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Pitfalls to avoid when using phage display for snake toxins

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Abstract

Antivenoms against bites and stings from snakes, spiders, and scorpions are associated with immunological side effects and high cost of production, since these therapies are still derived from the serum of hyper-immunized production animals. Biotechnological innovations within envenoming therapies are thus warranted, and phage display technology may be a promising avenue for bringing antivenoms into the modern era of biologics. Although phage display technology represents a robust and high-throughput approach for the discovery of antibody-based antitoxins, several pitfalls may present themselves when animal toxins are used as targets for phage display selection. Here, we report selected critical challenges from our own phage display experiments associated with biotinylation of antigens, clone picking, and the presence of amber codons within antibody fragment structures in some phage display libraries. These challenges may be detrimental to the outcome of phage display experiments, and we aim to help other researchers avoiding these pitfalls by presenting their solutions.

1. Introduction

Envenomings from snakes, scorpions, and spiders represent a serious neglected health issue in large parts of the developing world, causing pain and suffering to millions of victims with severe cases resulting in amputation (for snakebite) or even death (David Warrell et al., 2007; Williams et al., 2011). The cornerstone of envenoming therapies still consist of animal-derived antisera, which remain the only effective treatment options against snakebites, spider bites, and scorpion stings (Chippaux, 2012; Everardo Remi Rodríguez Rodríguez et al., 2015; Gutiérrez et al., 2011). However, since antisera suffer from drawbacks including immunogenicity due to their heterologous nature, complex production processes due to dependence on venoms and the immune systems of production animals, and batch-to-batch variation, an increasing amount of research is being focused on alternative approaches based on monoclonal antibodies and recombinant DNA technology (Laustsen et al., 2016a, 2016c; Richard et al., 2013; Rodríguez-Rodríguez et al., 2016; Roncolato et al., 2015). These novel approaches may hold the promise of delivering biotechnology-based therapies with improved efficacy, higher safety, and potentially lower cost of production (Laustsen et al., 2016a, 2016b; Rodríguez-Rodríguez et al., 2016).

One approach that has gained increasing attention within development of novel antivenoms is the use of phage display technology for discovery of antibodies and antibody fragments (Roncolato et al., 2015). Phage display technology exploits the linkage between antibody genotype and phenotype obtained by incorporating antibody genes, typically single-chain variable fragments (scFvs) or single-domain antibody fragments (V_H Hs or Nanobodies[®]) (Figure 1) into the DNA of bacteriophages displaying the antibody peptide sequence on its outer coat (Laustsen, 2016a) (Figure 2). In addition to phage display technology, several other approaches for development of novel antivenoms and antitoxins have been investigated, such as the use of small molecules (Lewin et al., 2016), the use of DNA epitopes strings (Harrison, 2004; Wagstaff et al., 2006), or the use of hybridoma

technology (Castro et al., 2014; Frauches et al., 2013). However, it is beyond the scope of this article to discuss these approaches in detail (see (Laustsen et al., 2016a, 2016d; Roncolato et al., 2015) for comprehensive reviews of these topics).

In phage display experiments, the M13 bacteriophage is often employed to create a library, where antibody fragments are displayed on the pIII coat protein of the M13 phage virion, while the antibody fragment encoding gene is incorporated into the DNA of the phage virion (Hoogenboom et al., 1998; Rodi and Makowski, 1999; Sidhu, 2000), see Figure 2. The phage display library is then used for *in vitro* selection by attaching the target antigen to a plate well or bead, and panning the phage virions onto the target (Parmley and Smith, 1988). Non-binding phages are then washed away after sufficient incubation time, and binding phages can be eluted, amplified in *E. coli* TG1, and either subjected to additional rounds of panning or analysis (typically ELISA or gel electrophoresis). After a few cycles of panning, monoclonal phage virions can be isolated and their DNA sequenced in order to reveal the sequence of the displayed antibody fragment (Laustsen, 2016a). Several factors influence the outcome of a phage display experiment, including the introduction of deselection steps to remove unwanted antibody fragments, the affinity of the displayed antibody fragments, the level of antibody display, antigen immobilization and presentation, and clonal variation. Clonal variation may further affect antibody fragment translation, folding, transport, and stability of the fusion, which may further create amplification biases towards phage virions displaying undesired antibody fragments (Barbas et al., 1991; Bass et al., 1990; Garrard et al., 1991; John McCafferty, 1996; Lowman et al., 1991).

Since McCafferty et al. reported the development of the first scFv phage display library in 1990 (McCafferty et al., 1990), a wealth of antibodies and antibody fragments have successfully reached the clinic for a wide range of indications (Nelson and Reichert, 2009; Schofield et al., 2007). In the field of antivenom development, phage display technology

was already introduced in 1995 when Meng et al. isolated the first murine scFv against different Mojave rattlesnake toxins (Meng et al., 1995). Since then, other researchers have reported the discovery of both human scFvs and camelid V_HHs against phospholipases A₂ and neurotoxins from both vipers and elapids (Chavanayarn et al., 2012; Kulkeaw et al., 2009; Richard et al., 2013; Roncolato et al., 2013; Stewart et al., 2007; Tamarozzi et al., 2006). Yet, antivenoms have still not entered the modern era of biopharmaceuticals, where protein-based therapies are produced recombinantly (Laustsen, 2016a; Laustsen et al., 2016a, 2016d). Part of the reason that this transition from serum-based therapies to recombinant antivenoms has not yet occurred is due to the difficulty of obtaining sufficient funding and resources for developing complex biologic therapies against conditions affecting mainly poor and rural communities of the tropical regions of the world. However, another part of the explanation could be the different technical obstacles encountered in phage display experiments, where animal venom toxins are used as targets. Many animal venom toxins are difficult to isolate in sufficient amounts and in high purity (Roncolato et al., 2015), the venoms may be difficult to procure, and the medically relevant venom components need to be identified (Laustsen et al., 2015c), which may be further complicated by toxin synergism (Laustsen, 2016b). In addition to some of the general considerations when using phage display technology, including loss of diversity during the panning and amplification rounds (Derda et al., 2011) and ineffective incorporation of the pIII-antibody protein (Roncolato et al., 2015), other specific challenges may be encountered in antitoxin discovery projects based on phage display.

In this paper, we report five non-obvious obstacles that we have encountered in our research within antitoxin phage display. These obstacles have significantly delayed our own research, and therefore we wish to help other researchers by discussing how they present themselves and their solutions. It should be noted that the results presented here are from our ongoing work on developing toxin-neutralizing antibodies and antibody fragments.

Thus, our antibodies and antibody fragments are at the time of writing still undergoing preclinical evaluation. However, irrespectively of whether our efforts of developing toxin-neutralizing antibodies will be successful, the challenges and solutions presented here are universal in nature for the identification of toxin-binding and/or toxin-neutralizing antibody fragments. Although these obstacles and solutions presented here do not exhaustively cover all challenges that may be encountered in phage display experiments, it is the hope that they may help the growing number of researchers already engaged or about to engage in development of next generation antivenoms against snake, scorpion, spider, and bee envenomings. Additional tricks for optimizing phage display experiments exists. These include switching of saturant (blocking buffer) between panning rounds, the use of chaotrophic agents during pannings, or performing pannings at low or elevated temperature to select for binder with thermostability (or to preserve antigens that are unstable at given temperatures). Other phage display challenges, including avidity, clonal variation, stability of the antigen, and the level of display, are discussed elsewhere (John McCafferty, 1996).

2. Materials and Methods

2.1. Venoms and toxins

Venom fractions containing Short neurotoxin 1, dendrotoxins, and α -elapitoxins from the venom of *D. polylepis* were purified according to the protocol given in (Laustsen et al., 2015d). *D. polylepis* venom and purified α -cobratoxin from *N. kaouthia* were obtained from Latoxan SAS, France. *A. laevis* venom was obtained from four specimens kept at the National Aquarium, Den Blå Planet, Denmark, frozen, lyophilized, and kept at -20 °C. PLA₂s were isolated according to a protocol similar to the one found in (Laustsen et al., 2015b). All toxins employed in the studies reported here were in a molecular range of 6 kDa to 14 kDa.

2.2. Biotinylation

Different toxins from *Naja kaouthia* and *Dendroaspis polylepis* were dissolved in phosphate buffered saline (PBS, Dulbecco's Phosphate Buffered Saline Sigma-Aldrich) to yield concentration between 0.5 to 6 µg/µl. Biotin linked to *N*-hydroxysuccinimide (NHS) via two different linkers (Aliphatic linker: EZ-Link™ Sulfo-NHS-LC-Biotin, No-Weigh™ Format, 21327, Thermo Scientific. PEG₄-linker: EZ-Link™ NHS-PEG₄-Biotin, No-Weigh™ Format, 21329, Thermo Scientific) was added in toxin to biotinylation reagent at ratios from 1:1.5 to 1:20 according to the protocol supplied by the manufacturer and left at room temperature for 30 min. Purification of the biotinylated toxins was achieved using buffer exchange columns (Vivacon 500, Sartorius, 2000 Da Molecular Weight Cut-Off). After three washes with 500 µL PBS, the columns were turned and the biotinylated toxins were eluted in 100-200 µL PBS.

The extent of biotinylation of the toxins was assessed using a Pierce™ Fluorescence Biotin Quantitation Kit (46610, Thermo Scientific), following the protocol supplied by the manufacturer.

Protein concentrations were determined using absorbances measured on a BMG labtech PHERAStar Fluorescence Spectrophotometer and individually calculated extinction coefficients (<http://web.expasy.org/protparam/>).

2.3. MS analysis

Purified biotinylated toxins and native toxins were adsorbed onto C18 micro-ZipTips (Millipore) to wash out any salt contaminants, after which the toxins were eluted with 2 µL 50% acetonitrile in water, containing 0.1% trifluoroacetic acid, directly onto an OptiTOF 384 plate. Immediately following, the eluate was mixed with 0.6 µL matrix (saturated α-cyanohydroxycinnamic acid in 50% acetonitrile, 0.1% TFA) and allowed to dry. The samples were analyzed by MALDI-TOF in a Proteomics Analyzer 4800 Plus mass

spectrometer (Applied Biosystems) operated in positive linear mode. Spectra were acquired using 1000 shots at a laser intensity of 4800.

2.4. Phage display selection protocol 1 (phospholipases A₂ from *Aipysurus laevis*)

Three phage display selection rounds using Tomlinson I + J libraries were carried out as described in the following. MaxiSorp™ plates (NUNC, Roskilde, Denmark) were coated with 100 µl of phospholipases A₂, *A. laevis* crude venom, or streptavidin (Sigma-Aldrich, Saint Louis, USA) dissolved in PBS at a concentration of 0.01 µg/µl and left overnight at 4 °C. Uncoated wells were left with PBS. The following day, wells with directly coated toxin were washed three times with PBS, filled to the brim with 2% milk powder (Fluka analytical) dissolved in PBS (2% M-PBS), and incubated at room temperature for two hours in order to block the wells. Streptavidin-coated wells were blocked for one hour with 2% M-PBS, after which 100 µl biotinylated toxin (1 µg) dissolved in 2% M-PBS was added and left for one hour to bind. After blocking and binding of biotinylated toxin, all wells were washed three times with PBS and approximately 10¹³ phages dissolved in 100 µl 2% M-PBS of library I and J respectively were added and incubated for one hour at room temperature with shaking (250 rpm), followed by one hour of incubation at room temperature without shaking. Supernatant was discarded and the plates were washed ten times with PBS containing 0.1% Tween[®] 20 (PBST, Sigma-Aldrich). Phages were eluted by adding 100 µl per well of trypsin-PBS (diluted 1:5 in PBS, Sigma-Aldrich). Supernatant was transferred to an Eppendorf tube containing 600 µl of *E. coli* TG1 grown in 2xTY to an OD₆₀₀ = 0.4 and was incubated at 37 °C for 30 minutes without shaking. The cells were then spun down at 11,600 x g for five minutes, resuspended in 50 µl 2xTY, and plated out on TYE-agar (containing 100 µg/ml ampicillin and 1% glucose). All plates were incubated (bottom up) overnight at 37 °C.

The following day, 2 ml 2xTY was added to the overnight grown plates and the cells were loosened with a spreader. From these solutions, 10 µl was transferred to 10 ml fresh 2xTY containing 100 µg/ml ampicillin and 1% glucose and incubated until $OD_{600} = 0.4$ was reached (approx. 1 hour). Following this, 5×10^{10} helper phage in 100 µl was added to the 10 ml and incubated at 37 °C without shaking for 30 minutes. The culture was spun down at 3,000 x g (Sigma Centrifuge 4K15) for ten minutes and the pellet resuspended in 25 ml of 2xTY containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 0.1% glucose, and incubated overnight at 30 °C. The following day, the overnight culture was spun down at 3,300 x g for 15 minutes. Then, 6.25 ml PEG/NaCl (20% Polyethylene glycol 6000, 2.5 M NaCl) was mixed well with supernatant and left on ice for one hour. This solution was then spun at 3,300 x g for 30 min, the PEG/NaCl was discarded, and the pellet resuspended in 2 ml PBS. The resuspended pellet was spun at 11,600 x g for ten minutes (to remove cellular debris) and 1 ml of the phage containing supernatant was transferred to a new 1.5 ml microcentrifuge tube. Two more rounds of selection were performed according to the procedure described above.

Isolation of monoclonal binders was achieved by plating out individual *E. coli* TG1 colonies on TYE-agar plates. Colonies from these plates were transferred using an inoculation loop into 100 µl 2xTY containing 100 µg/ml ampicillin and 1% glucose in 96 well plates and grown shaking overnight at 37 °C. The following day, 20 µl was transferred from the overnight plates to a second 96 well plate containing 200 µl of 2xTY with 100 µg/ml ampicillin and 1% glucose. These were grown for one hour at 37 °C. Then 25 µl 2xTY containing 100 µg/ml ampicillin, 1% glucose, and 10^9 helper phage was added to each well. The plate was incubated another hour at 37 °C, while shaking. Afterwards, the plate was centrifuged for ten min at 1,800 x g and the supernatant was aspirated off. The pellet was resuspended in 200 µl 2xTY containing 100 µg/ml ampicillin and 50 µg/ml kanamycin and grown overnight shaking at 30 °C. The following day, overnight cultures

were spun down at 1,800 x g for ten minutes and 50 µl of the supernatant was mixed with 50 µl 4% M-PBS and used in monoclonal phage ELISA as described in section 2.6.

2.5. Phage display selection protocol 2 (α -cobratoxin from *Naja kaouthia*)

Vials (NUNC, Immuno Tube MaxiSorp, PK3000) were coated with 250 µL 10 µg/mL streptavidin (Pierce® Streptavidin, 21125, Thermo Scientific) in PBS and left at 4 °C overnight. 100 mL of 2xYT media was inoculated with *E. coli* TG1 and incubated at 250 rpm and 37 °C overnight.

Next day, the vials were washed three times with 4 mL PBS and blocked with 4 mL 3% milk in PBS (M-PBS) for 1 hour at room temperature. Meanwhile, 420 µL of IONTAS phage library (an updated version of the library developed by Schofield et al. (Schofield et al., 2007)) was mixed with 420 µL 6% M-PBS and 840 µL 3% M-PBS and incubated for 1 hour at room temperature to pre-block the library. Simultaneously, 420 µL of Dynabeads M-280 Streptavidin (10 mg/mL, 11206D, Invitrogen by Life Technologies) was washed three times with 800 µL 3% M-PBS and re-suspended in 1 mL 3% M-PBS and incubated for 1 hour on a rotor. After one hour of blocking with M-PBS, 250 µL of 5 µg/mL biotinylated toxin (Dp4, Dp5, Dp6, Dp7, Dp8, and Dp20 from *D. polylepis* and α -cobratoxin (and α -CbtX) from *Naja kaouthia*) in 3% M-PBS was added to the vials. The vials were then incubated at room temperature for 1 hour. The Dynabeads M-280 Streptavidin centrifuged at 15,000 rpm for 5 min, the supernatant removed, and the beads were re-dissolved in 420 µL 3% M-PBS. The beads were then mixed with the pre-blocked library and incubated for 1 hour at room temperature on a rotor. Meanwhile, an appropriate amount (determined by OD measurement) of the overnight TG1 culture was added to a shake flask containing 100 mL 2xYT and set to grow at 37 °C at 250 rpm for 1-2 hours. After 1 hour on rotor, the phage library was separated from the beads by 1 min centrifugation at 15,000 rpm (Eppendorf Centrifuge 5417R), followed by placing the

Eppendorf tube on a magnetic rack, and using a pipette. The vials were washed five times with PBS, and 250 μ L of the separated phage solution was added to the vials, which were then incubated at room temperature for 1 hour. The vials containing the phages were washed six times with 4 mL PBS + 0.2% Tween[®] 20 and six times with 4 mL PBS. Then, 250 μ L of trypsin in Phage Elution Buffer (1/100 dilution of TPCK-trypsin from 10 μ g/mL stock in 50 mM Tris pH 8, 1 mM CaCl₂) was added to the vials, and the vials were incubated at room temperature for 15 min. The solutions from the vials were added to Falcon tubes containing 5 mL TG1 culture with an OD₆₀₀ of 0.5, which were incubated at 37 °C at 150 rpm for 1 hour. After incubation, 10 μ L of the cultures were added to Eppendorf tubes containing 990 μ L 2xYT. After mixing, two 2xYT-AG (2xYT containing 2% glucose and 100 μ g/mL ampicillin) plates were prepared for each culture by adding 10 μ L of the solutions and 100 μ L of the solutions, respectively to each plate. The plates were incubated at 30 °C overnight and counted next day to determine cfu. The remaining solutions in the Falcon tubes were centrifuged for 10 min at 2100 g (Heraeus Megafuge 40R Centrifuge). The supernatants were discarded, and the pellets were re-suspended in 50 μ L 2xYT and plated out on an 2xYT-AG plate. The plates (termed Output plates) were incubated at 30 °C overnight. As controls, 200 μ L of the TG1 culture was also plated out on both a 2xYT-AG plate and a 2xYT-KG plate (2xYT containing 2% glucose and 50 μ g/mL kanamycin). The 2xYT-AG plate was incubated at 30 °C overnight. The 2xYT-KG plate was incubated at 37 °C overnight.

Next day, the Output plates were scraped using 2 mL of a solution containing: 14 mL of 2xYT, 6 mL of 50% glycerol, and 100 μ g/ml ampicillin. The 2 mL were transferred to 50 mL Falcon tubes and rotated for 30 min. A sufficient amount (determined by OD₆₀₀) of the Output scraping was used to inoculate 10 mL of 2xYT-AG to obtain an OD₆₀₀ of 0.1. These new suspensions were incubated at 37 °C at 250 rpm for 1.5 hours. The remaining of the Output scrapings were stored in cryo tubes at -80 °C. Once the incubated solutions had

reached an OD₆₀₀ of 0.4-0.5, 100 µL of MK13KO7-trp helper phages (1/3000 dilution of 6 x 10¹⁴ phage/mL in 2xYT-AG) was added, and the solutions were incubated at 37 °C at 150 rpm for 1 hour. After 1 hour, the cells were centrifuged for 10 min at 2100 g. The supernatants were removed by decanting. The cells were re-suspended in Erlenmeyer flasks containing 40 mL of 2xYT-AK (2xYT containing 100 µg/mL of Ampicillin and 50 µg/mL of Kanamycin) and incubated at 25 °C at 280 rpm overnight.

Next day, the different solutions incubated in Erlenmeyer flasks were transferred to Falcon tubes and spun down at 10,500 g for 10 min (Sorvall Lynx 4000 Centrifuge). The supernatants were transferred to 50 mL Falcon tubes containing 10 mL 20% PEG-8000 + 2.5 M NaCl, mixed, and kept on ice for 1 hour to allow precipitation. After 1 hour, the phages were centrifuged at 15,000 g for 10 min (Sorvall Lynx 4000 Centrifuge). The supernatants were discarded, and the phage pellets were re-suspended in 500 µL PBS in Eppendorf tubes. The Eppendorf tubes were centrifuged at 9,300 g for 10 min (Eppendorf Centrifuge 5417R), and the supernatants were transferred to other Eppendorf tubes to get rid of cell debris. This was done twice more, since cell debris continued to be present.

Two more rounds of selection were performed according to the procedure described above with two exceptions: 1) In round 2 and 3, the phage libraries were not pre-incubated with Dynabeads M-280 Streptavidin. 2) Instead of streptavidin, 10 µg/mL NeutrAvidin (NeutrAvidin™ Biotin Binding Protein, 31000, Thermo Scientific) was used for coating in round 2 to deselect the streptavidin-binding phages.

2.6. ELISA protocol I

Polyclonal phage binders to the toxins, obtained from the Tomlinson I + J libraries after each of the rounds of selection, were investigated using ELISA. First, a MaxiSorp™ plate was coated with toxins or streptavidin overnight as described in section 2.5. The following day, wells coated with toxin were washed three times with PBS, filled to the brim

with 2% M-PBS, and incubated at room temperature for two hours to block the wells. Streptavidin-coated wells were blocked for one hour with 2% M-PBS, after which 100 μ l biotinylated toxin (1 μ g) dissolved in 2% M-PBS was added, and the well was left for one hour to allow biotin capture. Each well was then washed three times with PBST and two times with PBS to remove unspecific binders. Then, a mixture of 50 μ l 4% M-PBS and 50 μ l phage from each panning round was added to their respective wells and incubated for one hour on a plate shaker. Wells were then washed three times with PBS and two times with PBST, and 100 μ l of solution containing a 1:1000 dilution of anti-M13 monoclonal antibody (Horseradish Peroxidase conjugated, GE Healthcare) in 2% M-PBS was added to each well and incubated at one hour at room temperature on a plate shaker. Wells were washed three times with PBST and two times with PBS. Then 100 μ l of OPD solution (2 mg *ortho*-Phenylenediamine, DAKO, and 2.5 μ L H₂O₂ in 2.5 ml H₂O) was added to each well to achieve color development. Reactions were stopped by adding 100 μ l 0.5 M H₂SO₄ (Sigma-Aldrich) to each well and absorbances were measured at 490 nm (VersaMax Tunable Microplate reader, Molecular Devices).

2.7. ELISA protocol 2

The progress of the panning rounds in Phage Display Experiment 2 was monitored by ELISA. Each panning round was tested against the following antigens: Dp4, Dp5, Dp6, Dp7, Dp8, and α -cobratoxin, and synthesized epitopes (Epitope 1, 2, and 3) representing linear epitopes from type 1 and type 2 α -neurotoxins from *D. polylepis* (Laustsen, 2016a).

Wells (NUNC, MaxiSorp) were coated with 100 μ L 10 μ g/mL streptavidin (Pierce® Streptavidin, 21125, Thermo Scientific) in PBS and left at 4 C overnight. Control wells were coated with 10 μ g/mL NeutrAvidin (NeutrAvidin™ Biotin Binding Protein, 31000, Thermo Scientific).

Next day, the wells were washed three times with PBS and blocked with 200 μ L 2% M-PBS for 1 hour at room temperature on a mixer. The wells were then washed three times with PBS. 50 μ L of 1 μ g/ml of all different antigens and 50 μ L of 10 μ g/ml for selected peptides (Epitope 1, Epitope 2, Epitope 3) in 2% M-PBS was added to their respective wells, and the plates were incubated for 2.5 hours at room temperature on a mixer. The first control wells (NeutrAvidin) were left in or 200 μ L of 2% M-PBS, while the second control wells (streptavidin) wells were left in 50 μ L of 2% M-PBS on a mixer. The wells were washed three times with PBS. Solutions of 50 μ L of the different 50x phage libraries (dilution 1/100 in 2% M-PBS) found during selections rounds were added to their respective wells in duplicates. The wells were put on mixer for 1 hour. The wells were washed three times with PBS + 0.2% Tween[®] 20 and three times with PBS. 50 μ L of Mouse anti-M13 antibody (GE Healthcare) in 2% M-PBS (dilution 1/1000) was added to each well. The wells were put on mixer for 1 hour, followed by three washes with PBS + 0.2% Tween[®] 20 and three times with PBS. 100 μ L of Europium labeled anti-mouse antibody (Perkin Elmers) in 2% M-PBS (dilution 1/1000) was added to each well. The wells were put on mixer for 1 hour, washed three times with PBS + 0.2% Tween[®] 20 and three times with PBS. 100 μ L of DELFIA enhancement solution was added to each well. The wells were put on mixer for 5 minutes. The plates were read using a BMG labtech PHERAStar Fluorescence Spectrophotometer (excitation 340 nm, emission 615 nm).

3. Results and discussion

The pitfalls presented here were encountered during the course of approximately 50 different phage display selection experiments against snake venom toxins involving different displayed peptide, V_HH, and human scFv phage display libraries. From these, the results presented here will utilize six illustrative experiments with human scFv

libraries, of which four were run in parallel against phospholipases A₂ from the olive sea snake, *Aipysurus laevis*, according to section 2.4 in Materials and Methods, and two were run in sequence against α -cobratoxin, the medically most important toxin from the monocled cobra, *Naja kaouthia* (Laustsen et al., 2015a), according to section 2.5 in Materials and Methods.

3.1. Optimal display of antigens via biotinylation

Biotinylation of antigens involves the covalent attachment of a biotin molecule to the antigen via a chemical linker. Biotinylation may be employed for obtaining a better display of the antigen, as the linker will enable the antigen to be distanced from the solid-phase surface, when streptavidin coated wells, beads, or vials are used for panning, thereby avoiding conformational change of the antigen and reducing the possibility of steric hindrance in the antigen-antibody interaction. Although biotinylation may thus provide beneficial properties to a phage display selection experiment, this extra step may also bring about experimental complications, of which some will be discussed in the following.

3.1.1. Biotinylation versus direct coating

An important advantage of immobilization of biotinylated antigens to streptavidin via a linker in comparison with directly coated antigens is that the antigen may be better presented. This may allow for a larger part of its surface to interact with phage virions during pannings (Bronfman et al., 2003) and the achievement of better control of the concentration of bound antigen. Immobilization via a linker also allows the antigen to be distanced from the bottom and inner walls of the well, vial, or surface of a bead. This may contribute to better preservation of the native conformation of the antigen, which may in some cases be denatured when directly coated (see Figure 4A and 4B). However, the drawbacks of biotinylation include the addition of an extra (antigen consuming) step in the

discovery process and the possibility that biotinylation may interfere with a desired site on the antigen, which would have been optimal for selection.

In our experiments, we compared the outcomes from panning rounds with the Tomlinson I and J libraries (human scFv libraries) against both biotinylated PLA₂s captured by streptavidin (see Figure 5A and 5B) and directly coated PLA₂s from *A. laevis* (see Figure 5C and 5D). No normalization of the phage concentrations between different panning rounds was performed. Therefore the polyclonal ELISA results should mainly be used to evaluate signal ratios for a given panning round between target toxin and control. Based on the high signal intensities and signal ratios in ELISA assays between these different panning rounds, our results indicate that much better selection is obtained for the biotinylated PLA₂s than for the un-biotinylated PLA₂s, possibly due to better antigen presentation. Furthermore, when comparing the selectivity of polyclonal phage virions it is seen from the binding signals that phage virions from the Tomlinson I library selected against biotinylated PLA₂s were specific towards the biotinylated PLA₂s and did not bind to directly immobilized PLA₂s after the third round of panning (Figure 6A). In contrast, phage virions from the Tomlinson J library selected against biotinylated PLA₂s bind to both biotinylated PLA₂s and native PLA₂s (Figure 6B, panning round 3). The quality (e.g. diversity in displayed antibody fragments, naivety, and size) of a phage display antibody library is of high importance for the outcome of a phage display experiment. This is supported by the results presented in Figure 5 and Figure 6, from which it could be inferred that the Tomlinson Library I is of lower quality than the Tomlinson Library J in this given experiment, since selective PLA₂ binders are more quickly accumulated from this library. Nevertheless, it is seen in Figure 6A that a large decrease in signal intensities in the ELISA occur for the Tomlinson Library I between the second and third round of panning, indicating that successful positive selection of selective binder against biotinylated PLA₂ has occurred.

Our results thus suggest that immobilization of PLA₂s via biotinylation and streptavidin capture may provide better selection conditions for discovery of toxin-binding scFvs via phage display. As an alternative solution, other researchers have successfully employed amine binding plates for antigen immobilization via direct chemical coupling of toxins to the bottom of the wells (through a spacer) for the discovery of camelid V_HH inhibitors of α -cobratoxin (Richard et al., 2013). Outside of toxinology, immobilization to streptavidin-coated beads has successfully been used to identify scFv binders via solution-based panning (see e.g. (Haque and Tonks, 2012)), however, since these approaches have not been employed to discover antitoxins, they will not be discussed further here, but may be worth exploring for the interested researcher.

3.1.2. Over-biotinylation of antigen

Although biotinylation of the antigen may provide better selection condition during a phage display experiment, it is of paramount importance that the antigen is optimally biotinylated (one biotin per antigen for small toxins). Over-biotinylation may lead to masking of the antigen itself, thereby hindering the phage virions from accessing it (Figure 4C). In experiments performed with the IONTAS human scFv library (an updated version of the library developed by Schofield et al. (Schofield et al., 2007)) using α -cobratoxin as the antigen, two different batches of biotinylated α -cobratoxin were employed. In the first batch, α -cobratoxin had been biotinylated at a toxin-to-biotinylation reagent-ratio of 1:20 (leading to over-biotinylation, see Figure 7). In the second batch, α -cobratoxin had been biotinylated at a toxin-to-biotinylation reagent-ratio of 1:1.5 (primarily leading to singly biotinylated antigen). The extent of biotinylation was originally assessed using the PierceTM Fluorescence Biotin Quantitation Kit (46610, Thermo Scientific), according to which α -cobratoxin molecules had been biotinylated 0.7 times on average. However, when

assessed by MS (see Figure 7) it was evident that an average of 3-4 biotin moieties (each moiety having a mass of 0.5 kDa) had been conjugated to the antigen. This difficulty of over-biotinylation was unfortunately not immediately uncovered. Therefore, initial phage display selection experiments were performed, which did not yield polyclonal phage binders (Figure 8A). When the second batch of singly biotinylated α -cobratoxin was used, selection rounds yielded a good pool of α -cobratoxin binders that showed cross-recognition to homologous type 2 α -neurotoxins present in fractions Dp6 and Dp7 from *D. polylepis* venom (Figure 8B).

Again, our results demonstrate the applicability of the biotinylation approach for snake toxins to be used as antigens in phage display selection experiments, but also underline the importance of obtaining an optimal biotin-to-toxin ratio.

3.1.3. The effect of linker physico-chemistry

Many snake toxins have a small mass (three-finger toxins: 6-8 kDa and PLA₂s: 12-14 kDa), and since the biotinylation of a protein adds an additional 0.5 kDa to the molecule, the nature of the linker may have a profound effect on the physico-chemical properties of the antigen. Two different linkers were assessed in the biotinylation of different toxins (**Aliphatic linker:** EZ-Link™ Sulfo-NHS-LC-Biotin, No-Weigh™ Format, 21327, Thermo Scientific. **PEG₄-linker:** EZ-Link™ NHS-PEG₄-Biotin, No-Weigh™ Format, 21329, Thermo Scientific). In several experiments performed using the aliphatic linker, a white precipitate was clearly observed. In contrast, when using a hydrophilic PEG₄-linker, no precipitate could be seen, and NanoDrop® measurements of protein concentration in the purified solutions of toxins biotinylated with the PEG₄-linker confirmed that the biotinylated toxins were still in solution. It is therefore suggested that the hydrophobic nature of the aliphatic linker has a detrimental effect on the hydrophobicity of the biotinylated toxins leading to precipitation (Figure 9A). Thus, the use of more hydrophilic

linkers, such as the PEG₄-linker, may indeed be favorable for small snake toxins, as it allows the toxins to remain in solution (Figure 9B).

Additionally, the length of the linker may have an impact on antigen presentation, and it may occasionally be worthwhile to test different linker lengths to identify one that provides better display of the antigen. Taken together with the fact that streptavidin may bind up to four different biotin moieties, using biotinylation and streptavidin capture could at least theoretically lead to crowding of the presented toxins. This may potentially have negative effects on phage display selection experiments, as this may render entire or parts of the presented toxin antigen unavailable for binding interaction.

3.2. Clone picking

In phage display selection experiments performed with the Tomlinson I and J libraries on biotinylated PLA₂s from *A. laevis*, polyclonal ELISAs revealed that an accumulation of PLA₂-specific phage virions had taken place in the course of the three panning rounds (Figure 5A and 5B). From the different panning rounds (particularly round 2 and 3) phage virions were used to transfect *E. coli* TG1 cells, which were plated out to yield monoclonal TG1 colonies. Two different screening experiments were then performed on selected colonies. In the first experiment 288 primarily large colonies were picked and screened by monoclonal ELISA. This yielded a low rate of positive PLA₂-binders (3.8% of clones showing a signal three times higher than the background signal), see Figure 10A. Due to the somewhat unsuccessful outcome of this experiment, another 84 primarily small colonies were picked from the same plates and screened by monoclonal ELISA. In contrast, this yielded a high percentage of positive, selective PLA₂-binders (54.8% of clones showing a signal three times higher than the background signal), see Figure 10B. This observation is likely to be explained by the fact that phage production may be proportional to colony size.

Phage virions with truncations in (or absence of) their displayed antibody fragment create less metabolic strain on their host cells. These host cells therefore have a growth advantage in comparison to cells infected by phage virions displaying fully functional high affinity scFvs (Bruin et al., 1999). Clones with a growth advantage multiply faster, allowing them to produce more phage virions, since more phage virion producing cells will be present and since each phage virion is produced with less metabolic cost. Therefore, such “non-antigen binding” phage virions will dominate the total pool of phage virions despite having a lower affinity to the antigen target (Umlauf et al., 2015). Also, antibody fragments that are toxic to bacteria may be deleted due to negative selection pressure during amplification (Schofield et al., 2007). Thus, supported by observations reported in the scientific literature, our results indicate that an undesired growth bias may interfere with isolation of high affinity toxin binders in phage display experiments. This growth bias may however to some extent be dealt with by careful selection of small colonies over larger ones.

3.3. Amber codons in antibody libraries

A final pitfall that may be encountered when using certain libraries (such as the Tomlinson libraries) in phage display selection experiments is the presence of amber codons within the scFv fragment. Amber codons (TAG) are typically stop codons, which may however by some organisms and strains (such as the *E. coli* TG1 strain, which is a non-suppressor of the amber codon) be interpreted as a codon for glutamate. The amber codon is intentionally inserted after the myc-tag (see Figure 11) in e.g. the Tomlinson libraries, which allows for full biosynthesis of the scFv-pIII product in TG1 cells, but which will terminate protein synthesis in prior to gIII in non-suppressor strains (such as *E. coli* HB2151). This allows for easy expression of soluble scFvs following infection of a non-suppressor strain with phages directly after a phage display selection experiment. The

pitfall with amber codons, however, presents itself, when an amber codon is present within the scFv fragment – a phenomenon occurring due to the approaches used for construction of certain libraries (such as the Tomlinson library). When this occurs, the functional scFv-pIII product may be obtained from the TG1 strain and thus be enriched by phage display selection, but the construct will be prematurely terminated, when the soluble scFv expression is attempted in other strains. The presence of amber codons resulting in premature termination of protein synthesis has been reported by Wu et al. (Wu et al., 2007) and Roncolato et al. (Roncolato et al., 2015). In our phage display selection experiments against PLA₂s from *A. laevis*, we encountered the amber codon in the CDR2 region of the V_H of some of our scFv binders (Figure 11). In order to overcome this pitfall, it is a necessity to sequence the phagemid DNA and either introduce a glutamate encoding codon instead of the premature amber codon by site directed mutagenesis (Barderas et al., 2006), or simply to synthesize the entire scFv gene construct and transform it into the expression strain. This can be a cumbersome process, and undetected, it can lead to large amounts of wasted time.

4. Concluding remarks

The pitfalls presented here do not comprise an exhaustive set of challenges that may be encountered during phage display selection experiments with the purpose of identifying toxin-binding scFvs for the development of recombinant antivenoms. However, they do provide further insight into certain difficulties revolving around proper presentation of toxin antigens, how to avoid growth bias and select bacterial colonies expressing high affinity binder, and how to detect and possibly circumvent the difficulty that premature amber codons present in the scFv gene sequence represent. It is the hope that these findings may help to guide researchers in their efforts towards developing recombinant antivenoms against venoms from snakes, spider, scorpions, bees, and possibly other creatures, and that

526 such efforts may ultimately help envenomed victims in poor rural parts of the world, where
527 most envenomings occur.

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530 **References**

- 531 Barbas, C.F., Kang, A.S., Lerner, R.A., Benkovic, S.J., 1991. Assembly of combinatorial
532 antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. U. S.*
533 *A.* 88, 7978–7982.
- 534 Barderas, R., Shochat, S., Martínez-Torrecuadrada, J., Altschuh, D., Meloen, R., Ignacio
535 Casal, J., 2006. A fast mutagenesis procedure to recover soluble and functional
536 scFvs containing amber stop codons from synthetic and semisynthetic antibody
537 libraries. *J. Immunol. Methods* 312, 182–189. doi:10.1016/j.jim.2006.03.005
- 538 Bass, S., Greene, R., Wells, J.A., 1990. Hormone phage: an enrichment method for variant
539 proteins with altered binding properties. *Proteins* 8, 309–314.
540 doi:10.1002/prot.340080405
- 541 Bronfman, F.C., Tcherpakov, M., Jovin, T.M., Fainzilber, M., 2003. Ligand-induced
542 internalization of the p75 neurotrophin receptor: a slow route to the signaling
543 endosome. *J. Neurosci. Off. J. Soc. Neurosci.* 23, 3209–3220.
- 544 Bruin, R. de, Spelt, K., Mol, J., Koes, R., Quattrocchio, F., 1999. Selection of high-affinity
545 phage antibodies from phage display libraries. *Nat. Biotechnol.* 17, 397–399.
546 doi:10.1038/7959
- 547 Castro, J.M.A., Oliveira, T.S., Silveira, C.R.F., Caporrino, M.C., Rodriguez, D.,
548 Moura-da-Silva, A.M., Ramos, O.H.P., Rucavado, A., Gutiérrez, J.M., Magalhães,
549 G.S., Faquim-Mauro, E.L., Fernandes, I., 2014. A neutralizing recombinant single
550 chain antibody, scFv, against BaP1, A P-I hemorrhagic metalloproteinase from
551 *Bothrops asper* snake venom. *Toxicon Off. J. Int. Soc. Toxinology* 87, 81–91.
552 doi:10.1016/j.toxicon.2014.05.017
- 553 Chavanayarn, C., Thanongsaksrikul, J., Thueng-in, K., Bangphoomi, K., Sookrung, N.,
554 Chaicumpa, W., 2012. Humanized-Single Domain Antibodies (VH/VHH) that
555 Bound Specifically to *Naja kaouthia* Phospholipase A2 and Neutralized the
556 Enzymatic Activity. *Toxins* 4, 554–567. doi:10.3390/toxins4070554
- 557 Chippaux, J.-P., 2012. Emerging options for the management of scorpion stings. *Drug Des.*
558 *Devel. Ther.* 6, 165–173. doi:10.2147/DDDT.S24754
- 559 David Warrell, José María Gutiérrez, A. Padilla, 2007. Rabies and Envenomings: a
560 Neglected Public Health Issue: Report of a Consultative Meeting. World Health
561 Organization, Geneva.
- 562 Derda, R., Tang, S.K.Y., Li, S.C., Ng, S., Matochko, W., Jafari, M.R., 2011. Diversity of
563 phage-displayed libraries of peptides during panning and amplification. *Mol. Basel*
564 *Switz.* 16, 1776–1803. doi:10.3390/molecules16021776
- 565 Everardo Remi Rodríguez Rodríguez, Lidia Riaño Umbarila, Lourival D. Possani, Baltazar
566 Becerril, 2015. Recombinant Neutralizing Antibodies, A New Generation of
567 Antivenoms, in: *Toxinology - Scorpion Venoms*. Springer, Dordrecht Heidelberg
568 New York London, p. 580.
- 569 Frauches, T.S., Petretski, J.H., Arnholdt, A.C.V., Lasunskiaia, E.B., de Carvalho, E.C.Q.,
570 Kipnis, T.L., da Silva, W.D., Kanashiro, M.M., 2013. Bothropic antivenom based

- on monoclonal antibodies, is it possible? *Toxicon* 71, 49–56.
doi:10.1016/j.toxicon.2013.05.005
- Garrard, L.J., Yang, M., O'Connell, M.P., Kelley, R.F., Henner, D.J., 1991. Fab assembly and enrichment in a monovalent phage display system. *Biotechnol. Nat. Publ. Co.* 9, 1373–1377.
- Gutiérrez, J.M., León, G., Lomonte, B., Angulo, Y., 2011. Antivenoms for snakebite envenomings. *Inflamm. Allergy Drug Targets* 10, 369–380.
- Haque, A., Tonks, N.K., 2012. Use of Phage Display to Generate Conformation-Sensor Recombinant Antibodies. *Nat. Protoc.* 7, 2127–2143. doi:10.1038/nprot.2012.132
- Harrison, R., 2004. Development of venom toxin-specific antibodies by DNA immunisation: rationale and strategies to improve therapy of viper envenoming. *Vaccine* 22, 1648–1655. doi:10.1016/j.vaccine.2003.09.046
- Hoogenboom, H.R., de Bruijne, A.P., Hufton, S.E., Hoet, R.M., Arends, J.-W., Roovers, R.C., 1998. Antibody phage display technology and its applications. *Immunotechnology* 4, 1–20. doi:10.1016/S1380-2933(98)00007-4
- John McCafferty, 1996. Phage display: factors affecting panning efficiency. *Phage Display of Peptides and Proteins: A Laboratory Manual*, in: *Phage Display of Peptides and Proteins*. Academic Press Inc., San Diego, CA, USA, pp. 261–76.
- Kulkeaw, K., Sakolvaree, Y., Srimanote, P., Tongtawe, P., Maneewatch, S., Sookrung, N., Tungtrongchitr, A., Tapchaisri, P., Kurazono, H., Chaicumpa, W., 2009. Human monoclonal ScFv neutralize lethal Thai cobra, *Naja kaouthia*, neurotoxin. *J. Proteomics* 72, 270–282. doi:10.1016/j.jprot.2008.12.007
- Laustsen, A.H., 2016a. Recombinant antivenoms, 1st ed. University of Copenhagen, Copenhagen, Denmark.
- Laustsen, A.H., 2016b. Toxin synergism in snake venoms. *Toxin Rev.* 35, 165–170. doi:10.1080/15569543.2016.1220397
- Laustsen, A.H., Engmark, M., Milbo, C., Johannesen, J., Lomonte, B., Gutiérrez, J.M., Lohse, B., 2016a. From Fangs to Pharmacology: The Future of Snakebite Envenoming Therapy. *Curr. Pharm. Des.* 22.
- Laustsen, A.H., Gutiérrez, J.M., Lohse, B., Rasmussen, A.R., Fernández, J., Milbo, C., Lomonte, B., 2015a. Snake venomomics of monocled cobra (*Naja kaouthia*) and investigation of human IgG response against venom toxins. *Toxicon* 99, 23–35. doi:10.1016/j.toxicon.2015.03.001
- Laustsen, A.H., Gutiérrez, J.M., Rasmussen, A.R., Engmark, M., Gravlund, P., Sanders, K.L., Lohse, B., Lomonte, B., 2015b. Danger in the reef: Proteome, toxicity, and neutralization of the venom of the olive sea snake, *Aipysurus laevis*. *Toxicon, Omic perspectives of the toxin universe* 107, Part B, 187–196. doi:10.1016/j.toxicon.2015.07.008
- Laustsen, A.H., Johansen, K.H., Engmark, M., Andersen, M.R., 2016b. Snakebites: costing recombinant antivenoms. *Nature* 538, 41. doi:10.1038/538041e
- Laustsen, A.H., Lohse, B., Lomonte, B., Engmark, M., Gutiérrez, J.M., 2015c. Selecting key toxins for focused development of elapid snake antivenoms and inhibitors guided by a Toxicity Score. *Toxicon* 104, 43–45. doi:10.1016/j.toxicon.2015.07.334

- Laustsen, A.H., Lomonte, B., Lohse, B., Fernández, J., Gutiérrez, J.M., 2015d. Unveiling the nature of black mamba (*Dendroaspis polylepis*) venom through venomomics and antivenom immunoprofiling: Identification of key toxin targets for antivenom development. *J. Proteomics* 119, 126–142. doi:10.1016/j.jprot.2015.02.002
- Laustsen, A.H., Solà, M., Jappe, E.C., Oscoz, S., Lauridsen, L.P., Engmark, M., 2016c. Biotechnological Trends in Spider and Scorpion Antivenom Development. *Toxins* 8, 226. doi:10.3390/toxins8080226
- Laustsen, A.H., Solà, M., Jappe, E.C., Oscoz, S., Lauridsen, L.P., Engmark, M., 2016d. Biotechnological trends in spider and scorpion antivenom development. *Toxins* Accepted.
- Lewin, M., Samuel, S., Merkel, J., Bickler, P., 2016. Varespladib (LY315920) Appears to Be a Potent, Broad-Spectrum, Inhibitor of Snake Venom Phospholipase A2 and a Possible Pre-Referral Treatment for Envenomation. *Toxins* 8. doi:10.3390/toxins8090248
- Lowman, H.B., Bass, S.H., Simpson, N., Wells, J.A., 1991. Selecting high-affinity binding proteins by monovalent phage display. *Biochemistry (Mosc.)* 30, 10832–10838.
- McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J., 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348, 552–554. doi:10.1038/348552a0
- Meng, J., John, T.R., Kaiser, I.I., 1995. Specificity and binding affinity of an anti-crotoxin combinatorial antibody selected from a phage-displayed library. *Biochem. Pharmacol.* 50, 1969–1977.
- Nelson, A.L., Reichert, J.M., 2009. Development trends for therapeutic antibody fragments. *Nat. Biotechnol.* 27, 331–337. doi:10.1038/nbt0409-331
- Parmley, S.F., Smith, G.P., 1988. Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. *Gene* 73, 305–318. doi:10.1016/0378-1119(88)90495-7
- Richard, G., Meyers, A.J., McLean, M.D., Arbabi-Ghahroudi, M., MacKenzie, R., Hall, J.C., 2013. In Vivo Neutralization of α -Cobratoxin with High-Affinity Llama Single-Domain Antibodies (VHHs) and a VHH-Fc Antibody. *PLoS ONE* 8, e69495. doi:10.1371/journal.pone.0069495
- Rodi, D.J., Makowski, L., 1999. Phage-display technology – finding a needle in a vast molecular haystack. *Curr. Opin. Biotechnol.* 10, 87–93. doi:10.1016/S0958-1669(99)80016-0
- Rodríguez-Rodríguez, E.R., Olamendi-Portugal, T., Serrano-Posada, H., Arredondo-López, J.N., Gómez-Ramírez, I., Fernández-Taboada, G., Possani, L.D., Anguiano-Vega, G.A., Riaño-Umbarila, L., Becerril, B., 2016. Broadening the neutralizing capacity of a family of antibody fragments against different toxins from Mexican scorpions. *Toxicon* 119, 52–63. doi:10.1016/j.toxicon.2016.05.011
- Roncolato, E.C., Campos, L.B., Pessenda, G., Costa e Silva, L., Furtado, G.P., Barbosa, J.E., 2015. Phage display as a novel promising antivenom therapy: A review. *Toxicon* 93, 79–84. doi:10.1016/j.toxicon.2014.11.001

- Roncolato, E.C., Pucca, M.B., Funayama, J.C., Bertolini, T.B., Campos, L.B., Barbosa, J.E., 2013. Human antibody fragments specific for *Bothrops jararacussu* venom reduce the toxicity of other *Bothrops* sp. venoms. *J. Immunotoxicol.* 10, 160–168. doi:10.3109/1547691X.2012.703253
- Schofield, D.J., Pope, A.R., Clementel, V., Buckell, J., Chapple, S.D., Clarke, K.F., Conquer, J.S., Crofts, A.M., Crowther, S.R., Dyson, M.R., Flack, G., Griffin, G.J., Hooks, Y., Howat, W.J., Kolb-Kokocinski, A., Kunze, S., Martin, C.D., Maslen, G.L., Mitchell, J.N., O’Sullivan, M., Perera, R.L., Roake, W., Shadbolt, S.P., Vincent, K.J., Warford, A., Wilson, W.E., Xie, J., Young, J.L., McCafferty, J., 2007. Application of phage display to high throughput antibody generation and characterization. *Genome Biol.* 8, R254. doi:10.1186/gb-2007-8-11-r254
- Sidhu, S.S., 2000. Phage display in pharmaceutical biotechnology. *Curr. Opin. Biotechnol.* 11, 610–616. doi:10.1016/S0958-1669(00)00152-X
- Stewart, C.S., MacKenzie, C.R., Hall, J.C., 2007. Isolation, characterization and pentamerization of alpha-cobrotoxin specific single-domain antibodies from a naïve phage display library: preliminary findings for antivenom development. *Toxicon Off. J. Int. Soc. Toxinology* 49, 699–709. doi:10.1016/j.toxicon.2006.11.023
- Tamarozzi, M.B., Soares, S.G., Marcussi, S., Giglio, J.R., Barbosa, J.E., 2006. Expression of recombinant human antibody fragments capable of inhibiting the phospholipase and myotoxic activities of *Bothrops jararacussu* venom. *Biochim. Biophys. Acta* 1760, 1450–1457. doi:10.1016/j.bbagen.2006.04.008
- Umlauf, B.J., McGuire, M.J., Brown, K.C., 2015. Introduction of plasmid encoding for rare tRNAs reduces amplification bias in phage display biopanning. *BioTechniques* 58, 81–84. doi:10.2144/000114256
- Wagstaff, S.C., Laing, G.D., Theakston, R.D.G., Papaspyridis, C., Harrison, R.A., others, 2006. Bioinformatics and multiepitope DNA immunization to design rational snake antivenom. *PLoS Med* 3, e184.
- Williams, D.J., Gutiérrez, J.-M., Calvete, J.J., Wüster, W., Ratanabanangkoon, K., Paiva, O., Brown, N.I., Casewell, N.R., Harrison, R.A., Rowley, P.D., O’Shea, M., Jensen, S.D., Winkel, K.D., Warrell, D.A., 2011. Ending the drought: New strategies for improving the flow of affordable, effective antivenoms in Asia and Africa. *J. Proteomics, “Omic” studies on Neglected Tropical Diseases* 74, 1735–1767. doi:10.1016/j.jprot.2011.05.027
- Wu, S., Ke, A., Doudna, J.A., 2007. A fast and efficient procedure to produce scFvs specific for large macromolecular complexes. *J. Immunol. Methods* 318, 95–101. doi:10.1016/j.jim.2006.10.005

Figure legends

Figure 1. Overview of the different antibody formats employed to construct phage display antibody libraries and how these formats relate to human (scFv) and camelid (V_HH) antibodies, respectively.

Figure 2. Schematic representation of the M13 bacteriophage containing single-stranded DNA (ssDNA) and displaying an scFv on the pIII protein.

Figure 3. Schematic representation of a phage display selection experiment. **(1)** First the scFv displaying phage library is panned against the target toxin, which is bound to a well. **(2)** Non-binding phage particles are washed away. **(3)** Binding phage particles are eluted. **(4)** Phage particles are amplified and either submitted to another round of selection or **(5)** analyzed by polyclonal ELISA.

Figure 4. Schematic representation of antigen coating strategies and problems during phage display selection. **(A)** When an antigen is coated directly onto the bottom of a well (or surface of a bead), the antigen may change its native conformation. **(B)** Biotinylation of the antigen may provide a way to distance the antigen from the solid-phase surface and into solution for better accessibility and to avoid conformational change of the antigen. **(C)** Over-biotinylation of antigens can lead to masking of the antigen epitopes, hampering their recognition by antibody paratopes.

Figure 5. Polyclonal ELISA results from Phage Display Experiment 1 with the Tomlinson Libraries I and J against PLA₂s from *A. laevis* and control (uncoated) (see Materials and Methods 2.4). **(A)** Tomlinson Library I panned against biotinylated PLA₂ toxins. **(B)** Tomlinson Library J panned against biotinylated PLA₂ toxins. **(C)** Tomlinson Library I panned against directly coated PLA₂ toxins. **(D)** Tomlinson Library J panned against directly coated PLA₂ toxins.

Figure 6. Polyclonal ELISA results from Phage Display Experiment 1 with the Tomlinson Libraries I and J against biotinylated PLA₂s and directly coated PLA₂s from the *A. laevis*, streptavidin, and control (uncoated) **(A)** Tomlinson Library I. **(B)** Tomlinson Library J.

Figure 7. MALDI-TOF mass spectra for α -cobratoxin from *Naja kaouthia* biotinylated with different toxin-to-biotinylation reagent-ratios showing that over-biotinylation (more than one biotin moiety per toxin molecule) occurs at higher toxin-to-biotinylation reagent-ratios. **(A)** Native α -cobratoxin. **(B)** Biotinylated α -cobratoxin, toxin-to-biotinylation reagent-ratio: 1:1. **(C)** Biotinylated α -cobratoxin, toxin-to-biotinylation reagent-ratio: 1:2. **(D)** Biotinylated α -cobratoxin, toxin-to-biotinylation reagent-ratio: 1:5 (over-biotinylation). **(E)** Biotinylated α -cobratoxin, toxin-to-biotinylation reagent-ratio: 1:10 (over-biotinylation). **(F)** Biotinylated α -cobratoxin, toxin-to-biotinylation reagent-ratio: 1:20 (over-biotinylation).

Figure 8. Polyclonal phage ELISA for panning rounds 2 and 3 for the IONTAS scFv phage display library panned against α -cobratoxin from *Naja kaouthia* following Phage display selection protocol 2 (see Materials and Methods 2.5). **(A)** ELISA results from panning rounds using over-biotinylated α -cobratoxin (toxin-to-biotinylation reagent-ratio of 1:20). **(B)** ELISA results from panning rounds using α -cobratoxin with more optimal biotinylation (toxin-to-biotinylation reagent-ratio of 1:1.5). **Dp5-Dp8:** Fractions 5, 6, 7, and 8 from *Dendroaspis polylepis* containing Short neurotoxin 1, dendrotoxins, and α -elapitoxins (numbering according to (Laustsen et al., 2015d)). **Cbtx:** α -cobratoxin from *N. kaouthia*.

Figure 9. Schematic representation of the effect of linker chemistry on the biotinylated antigen/toxin employed for phage display selection. **(A)** The solubility of small toxins may be highly affected by aliphatic linkers, which can lead to precipitation of the biotinylated antigen, making it useless for coating. **(B)** Using hydrophilic linkers (such as PEG-linkers) may retain the solubility of the toxin, thereby providing better antigens for coating.

Figure 10. Monoclonal ELISA results for clones selected from various panning rounds in Phage Display Experiment 1 (see Materials and Methods 2.4). **(A)** In the first attempt, 288 clones were picked from primarily large colonies, of which only a very low number displayed binding to PLA₂. **(B)** In the second attempt, 84 clones were picked from small colonies, of which more than 50% displayed selective binding to PLA₂. Note: Clones were ranked and numbered according to their signal intensities.

764 **Figure 11.** Conceptual sequence of one of our scFvs displaying the different elements
765 of the scFv, the presence of a desired amber codon after the myc-tag, and an undesired
766 amber codon in the CDR2 region of the V_H.

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768 Figure 1

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There is no ethical issue to report, in connection with the manuscript.

Sincerely yours,

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