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YEAST DISPLAY SCREENING OF SINGLE-DOMAIN ANTIBODIES AGAINST SNAKE VENOM TOXINS

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Abstract

Snakebite envenoming remains a neglected tropical disease, and conventional animal-derived antivenoms are limited by cost, cross-reactivity and adverse reactions. Single-domain antibodies, the variable fragments of camelid heavy-chain antibodies, are small and stable and have attracted interest as recombinant alternatives. This review, which reports no new data, summarises published work on how yeast surface display is used to select such binders against venom toxins. It covers venom composition, antibody format, library construction, flow-cytometric sorting, reported neutralisation studies and developability concerns. The literature shows that yeast display offers quantitative control over selection, although most examples against venom components are preliminary and clinical translation will depend on broad neutralisation across species.

1. INTRODUCTION

Snakebite envenoming kills and disables more people than most recognised tropical diseases, yet it attracted little international funding until the World Health Organization placed it on its list of neglected tropical diseases. Modelling studies suggest that hundreds of thousands of people are envenomed every year and tens of thousands die, with many more left with amputations, contractures or chronic kidney disease [1, 2, 3]. The burden falls mainly on rural agricultural workers in South Asia, sub-Saharan Africa and Latin America, who are poor and often far from a hospital [4, 5]. A global strategy published in 2019 set

targets to halve deaths and disability, and better antivenom is central to it [6].

The therapeutic standard has barely changed in a century. Horses or sheep are immunised with venom, and the purified immunoglobulins or fragments from their plasma are given intravenously. Such products save lives, but they are expensive to make and store, they contain many antibodies unrelated to the toxins that matter, and they may provoke early anaphylactoid reactions or serum sickness [7, 8, 9]. Because they are raised against the venom of particular species, their ability to neutralise related species is variable. These problems have prompted a search for recombinant antibodies, made in microbial or mammalian cells and chosen for activity against the toxins that cause most harm [10].

Among candidate formats, single-domain antibodies have been particularly attractive. This review, which reports no new data, brings together published literature on how yeast surface display is used to isolate and improve single-domain antibodies against snake venom toxins. Related methods such as phage display are discussed where they set a benchmark. The sections that follow describe venom toxins as targets, the structure and properties of the binders, the mechanics of yeast display, screening designs and reported results, and the practical hurdles standing between a promising clone and a usable drug. The principal findings are collated in table 1.

2. VENOM TOXINS AS TARGETS

Snake venoms are mixtures of dozens to hundreds of proteins and peptides, but a small number of toxin families account for most of their lethal and disabling effects. Proteomic surveys of many species show that three-finger toxins and phospholipases A_2 dominate the venoms of elapids such as cobras, kraits and mambas, whereas snake venom metalloproteinases, serine proteinases and phospholipases A_2 predominate in vipers [11, 12, 13]. Three-finger toxins block nicotinic acetylcholine receptors and cause paralysis. Metalloproteinases degrade basement membranes and clotting factors, producing haemorrhage, coagulopathy and local tissue destruction. The relative abundance of each family differs between and even within species.

Table 1: Selected published work on display-derived single-domain and related antibodies against venom or related toxins

Study or source|approach	Reported finding	Ref.
Boder and Wittrup Original yeast display	Antibody fragments displayed through Aga2p fusion and sorted by flow cytometry	[14]
Feldhaus et al. Nonimmune library	Binders isolated from a naive yeast-displayed library without immunisation	[15]
McMahon et al. Synthetic nanobody library	Conformationally selective nanobodies obtained against membrane protein targets	[16]
Uchanski et al. Immune library	Improved yeast platform for screening nanobody immune libraries	[17]
Richard et al. Llama VHH	Single-domain antibodies and a VHH-Fc protected mice against α-cobratoxin	[18]
Hmila et al. Bivalent VHH	Bivalent and chimeric formats raised neutralisation of scorpion toxin AahI'	[19]
Laustsen et al. Phage display	Oligoclonal human IgG neutralised dendrotoxin-mediated mamba venom toxicity in mice	[20]
Boder et al. Directed evolution	Antibody fragments evolved to monovalent femtomolar affinity by repeated sorting	[21]
Ledsgaard et al. Cross-reactivity	Cross-reactivity of antibodies is a key parameter in antivenom research	[22]
Alirahimi et al. Review	Nanobodies discussed as emerging therapeutic agents in envenomation	[23]

Geography complicates the picture further. Venom composition in a single species can differ between regions, between juveniles and adults, and even between individual animals, with the differences sometimes large enough to alter the clinical syndrome that follows a bite. Proteomic surveys have catalogued these shifts for many medically important species, and the resulting databases show that no single venom profile can be assumed for a given species name [12, 13]. For an antibody

developer this means that a binder validated against one reference venom is only a starting point. Panels of venoms from several localities, tested in parallel, are a more honest basis for claims of broad activity than a single laboratory standard [8, 11].

This composition has practical consequences for antibody design. An effective product must neutralise the few toxin families that account for clinical severity, and it must do so across geographically separated populations of the same species, where venom composition changes. Antivenomics, which uses affinity chromatography and mass spectrometry to measure how well an antivenom binds each venom component, has revealed that conventional products often bind poorly to low-molecular-mass toxins, which are weakly immunogenic in horses [9, 13]. Those small toxins, including the short and long neurotoxins, are among the most lethal, which is a strong argument for recombinant binders chosen directly against them [10, 18].

Clinical syndromes offer a rational way to rank targets. Neurotoxic envenoming, typical of elapid bites, presents with ptosis, bulbar weakness and respiratory failure within hours, and its treatment is time critical. Haemotoxic envenoming from vipers causes incoagulable blood, bleeding and shock, while cytotoxic effects produce swelling and necrosis around the bite that can lead to permanent tissue loss [1, 8]. Each syndrome points to a different toxin family and to a different timescale for treatment. A binder aimed at a post-synaptic neurotoxin needs to act fast and reach the neuromuscular junction, whereas one aimed at a metalloproteinase may have more time to work but must penetrate damaged tissue [9].

3. SINGLE-DOMAIN ANTIBODIES

Camelids produce a class of antibodies lacking light chains, first described in dromedary serum in 1993 [24]. The antigen-binding unit of such a heavy-chain antibody is a single variable domain, termed VHH or nanobody, of about 15 kDa. Though a tenth the size of an IgG, it binds antigens with affinities comparable to those of conventional antibodies, and it folds as a single polypeptide that tolerates heat and chemical stress better than most paired fragments [25, 26]. Its third complementarity-determining region is often longer than in conventional antibodies and can extend into clefts that are hard for larger molecules to enter, such as enzyme active sites [27].

Generating the raw material for an immune library follows a well-documented protocol. A llama or alpaca receives a series of injections of toxin or venom with adjuvant, peripheral blood lymphocytes are collected after the response has matured, and RNA is converted to cDNA from which the VHH repertoire is amplified by PCR and cloned into the display vector [17, 28]. Library sizes of 10^7 to 10^8 independent clones are typical and adequate, since the immune response has already enriched for relevant sequences [25, 28]. Detoxified or recombinant toxin is often used for immunisation when handling live venom is judged unsafe for the animal, with the caveat that structure may change on detoxification.

For venom toxins these traits have both appeal and drawbacks. The small size allows rapid tissue distribution, and for neurotoxins that act within minutes at nerve endings this may help. Expression in bacteria or yeast is cheap, and constructs can be linked into bivalent or bispecific formats to raise avidity or cover two toxins [18, 19]. The drawback is pharmacokinetic. A nanobody is cleared by the kidney within hours, which is shorter than the time needed to neutralise toxins released slowly from a bite site. Fusing the domain to an Fc region or to albumin-binding modules can extend half-life, but at the cost of larger and more complex molecules [18, 25].

Immunogenicity is less of a worry than the term foreign might suggest, since nanobody frameworks resemble human VH3 family sequences, and humanisation by framework substitution has been done for therapeutic candidates [25]. Evidence in the venom field is limited, though. Most reported anti-toxin nanobodies were tested in mice, and even there many were given before or together with the toxin and not afterwards. The few studies on binder formats outside the camelid scaffold, such as human IgG obtained from phage display libraries against mamba

dendrotoxins, show that oligoclonal mixtures can protect animals from venom lethality, which reinforces the idea that a small set of well-chosen binders may suffice [10, 20].

4. YEAST SURFACE DISPLAY

In the yeast surface display system developed in the 1990s, the protein of interest is fused to the Aga2p subunit of the a-agglutinin mating adhesion complex and secreted by *Saccharomyces cerevisiae*, where it attaches by disulfide bonds to Aga1p anchored in the cell wall [14]. A tag at one terminus reports expression. Each cell therefore carries tens of thousands of copies of a single variant on its surface, and the genotype is housed within the same cell. Since yeast cells are large enough for flow cytometry, the library can be analysed and sorted with quantitative precision [14, 29, 30].

Library construction uses one of two broad routes. Immune libraries are generated from the B-cell repertoire of a camelid immunised with venom or purified toxin, which enriches for binders already matured in vivo; naive or synthetic libraries are assembled from germline-based frameworks with randomised loops, allowing selection without animals. A nonimmune human library displayed on yeast showed that binders could be isolated without immunisation [15], and a synthetic nanobody library later yielded conformationally selective binders to membrane proteins [16]. Immune nanobody libraries have likewise been displayed on yeast with an improved vector design [17]. Transformation efficiency usually caps yeast libraries at around 10^8 to 10^9 clones, below the sizes available by phage display [29, 31].

The distinctive advantage over phage is the ability to see what is happening. Labelled antigen at different concentrations lets sorting windows be placed on the affinity of the displayed clone, and a second fluorescent reagent against the expression tag normalises binding to the amount of protein on each cell, which removes a bias towards well-expressed variants [29, 30]. Counter-selection can be added by labelling a second antigen with another colour, so that cells binding both are excluded when a family-specific binder is wanted, or retained when breadth is the goal. Directed evolution on yeast has pushed antibody fragments into the femtomolar range of monovalent affinity [21].

It helps to set yeast display beside phage display, which has produced most of the venom-neutralising antibodies reported to date. Phage libraries can reach 10^{10} to 10^{11} members, and panning is simple, cheap and scalable, but the enrichment is a bulk process that gives little information about individual clones until the end [20, 31]. Yeast display trades library size for resolution: each round can be inspected, gates can be set from the data, and affinity-matured variants can be recovered along with kinetic information. Many groups combine them, using phage for the first broad enrichment and yeast to refine the output, which draws on the strengths of both systems without demanding the impossible from either [29, 31].

Eukaryotic expression is the second commonly cited benefit. Proteins pass through the endoplasmic reticulum, where chaperones and quality control favour correctly folded, disulfide-bonded molecules, so unstable clones tend to be displayed poorly and are removed from the pool before sorting begins [29, 30]. This is useful for toxin-binding nanobodies, which often depend on a disulfide between framework regions. The system has costs of its own, including glycosylation sites that differ from those in mammalian cells, and avidity effects at high display density that can make weak binders look better than they are. Careful work therefore verifies hits as soluble monomers and not merely as surface-displayed clones [16, 17, 29].

5. SCREENING STRATEGIES AND REPORTED OUTCOMES

A typical campaign begins with a library, immune or synthetic, transformed into yeast and induced for display. Magnetic selection with biotinylated toxin on beads often reduces the library cheaply in a first round, after which fluorescence-activated cell sorting separates the high-binding fraction in two or three further rounds, with stringency raised each time by lowering the antigen concentration or extending a wash [16, 17, 29]. Individual clones are then sequenced and

expressed as soluble protein for affinity measurement by biolayer interferometry or surface plasmon resonance. Table 1 sets out published examples of display systems, targets and functional tests, and the distribution of the entries by theme is shown in fig. 1.

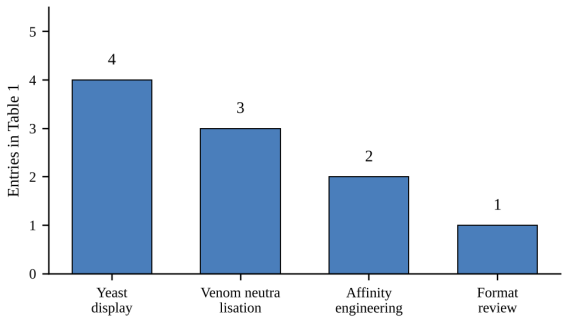


Fig. 1: Number of entries in table 1 by theme

The strongest functional evidence in the field currently comes from non-yeast systems, which should be stated plainly. Llama VHHs and a bivalent VHH-Fc selected against α-cobratoxin protected mice from lethal challenge in vivo [18]. Anti-scorpion toxin VHHs, including bivalent constructs, neutralised a potent insect-selective and mammalian neurotoxin, and showed that format engineering raised protection [19]. Human antibodies against dendrotoxins selected by phage display neutralised black mamba venom in mice when mixed in oligoclonal combinations [20]. These studies provide the standard that yeast-derived binders are expected to meet and they also show that selection platform matters less than the quality of the epitope and the final format.

Affinity maturation is where yeast display shows its particular strength. Error-prone PCR, DNA shuffling or targeted randomisation of the complementarity-determining regions creates a daughter library around a promising clone, and successive sorts at decreasing antigen concentration select for slower off-rates, often by competing with unlabelled antigen over hours [29, 30]. The classic demonstration pushed a single-chain antibody fragment from nanomolar to femtomolar affinity [21]. For a toxin target the benefit is practical: high-affinity binders can neutralise at lower doses, which lowers the quantity of product needed per treatment and, therefore, its cost. Past a certain point, however, extra affinity brings little extra protection if the epitope is wrongly placed [10, 22].

Reports that use yeast display for venom targets are fewer, and are often described as proofs of principle. The method suits situations in which a precise measurement of binding is needed, for example selecting clones that recognise a conserved site while ignoring a variable one, or evolving a weak initial hit to a more potent version through mutagenesis and repeated sorting [21, 30]. The same logic underlies the work on conformation-selective nanobodies, where sorting against two states of a target separated clones that distinguished them [16]. Applying that principle to toxins, in which a binder might be selected to discriminate between a receptor-bound and a free toxin, has been proposed but not widely demonstrated [10, 23].

Functional testing needs to move beyond binding. Enzymatic assays can show whether a clone inhibits phospholipase or metalloproteinase activity, receptor-binding assays can reveal blockade of nicotinic acetylcholine receptors by three-finger toxins, and cell-based or mouse assays test protection against cytotoxicity or lethality [18, 19, 23]. Binding without neutralisation is common, because an antibody can attach to a toxin at a site that does not matter. Regulatory guidance for antivenoms emphasises the median effective dose test in mice, and any recombinant replacement will be compared against it in some form [7]. Screening cascades that add functional filters early save effort that would be lost on clones that bind well but protect poorly [10, 22].

Steering selection towards functionally relevant epitopes is a design challenge. One approach is to sort in the presence of an excess of the natural receptor or a known neutralising antibody, so that clones competing for that site are retained and those binding irrelevant surfaces are lost. Another is to use bioinformatics to nominate conserved, surface-exposed segments of a toxin family and select for binders to peptides or recombinant fragments derived from them, in line with earlier multi-epitope immunisation work for antivenom design [32]. Cross-reactivity measurements then show

how far the binders extend across a family [22, 23]. These approaches remain more discussed than reported in yeast display for venom targets.

6. DEVELOPABILITY AND LIMITATIONS

Even a potent clone faces developability questions. Recombinant antibody fragments must be expressed at high yield, remain monomeric after storage, tolerate freeze-drying and withstand heat in settings without a cold chain, where antivenom is most needed. Nanobodies generally perform well here, and many can refold after thermal denaturation [25, 26]. Aggregation-prone clones can be removed by including a stress step, such as heating the displayed library or the purified protein before sorting. Manufacturing cost matters as well, since an affordable alternative must compete with animal-derived products already priced low in many regions [4, 6, 10].

Regulatory expectations will shape the path to approval. Existing guidance for antivenoms sets standards for potency, preclinical efficacy and safety, and for batch consistency, and was written with polyclonal animal-derived products in mind [7]. A recombinant mixture would be treated as a biologic, bringing requirements for host-cell protein and endotoxin limits, defined composition and demonstration that each component contributes to protection. These requirements are heavier than for a plasma product, but they are also an opportunity, since a defined composition is easier to standardise and compare than a polyclonal pool [6, 10]. Early dialogue with regulators in the countries where the product will be used has been recommended repeatedly in policy papers.

The larger scientific constraint is breadth. A product that protects against one cobra species but not another will have limited value in regions where several species occur, and many venoms contain isoforms that differ at the very residues recognised by an individual binder. Most proposals therefore combine several binders into a defined mixture, designed from toxin classes shown to account for lethality [10, 20, 22]. Selecting clones that bind conserved epitopes, and testing them against panels of venoms, is more demanding than demonstrating protection against a single toxin. Most published nanobody studies still stop at a single toxin or species, and the evidence on cross-species neutralisation is thinner than the enthusiasm suggests.

Future work will probably combine the strengths of the available platforms. Immune libraries from camelids exposed to representative venoms can be sorted by yeast display with quantitative gating for breadth, followed by affinity maturation, formatting into long-lived constructs, and testing against panels of regional venoms in rescue models [10, 16, 17, 23]. Progress will also depend on non-technical factors, including regulatory pathways for recombinant antivenoms, cost-effective manufacture in the regions affected, and commitment of funders [5, 6]. For readers weighing the technology, the useful message is that yeast display is a mature selection method that deserves wider use on venom targets, and that its value will be settled by neutralisation data, not by binding alone.

7. CONCLUSION

Yeast surface display combines eukaryotic folding, display of the antibody on the cell, and flow-cytometric sorting that can be tuned to affinity, stability or specificity, and these features suit the isolation of single-domain antibodies against snake venom toxins [14, 29, 30]. Nanobodies bring small size, stability and low production cost, and animal and phage-based studies show that single-domain and related fragments can neutralise elapid and scorpion neurotoxins in mice [18, 19, 25]. Evidence for yeast-derived binders against venom itself is still sparse (fig. 1), and most of the supporting method papers concern other targets [15, 16, 17].

The route from clone to medicine depends on breadth, pharmacokinetics and affordable manufacture. Mixtures of binders directed at conserved epitopes of the main toxin families, formatted for longer circulation and tested against regional venoms in treatment rather than pre-incubation models, would address the weaknesses of both current antivenoms

and early recombinant candidates [7, 10, 20, 22]. Pharmacists, toxicologists and biotechnologists have a shared interest in setting standards for such testing early, so that comparison between platforms is possible and the burden of snakebite is addressed with products suited to the places where it is heaviest [4, 5, 6].

8. ABBREVIATIONS

VHH-Variable domain of heavy-chain-only antibody, Fc-Fragment crystallisable, IgG-Immunoglobulin G, FACS-Fluorescence-activated cell sorting, PLA_2-Phospholipase A_2, 3FTx-Three-finger toxin, SVMP-Snake venom metalloproteinase, WHO-World Health Organization

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10. AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

11. CONFLICT OF INTERESTS

Declared none

12. REFERENCES

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