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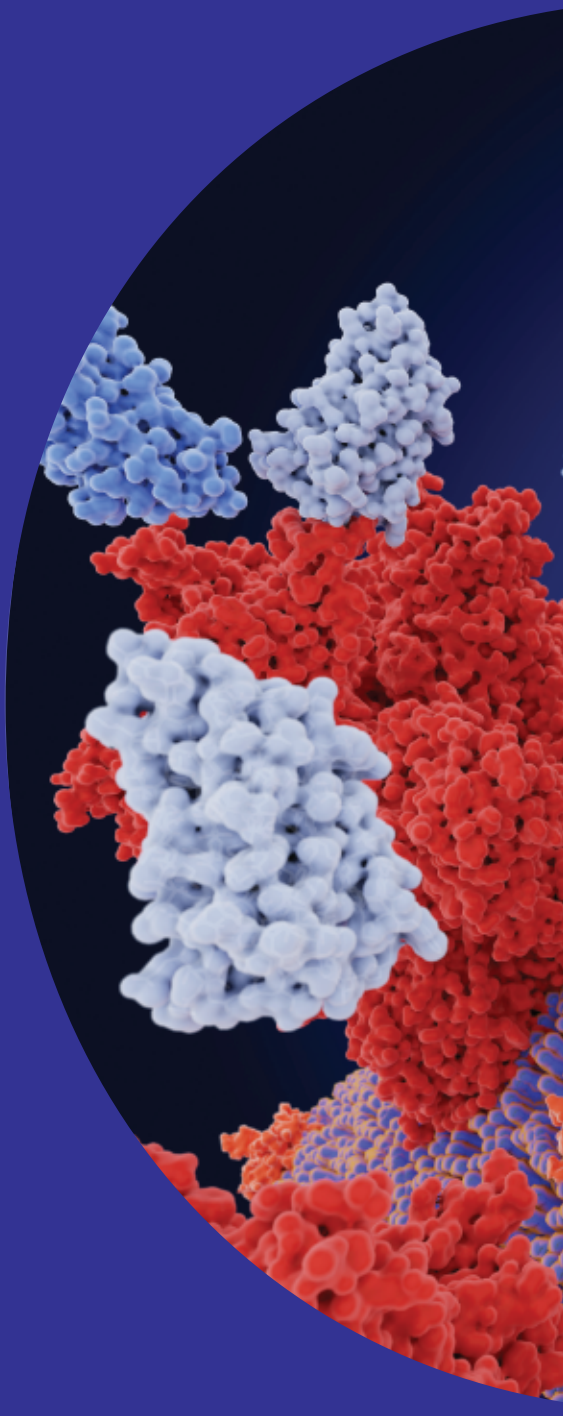

VHH Antibodies: Novel Engineering Strategies Beget Diverse Applications

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Contents

Structural and biochemical properties of VHH antibodies

VHH antibodies, also known as single domain antibodies (sdAb) or heavy-chain antibodies (hcAb), are a monomeric variable antibody domain. The variable domains have a molecular weight of approximately 15kDa (about 10% that of conventional antibodies) and in nature are unique to camelids, including llamas and alpacas. VHH antibodies are found in the IgG2 and IgG3 subtypes which evolved without a CH1 domain and consist of a VH binding domain, extended hinge, and CH2 and CH3 domains (Figure 1). Recently, the advantages of VHH have become apparent in research, diagnostic, and therapeutic applications.

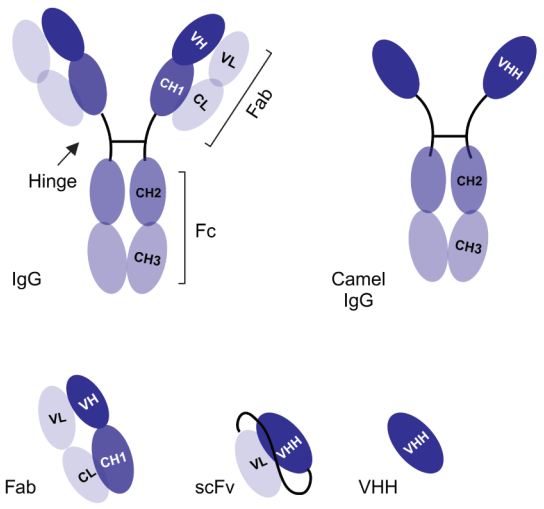

Figure 1. Comparison of canonical IgG molecules, hcAb camelid IgG molecules, common Fv formats Fab and scFv, and the monomeric VHH domain.

VHH antibodies exhibit unique characteristics in terms of framework and complementarity-determining regions (CDRs). The three complementarity determining regions (CDR1, CDR2 and CDR3) form the antigen-binding paratope. CDR1 and CDR2 have canonical structures and are found in the variable (V) region. CDR3, which has a non-canonical conformation, includes some of the V region, all of the diversity (D, heavy chain only) and joining (J) regions, and a piece of the constant (C) regions. While VHH domains lack VH-VL combinatorial diversity, the hcAb fragments have a longer CDR3 than either human or mouse VH antibodies and show lower conservation in the hypervariable regions. Compared with conventional camelid VH, llama VHHs differ by four amino acids in the FR2; positions (37, 44, 45, and 47). In the conventional VHs, these FR2 amino acids were conserved during evolution and are involved in forming the hydrophobic interface with the VL.

The CDR1 and CDR2 regions also show increased variability. Germline VHH antibodies have a conserved Cys22 and Cys92 disulfide bridge in all Ig domains, which provides stability, induces canonical Ig-like folding, and induces constrains in the orientation of the relationship between CDR1 and CDR3 regions. Combined, this allows VHH domains to evolve several novel binding modes to antigens that may be inaccessible to VH-VL binding. A common and high-value binding conformation would allow for CDR3 of the VHH to protrude beyond the typical interface and binds to clefts or grooves on a protein; this unique binding mode is particularly valuable in inhibition of enzymes or membrane-bound sites.

The hydrophilic nature of the VHH framework, in contrast to the hydrophobic VH framework, reduces the inclination for VH-VL mispairing in bispecific constructs. This characteristic causes the CDR3 of VHH antibodies to fold over onto the hydrophilic region, increasing stability.

Methods of VHH library construction

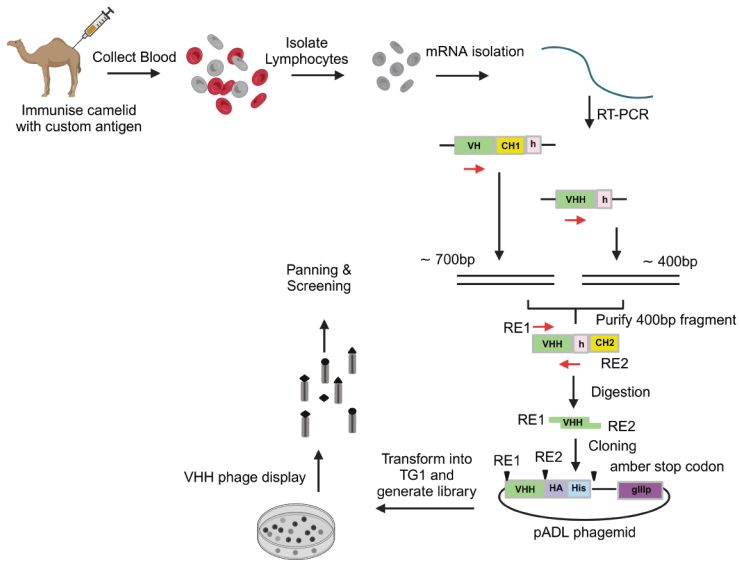

Figure 2. Overview of the Abcore immune library workflow, from injection and isolation to phage display library.

Immunized VHH Library Construction and Panning

Immunization allows for endogenous affinity maturation processes to take place within the animal. Typical antigen is protein-based with the addition of adjuvant, but nanoparticles, liposomes, VLPs, whole cells, or genetic material has also generated robust immune responses in camelids. The process of constructing and panning immunized VHH libraries involves RNA isolation and cDNA amplification of llama peripheral blood mononuclear cells (PBMCs).

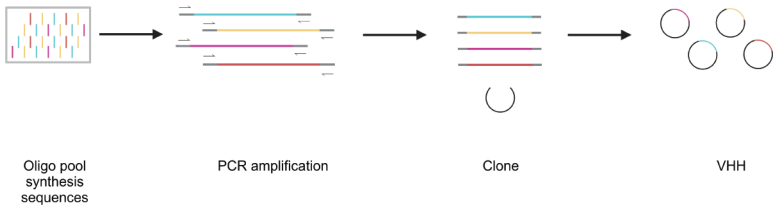

Figure 3. DNA library cloning. Many unique sequences are represented in one pool.

PCR amplification of the cDNA allows for isolation of the VHH band; due to sequence homology, the VH band and CH1 region are also amplified, but a purification step allows VHH product to be isolated (Figure 2). Additional PCR amplification steps depend on the vector being used, such as inserting the VHH library into yeast-displayed or phage-displayed fusions. Briefly, a molecular target is fixed to plates. Most commonly, protein is adsorbed onto polystyrene or biotinylated protein is bound to streptavidin-coated beads. Phage pools are added to the plates, and then unbound and weakly-binding phage are washed away. The remaining phage are eluted and amplified in bacteria, and the entire process is repeated multiple times to enrich for higher-affinity binding sequences (Figure 4).

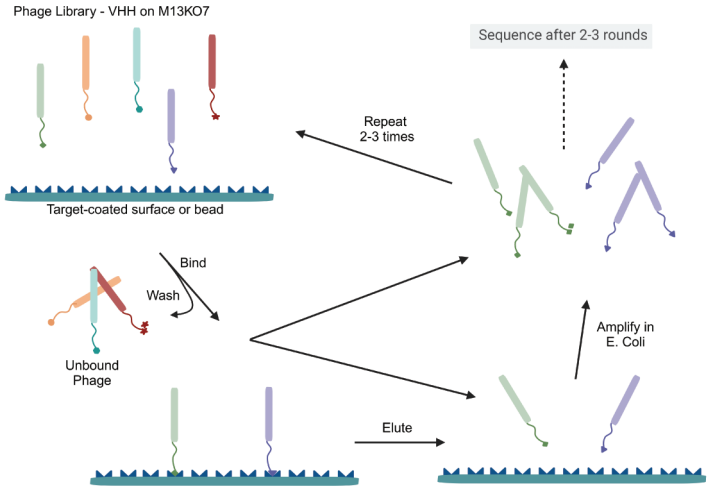

Figure 4. The phage display panning process.

Synthetic VHH Libraries

Recent advancements have led to the development of high-quality commercial synthetic libraries for naïve humanized sdAbs. Advantages of synthetic libraries include diversity control in CDR regions, such as partially-degenerate codons, and the ability to tie in computational prediction methods with the diversification process, such as PyRosetta or AlphaFold pipelines. Synthetic libraries have difficulty sampling different lengths in CDR3, which is a major contributor to the diversity of binding mode in VHH interaction. Synthetic library sizes are determined in the design phase and typically range in diversity from 1E7 to1E10 unique members.

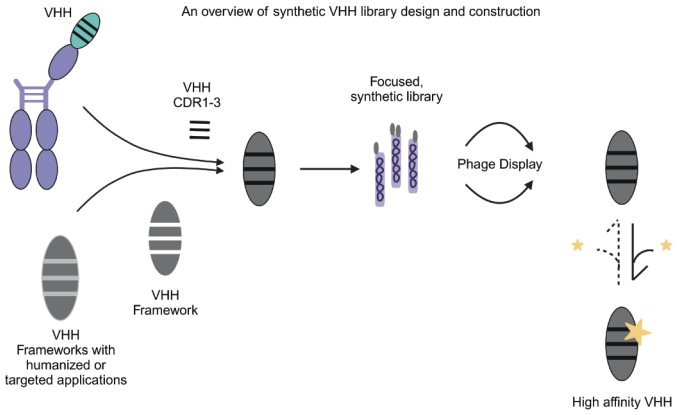

Figure 5. Synthetic VHH library design and construction.

Considerations for selecting VHH over conventional antibodies

Advantages of VHH Antibodies

1. **Smaller size:** The smaller size of VHH antibodies allows better access to antigen epitopes that may otherwise be inaccessible to larger antibodies. The smaller molecular weight of VHH antibodies changes several biophysical characteristics related to tissue perfusion and membrane permeability. Limited evidence also exists for an adsorptive endocytosis allowing for cell penetration and the potential to cross the blood-brain barrier.
2. **Thermostability and pH stability:** Compared with conventional antibodies where the paratopic interface between VL and VH is stabilized by hydrophobic residues in the FR2, hcAbs have evolved hydrophilic residues that are often charged in llama VHHs. VHH domains have less complex folding patterns than conventional antibodies, and

some VHH clones recover markedly better from denaturing conditions or heat shock than IgG molecules.

3. **Simplified workflow:** VHH antibodies simplify the discovery workflow as they do not require any crossed or paired workflows in cloning, expression, or assay projects. This single-domain advantage extends to functionalization workflows such as labeling and conjugation.
4. **Modulation of binding valency:** VHH antibodies can be engineered for a non-canonical binding valency depending on the desired functionality, such as activation, clustering, blocking, and specificity to multiple targets. Bivalent and tetravalent technologies exist as well as bispecific and trispecific recognition motifs. These technologies have emerging roles in therapeutics, synthetic biology, and diagnostic technologies.

Disadvantages and Limitations of VHH Antibodies

1. **Requirement for llamas or alpacas:** Generating immunized libraries of VHH antibodies requires the housing and care of llamas or alpacas, leading to higher costs and space requirements compared to mouse models.
2. **Small interface size:** Due to the single-domain nature that imparts so many unique advantages, VHH antibodies exhibit lower affinity in binding to small antigens and peptides. This is most frequently due to a lower total buried surface area in the complex and may be addressed by engineering valency or affinity maturation techniques.
3. **Possible anti-drug antibody (ADA) effects:** Even with humanized VHH antibodies, the CDR3 loops themselves contribute more to immunogenicity than the innate framework residues, leading to potential ADA effects.
4. **Short serum half-life and rapid clearance:** VHH antibodies have a shorter serum half-life and are rapidly cleared. This limitation can be overcome through half-life extension modifications. In therapeutic contexts, however, this is less commonly an issue due to the fusion of VHH domains to a larger domain to trigger recognition such as an Fc domain.
5. **Structural integrity and stability:** Due to the limited total size, VHH antibodies have a maximum tolerance for manipulation in some binding modes. The smaller domain limits the number of first- and second-shell residues that may be engineered for improvements in affinity or specificity campaigns.

Conclusion

The unique features of VHHs, including that they are: small; soluble; high affinity; thermostable; and have high expression yields in multiple systems, make them the ideal tool for a variety of applications including research, immunotherapy, diagnostics, in vivo imaging of tumors, and synthetic biology.

About Abcore

Abcore is an industry leader in VHH discovery and custom antibody production located in sunny Southern California, USA. Abcore has developed a complete single-domain antibody platform based on antibody discovery via phage display and has made a variety of naïve and immunized phage display libraries that can be used for animal-free antibody discovery. Abcore offers a breadth of services across custom polyclonal, monoclonal, recombinant, and single-domain antibody production and is the largest camelid facility in the United States. As an established CRO, Abcore has worked with some of the world's largest biotechnology and pharmaceutical companies.

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