10 measurements between 250 nm and 190 nm with a bandwidth of 1 nm and intervals of 1 nm, with a scan speed of 50 nm/sec. The measurements were carried out at a temperature of 15 °C, and the spectra were processed and smoothened using SpectraManager software (JASCO) and GraphPad Prism (GraphPad Software).

**Electrophysiology**

The toxin’s ability to inhibit the muscle-type nicotinic acetylcholine receptor (nAChR) was assessed using a Qube 384 automated electrophysiology platform (Sophion Bioscience A/S, Ballerup, Denmark) as described elsewhere [7]. In brief, the human-derived rhabdomyosarcoma RD cell line (ATCC cat. #CCL-136) was used, which endogenously expresses the muscle-type nicotinic acetylcholine receptor (consisting of the α1, β1, δ, γ, and ε subunits). The cells were patched with an extracellular solution containing 145 mM NaCl, 2 mM CaCl$_2$, 4 mM KCl, 1 mM MgCl$_2$, 10 mM HEPES, and 10 mM glucose, pH adjusted to 7.4 and osmolality adjusted to 296 mOsm and an intracellular solution containing 140 mM CsF, 10 mM EGTA, 10 mM NaCl, 10 mM HEPES, pH adjusted to 7.3 and osmolality adjusted to 290 mOsm. The toxins were prepared in the extracellular solution supplemented with 0.1% human serum albumin. Current mediated by nAChR was elicited by the addition of 70 μM acetylcholine (ACh), and a wash with 2 U acetylcholinesterase was used to ensure complete ACh removal. The cells were preincubated with the toxins before the addition of ACh in combination with the
toxins. Rα-cobratoxin was tested at two different concentrations, 150 nM and 15 nM, while α-cobratoxin also was tested at 1.5 nM.

A previously isolated IgG antibody against α-cobratoxin, 2554.01_D11 [8], was used to neutralize the effect of both Rα-cobratoxin and α-cobratoxin as described elsewhere [7]. To test the neutralization, 100 nM IgG was preincubated with the toxins at various concentrations for at least 30 min at room temperature prior to the application. The analysis of the data was performed using the Sophion Analyzer (Sophion Software).

Phage display selection

Selection of the scFvs was performed by panning the IONTAS phage display library [29] (diversity of 4 × 10^10 human scFv clones, kindly donated by IONTAS Ltd., Pampsisford, UK) against biotinylated native and recombinant α-cobratoxin. Panning was carried out as described elsewhere [29], except that the biotinylated toxins were captured on streptavidin-coated beads (Dynabeads M-280, Invitrogen, Waltham, MA, USA) instead of direct coating to a 96-well microtiter plate. The concentration of the target toxins was decreased stepwise through the three rounds of selections, starting at 100 nM in the first round and ending at 25 nM in the third round.

Subcloning and primary screening of scFvs

Subcloning of the α-cobratoxin-binding selection output into the pSANG10–3 F expression vector and primary screening of candidates was performed as described elsewhere [6]. In brief, scFv genes from the selection outputs were subcloned from the pSANG4 phagemid vector to the pSANG10–3 F expression vector using NcoI and NotI restriction endonuclease sites and transformed into E. coli strain BL21(DE3). This expression vector allows for the production of scFvs with a C-terminal His6 tag followed by a 3xFLAG tag. From the two subcloned selection outputs, colonies from the Rα-cobratoxin and the α-cobratoxin selections were picked and expressed in 96-well plates. The scFvs were assessed for their binding to biotinylated α-cobratoxin and Rα-cobratoxin (5 µg/mL) indirectly immobilized on black MaxiSorp plates (Nunc A/S, Roskilde, Denmark) coated with streptavidin (10 µg/mL) using a DELFIA assay [7] (Perkin Elmer, Waltham, MA, USA). After thorough washing with PBS-T (PBS, 0.1% Tween-20) and PBS, scFv binding to the immobilized toxins was detected using a 1 in 1500 dilution of anti-FLAG M2 (F1804, Sigma, Saint-Louis, USA) previously conjugated with Europium (DELFIA Eu-N1 ITC chelate, 1244–301, Perkin Elmer).

Secondary screening and sequencing of scFvs

Clones from the α-cobratoxin (24 clones) and Rα-cobratoxin (51 clones) selection were cherry-picked and assessed for their binding to α-cobratoxin, Rα-cobratoxin, streptavidin, and the ubiquitin tag using a DELFIA assay as described above. From those, 20 clones from each selection were sequenced (Eurofins Genomics Sanger sequencing service, Ebersberg, Germany). The antibody framework and CDR regions were annotated and analyzed to identify 11 unique clones from the α-cobratoxin selection and 10 unique clones from the Rα-cobratoxin selection.

Expression-normalized capture DELFIA on native and denatured toxins

A DELFIA sandwich immunoassay was carried out on the unique clones as described elsewhere [6] using a biotinylated antigen concentration of 100 nM. Briefly, black Maxisorp plates (Nunc) were coated overnight with anti-FLAG M2 antibody (Sigma, 2.5 µg/mL in PBS, 50 µL per well). After blocking with 3% M-PBS (skim milk in PBS), washing with PBS, and addition of 25 µL of 6% M-PBS to each well, 25 µL of individual auto-induction culture supernatants containing expressed scFv was added for each scFv to the assay plate and incubated for 1 h. Plates were washed three times with PBS-T and three times with PBS. Binding of biotinylated antigen (100 nM of each antigen in 3% M-PBS, 50 µL per well) was allowed to occur for 1 h, which was followed by a detection step using Europium-labeled streptavidin (Perkin Elmer, 1244–360, 1 µg/mL in M-PBS, 50 µL per well) for 30 min. For the DELFIA using denatured toxins, the necessary amount of snake toxins (α-cobratoxin and long-neurotoxin 1 from N. naja) and Rα-cobratoxin were boiled for 15 min in presence of 4 mM DTT before being diluted into PBS to their final concentration (leading up to a final DTT concentration of 0.1 mM) and added to the wells.

scFv expression and purification

The top 3 binders were expressed and purified for further characterization as described elsewhere [30]. The scFvs were purified using HisTrap FF 1 mL columns (17531901, Cytiva, Marlborough, MA, USA) on an NGC Quest 10 system (Bio-Rad, Hercules, CA, USA). Protein concentration was determined by absorbance at 280 nm and the molar extinction coefficient predicted by ProtParam (web.expasy.org).

Determination of binding affinities with BLI (biolayer interferometry)

The binding affinities of the selected clones were measured using the Octet K2 system (FortéBio, Fremont, CA, USA). Measurements were performed at 30 °C in 96-well black microplates (655209, Greiner Bio-One, Kremsmünster, Upper Austria) that were agitated at 1000 rpm. The biotinylated α-cobratoxin (ligand, 50 nM) was captured on a Streptavidin (SA) Biosensor (18–5019, Sartorius, Göttingen, Germany). After a brief acid conditioning with glycine buffer (10 nM, pH 2.0), the toxin-coupled biosensor was neutralized in kinetic buffer (18–1105, Sartorius). Steady-state measurements consisted of equilibration of the toxin-coupled biosensor and the reference biosensor in kinetic buffer for 600 s, followed by a baseline reading for an additional 120 s. The sensors were then dipped into wells containing the scFvs at concentrations ranging from 2 µM to 4 nM in kinetic buffer for 600 s. The sensors were then dipped into wells containing kinetic buffer for 150 s to see if there was a dissociation rate before being regenerated with glycine buffer. To determine affinity, steady-state analysis was performed plotting the binding response at “equilibrium” (599 s) against the scFv concentration. Equilibrium dissociation constant (Kd) values were determined as the scFv concentration, at which half of the toxin sites are occupied at equilibrium using the One-site binding equation from GraphPad Prism 9.

In vitro blocking DELFIA

In vitro neutralization of the α-cobratoxin interaction with the α7 nicotinic acetylcholine receptor (α7-nAChR), recombinantly expressed as in [31], by the selected clones was performed using a similar DELFIA protocol to that described above, but with some modifications as described elsewhere [31]. Briefly, Maxisorp 96-well plates were coated overnight at 4 °C with 500 ng of α7-nAChR/well. Mixtures of serially diluted anti-α-cobratoxin clones and a fixed amount of biotinylated α-cobratoxin (0.1 µg/mL) were pre-incubated at room temperature for 30 min prior to being added to the coated plates. Wells containing only the biotinylated α-cobratoxin with no added anti-α-cobratoxin scFv or wells containing blocking buffer only (1 x PBS, 1% BSA) were used as controls to determine the percentage of inhibition of the binding between α-cobratoxin and α7-nAChR. Biotinylated α-cobratoxin bound to α7-nAChR was detected using Europium-labeled Streptavidin (Perkin Elmer, 1244–360, 1 µg/mL in assay buffer, 100 µL per well for 30 min). Each concentration was run in duplicate and presented as mean ± SEM values. The IC50 value of each scFv was determined by fitting dose-response curves to the data with GraphPad Prism 9 ([Inhibitor] vs. response - Variable slope equation).
Results and discussion

Patch-clamp-based characterization of the recombinant toxin demonstrates functionality

The recombinant α-cobratoxin, a 7.8 kDa three-finger toxin from N. kaouthia (71 amino acid residues, five disulfide bridges), was expressed in E. coli using the csCyDisCo system designed for the production of disulfide-rich peptides and proteins in the cytosol of E. coli [16]. After expression using auto-inducing medium, the recombinant α-cobratoxin-AviTag (Rx-cobratoxin-AviTag) fusion protein was purified from crude lysate by metal-affinity chromatography (Fig. 1B). Premature termination of protein translation or in vivo cleavage after the ubiquitin tag was observed, as reported by others [32], leading to two purified proteins; one corresponding to the full-length Ubi-His₁₀-α-cobratoxin and the other corresponding to the N-terminal fragment (Ubi-His₁₀). Before undergoing a TEV protease cleavage with the SuperTEV endoprotease, an SDS-PAGE analysis of the eluted protein under reducing and non-reducing conditions was performed to verify disulfide-bond formation (Fig. 1C). The species migrating with a molecular weight corresponding to that of the full-length protein (Ubi-His₁₀-α-cobratoxin; theoretical mass: 22.3 kDa) shifted up when using a reducing SDS-PAGE loading buffer, confirming the presence of disulfide bridges. Since all molecules present in the band representing Ubi-His₁₀-α-cobratoxin shifted upon reduction, it was concluded that disulfide bond formation had occurred. As observed in many other cases [33-36], TEV protease cleavage was incomplete and estimated to be around 50% based on SDS-PAGE analysis. To confirm the proper folding of the recombinant Rx-cobratoxin, its secondary structure was evaluated through circular dichroism analysis, demonstrating that the protein had a structural fold (suppl. Fig. S1). The in vitro biotinylated Rx-cobratoxin was further purified by size exclusion chromatography (SEC). As judged by a streptavidin gel-shift assay (Fig. 1D), the biotinylation level was estimated to be above 30%.

To assess whether the protein preparation contained properly folded Rx-cobratoxin, thereby allowing its use as antigen in a phage display selection campaign, the ability of the recombinant toxin to inhibit the nicotinic acetylcholine receptor (nAChR) was investigated using planar patch-clamp. The Rx-cobratoxin was tested at two different concentrations in parallel with native α-cobratoxin to test their ability to inhibit acetylcholine-induced current in a human cell line endogenously expressing the nAChR. At 150 nM, both Rx-cobratoxin and α-cobratoxin were able to fully inhibit the current, while more than 90% of the response was inhibited by 15 nM of Rx-cobratoxin (Fig. 2A). Complete inhibition was still observed using 15 nM of native α-cobratoxin, and even at 1.5 nM of native α-cobratoxin, significant inhibition of the receptor was measured. In these experiments, both native α-cobratoxin and Rx-cobratoxin could be neutralized by a human monoclonal IgG previously reported against native α-cobratoxin [8] (Fig. 2B). The IgG could, however, neutralize a higher concentration of Rx-cobratoxin than native α-cobratoxin. This observation could potentially be explained by Rx-cobratoxin being a mixture of correctly and incorrectly folded species. Nevertheless, based on its clear ability to inhibit nAChR, it was deemed that Rx-cobratoxin was of sufficient quality to be used as antigen in further phage display selection experiments.

Phage display selection on recombinantly expressed α-cobratoxin yields scFv binders

A naïve human single-chain variable fragment (scFv) phage display library containing 4 × 10¹⁰ clones was used for phage display selection [29]. Three rounds of selection were performed on streptavidin-coated magnetic beads functionalized with the biotinylated recombinant α-cobratoxin (TPL0442 selection) or the biotinylated α-cobratoxin from N. kaouthia (TPL0441 selection). The antigen concentration was decreased between each round to increase the stringency (from 100, 50–25 nM). After the third round, antibody-encoding genes (scFv format) from both selections were isolated and subcloned into a bacterial expression vector [37]. In total, 128 clones from the α-cobratoxin selection and 92 clones from the Rx-cobratoxin selection were picked and analyzed by a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) as previously described [6] (Fig. 3A and B). DELFIA is an alternative to traditional Enzyme-linked Immunosorbent Assay (ELISA), where HRP-labeled immunoreagents are replaced by lanthanide-labeled immunoreagents. Immunoreagent binding is measured by time-resolved fluorometry (TRF) instead of absorbance.

Fig. 2. *In vitro* inhibition of nAChR by recombinant and native α-cobratoxin. A) Representative current traces showing the nAChR-mediated current inhibited by increasing concentrations of native and recombinant α-cobratoxin. At 150 nM and 15 nM α-cobratoxin, the current is completely inhibited, and the current traces are therefore superimposed. B) Top: Inhibition of the nAChR response by increasing concentrations of native and recombinant α-cobratoxin. Bottom: IgG 2554_01_D11 (against native α-cobratoxin) can neutralize the effect of both the native and recombinant α-cobratoxin.
**Fig. 3.** Affinity ranking of scFvs and their sequences. A) Direct DELFIA against native $\alpha$-cobratoxin and Ra-cobratoxin of monoclonal scFvs from selections TPL0441 and TPL0442. B) Direct DELFIA against native $\alpha$-cobratoxin (dark blue), Ra-cobratoxin (light blue), and streptavidin (green) of cherry-picked monoclonal scFvs from selections TPL0441 and TPL0442. C) CDR sequences of the best binders selected from selections TPL0441 and TPL0442 (IMGT numbering). D) Direct and ENC DELFIA of the top ten monoclonal scFv-containing supernatants from both selections.
(ELISA). Of these first screened clones, 10 clones displaying a specific binding signal against α-cobratoxin from TPL0441 selection and 10 clones from TPL0442 selection were picked for DNA sequencing and further characterization (Fig. 3C and supplementary file 1). Interestingly, a high proportion of these clones (>60% for TPL0441 and 10% for TPL0442) showed a six amino acid disulfide loop, C-X₄-C, in the V₃H CDR3 sequences, as noticed previously [7]. These scFvs were then evaluated in an expression-normalized capture (ENC) DELFIA assay, which reduces the influence of the clone expression levels on the signal (Fig. 3D). The three α-cobratoxin-binding scFvs that yielded the highest binding signals (two from TPL0441 selection and one from TPL0442 selection) were expressed for further characterization.

Isolated scFvs have similar affinity to a positive control antibody

Binding affinities of TPL0441_01_F04, TPL0441_01_H05, and TPL0442_01_G02 to α-cobratoxin were evaluated by steady-state analysis of biolayer interferometry binding curves alongside a positive control antibody 368_01_C05 (Fig. 4). The positive control antibody was previously selected against the native α-cobratoxin from N. kaouthia and was characterized in vitro and demonstrated to have partially neutralizing activity in vivo [7]. The affinities of TPL0441_01_H05 and TPL0442_01_G02 were similar to each other (around 10 nM) and very close to the affinity of the positive control antibody (8.4 nM). The weakest binder, TPL0441_01_F04, showed a submicromolar affinity resulting from a fast dissociation from the α-cobratoxin coated tip in the biolayer interferometry setup.

Fig. 4. Estimation of dissociation constants with biolayer interferometry. A) Affinity of three selected scFvs to α-cobratoxin was measured using streptavidin-coated biosensors to capture the biotinylated α-cobratoxin. The reported concentrations (above the highest positioned curve) represent the highest analyte concentration used in the threefold dilution series. B) Steady-state analysis of the binding curves presented in A).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Kᵢ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPL0441_01_F04</td>
<td>269.8 ± 89.5 x 10⁻⁹</td>
</tr>
<tr>
<td>TPL0441_01_H05</td>
<td>13.5 ± 3.6 x 10⁻⁹</td>
</tr>
<tr>
<td>TPL0442_01_G02</td>
<td>11.5 ± 11.2 x 10⁻⁹</td>
</tr>
<tr>
<td>368_01_C05</td>
<td>8.4 ± 6.5 x 10⁻⁹</td>
</tr>
</tbody>
</table>
The selected scFvs block the α-cobratoxin:receptor binding interaction

To assess if the scFvs selected against the recombinant α-cobratoxin prevented the interaction between the nAChR and the native toxin, a receptor-blocking assay was performed with the three selected clones and our positive control scFv. Results showed that the three selected scFvs were able to prevent binding between the receptor subunit and α-cobratoxin (Fig. 5). The IC50 values were determined to be in the nM to sub-µM range, with TPL0441_01_H05 being the most potent inhibitor (IC50 of 18.9 nM) and TPL0442_01_G02 being the least potent (IC50 of 240 nM). Interestingly, TPL0441_01_F04, the weakest binder, which has a sub-µM affinity for α-cobratoxin (32-fold weaker than the positive control antibody), showed a better blocking potency than TPL0442_01_G02, which has a nM affinity, indicating that affinity does not always directly correlate with neutralization capacity, as previously observed [7,8].

The toxin-antibody binding interaction relies on a structural epitope

Upon determination of the binding affinities and the IC50 values of the selected clones, a DELFIA-based assay was employed to investigate the binding mode(s) of the scFvs to the native and denatured toxin (Fig. 6). In addition to α-cobratoxin from N. kaouthia, another three-finger toxin from the Naja genus with 81% sequence identity, the long neurotoxin 1 from N. nivea, was also employed. Here, TPL0442_01_G02, TPL0441_01_F04, and 368_01_C05 lost their ability to bind to α-cobratoxin when the toxin was denatured, demonstrating that those scFvs interact with one or more structural epitopes on the toxin. Notably, the two clones selected against the native α-cobratoxin also showed binding to the denatured long neurotoxin 1 from N. nivea, but not to its native form. This might indicate that a linear epitopic element may exist in this toxin, which has been unfolded upon denaturation, but which is not accessible to the scFv when the toxin is folded in its three-dimensional structure. In regard to clone TPL0441_01_H05, binding to α-cobratoxin was not detected using the capture DELFIA, even though biolayer interferometry showed measurable binding. This could be due to the scFv orientation in the capture DELFIA assay, where the scFv is immobilized in the well as opposed to the biolayer interferometry assay, where the toxin is immobilized to the streptavidin coated tip.

Conclusion

In this study, it is demonstrated that α-cobratoxin from N. kaouthia can be recombinantly expressed in a form that allows for the selection of monoclonal scFv antibodies with similar binding affinities and functional neutralization potency as those scFvs selected against the native toxin. Thereby, it is exemplified that neutralizing monoclonal antibodies against snake toxins can be obtained entirely in vitro and without the need of snakes and animals for immunization. Importantly, the methods presented here not only remove a potential procurement bottleneck for snake venoms but may also enable the expression and study of non-natural toxins, such as toxin mutants, toxoids, tagged toxins, or fusion proteins. While such other toxins might find utility as molecular tools for research, it is also envisaged that they can help unravel new biology in the field of toxin and venom evolution. However, it is also noted that the recombinant expression of three-finger toxin is complicated by the presence of many cysteine residues in these toxins and that methods that could improve correct protein folding are warranted.

Funding

This research was funded by the Villum Foundation (Grant No. 00025302, A.H.L.), the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (Grant agreement No. 850974, A.H.L.), the Independent Research Fund
Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability
Data will be made available on request.

Appendix A. Supplementary material
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.04.002.

References

