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Nanobodies Production from Bacterial Sources

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Review article

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ABSTRACT

Nanobodies provide the incredible specificity of antibodies within a single immunoglobulin VHH domain. This unique feature enables applications ranging from biochemical tools to therapeutic agents. In the case of nanobody production, the tendency is toward more straightforward but dependable (bacterial) technologies that can replace more time-consuming eukaryotic based processes. Nanobody library can be synthesized in different ways, such as immunizing the camels or llamas or constructing synthetically. Other plasmid systems are used for carrying the nanobody-specific gene. Bacterial and cell surface displays are emerging technologies for isolating interested proteins from libraries. Here immune, naïve and synthetic libraries are displayed on different bacterial sources. A steady increase in product titers and the corresponding change in impurity composition are challenges for developing and optimising nanobody production processes. Additionally, increasing demands on product quality result in higher complexity of processes and analytics, thereby increasing the costs for product work-up. The concentration and composition of impurities are critical for efficient process development. These impurities can show significant variations, so we have to control this impurity by control of upstream processing.

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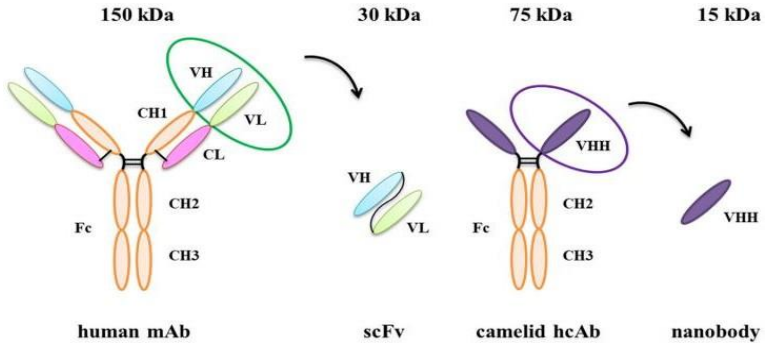
INTRODUCTION

Nanobodies (Nbs) are recombinant single-domain antibodies obtained from the variable VHH domains of Heavy Chain Antibodies (HCAbs) present in camelids (e.g., dromedaries, llamas, alpacas.). The recombinant antigen specific single-domain VHH with dimensions in the nanometer range is also called Nanobody (Nb) or single-domain Antibody (sdAb) (Figure 1).

Figure 1. General structure of Nanobody.



The VHHs have acquired essential modifications to be soluble and functional without the light chain, more extended and mixed Complementarity Determining Regions (CDRs), strict monomer behavior, reversible folding properties and more excellent resistance to proteolysis and thermal denaturation than VH domains from classical antibodies. These intrinsic biophysical properties enable their expression in bacteria, yeast and mammalian hosts. Their small size (ca. 15 k Da; 24 nm in diameter) and long CDRs also permit the binding of less available epitopes than those recognized by classical antibodies, comprising active sites of enzymes and internal areas in the surface proteins of pathogens. VHHs are also comparable to human VH3 sequences, which is essential for low immunogenicity in therapeutic uses of Nbs. These properties have made Nbs particularly desirable molecules for many applications, comprising cell biology studies, protein crystallography, therapy and *in vivo* diagnosis of human diseases. Therefore, Nb selection, characterisation and production approaches are of significant interest. A notorious exception to the classic mammalian IgG structure is the sera of Camelidae. In addition to traditional heterotetrameric antibodies. IgG antibodies, known as Heavy-Chain Antibodies (HCAbs), do not contain an L-chain polypeptide and are characterized lack of the first constant domain (CH1). At its N-terminal region, the H chain of the homodimeric protein includes a dedicated variable domain, termed VHH, sufficient to associate with its cognate antigen. The VHH in an HCAb is the structural and functional equivalent of the Fab classical antibodies' Fab fragment (antigen-binding fragment). Synthetic library starts from a consensus scaffold derived from the genes of llamas, camelids, and alpacas family members. The nanobody library plays a crucial role in the availability of nanobodies. The VHH library mainly focuses on the CDR3 region, the most diverse and antigen-binding region present in VHH. The amino acid sequence of the VHH region plays a vital role in identifying the antigen. For the highly variable positions in each CDR, introduce more aggressive randomisation. The VHH library can be constructed by analysing the nanobody sequence and based on this, the introduction of highly variable positions such as CDR1 and CDR2 can be chosen [1]. For nanobody production, the trend is towards the more straightforward and reliable bacterial systems that can substitute more cumbersome processes requiring

eukaryotic systems. The bacterial periplasm helps create oxidizing conditions that favour forming a disulphide bond stabilising the nanobody structure. Different bacterial sources include *E.Coli*, *Staphylococcus carnosus, Pichia pastoris*, *Salmonella*, *Staphylococcus aureus*, *Magnetospirrilum gryphiswaldense* and *Saccharomyces cerevisiae* used for the nanobodies production. Different techniques used to display the nanobody or VHH fragments. Techniques include size exclusion chromatography, ELISA, phage display techniques and bacterial display techniques used to detect the nanobody fragments.

LITERATURE REVIEW

Nanobody library

The single-domain nature of VHH gives rise to several unique features compared to conventional antibodies. They have proved their great importance as a therapeutic molecule. Nanobodies are naturally found in the Camelidae family, including the dromedaries, alpacas and llamas. A nanobody library is also known as a VHH library as they formed from the VHH heavy chain region of the antibody. Antigen-specific nanobodies can be selected from constructed VHH libraries by various technologies, such as phage display, cell surface display, high-throughput DNA sequencing and mass spectrometric identification. The VHH libraries can be classified into three main types: Immune, naive and semisynthetic/synthetic [2].

Immune library: The immune library is the most widely used strategy for VHH screening, which provides highly abundant and generates high-affinity target-specific binders.

Construction of immune library: Dromedaries are the natural source of nanobodies. The immunisation of the dromedaries with the target antigen leads to the production of nanobodies against the target antigen. mRNA isolated after three to four days of immunization by collecting peripheral anticoagulant blood containing our target nanobodies. Using reverse transcriptase enzyme, convert mRNA to cDNA using a specific pair of primers in PCR. After that, the PCR fragment is ligated into the vector by cutting both the vector and the PCR fragments with a specific pair of restriction enzymes. After ligation, they are transformed into a host cell by electroporation. Then extract the colonies and store them at the required temperature. The immune library formed. Different techniques may be used to screen the library, such as phage display, bacteria display, cell surface display, ribosome display, etc. The GFPplus antigen was expressed in bacteria and purified. A phage display nanobody library was made in the vector pHEN4. The Phage Display-Derived Nanobodies (P-Nbs) were amplified by PCR, digested with PstI and BstEII and ligated into the pHEN6 vector. Plasmids were transformed into *E. coli* RR1dM15 cells. An alternate cloning technique based on Golden Gate Cloning and negative selection transformants with an unmodified phage display vector by the presence of a lethal ccdB gene with an unmodified phage display vector [3].

Advantages of an immune library

- Immune libraries are complex and sub-cloned to most display formats such as phage display, bacterial display and yeast cell surface display.
- Affinity maturation of the HcAbs occurs during the immunisation of the camelids.
- Subsequent affinity maturation steps can be avoided.

Challenges

- Time-consuming process.

- Sometimes, it is challenging to immunise camels due to the high toxicity and pathogenicity of the antigens.
- Antigens produce the inclusion bodies with strongly folded proteins.
- Self-immune antigens can occur.
- Non-immunogenic, small molecular weight compounds unable to immunise the camels.

Naïve library: The design of recombinant VHH phage display libraries from immunised animals and their subsequent panning has been preferred for isolating high-affinity-specific binders. The method is laborious and numerous synthetic and naïve VHH libraries from llamas and sharks have recently been proposed. This is because the wide variety of libraries would allow the identification of binders for each potential antigen [4]. Such diversity can be achieved in (semi)synthetic libraries by hyper mutation of the variable regions within a single VHH backbone, similar to what has been successfully done in the case of scF.

Challenges

- High specificity and affinity can only be achieved by selecting from very large functional libraries.
- A substantial amount of blood samples collected from many individual animals is a prerequisite to ensuring the diversity of the naïve library.

Synthetic library: A non-immune recombinant antibody library with a high diversity of non-immune antibodies constructed on a nanobody architecture enables successful *in vitro* antibody selection against practically any antigen. Antibodies that can be used in standard immunological assays should be boosted in such a library, as should antibodies that are active in the intracellular environment. To begin, an *E. coli* fusion experiment might be used to generate a series of highly functional VHH scaffolds with excellent intracellular expression and stability. A synthetic library by meticulously regulating each CDR1 and CDR2 using a collection of amino acids that somewhat matches natural diversity while reducing the presence of the most hydrophobic residues to minimize aggregation propensity.

Plasmid construction: Plasmids are small circular DNA molecules present in many species of bacteria with an "origin of replication" that regulates plasmid replication and ensures that the cloned cell contains multiple copies of the plasmid that are dispersed across the daughter cells when the cell splits. A plasmid expression vector delivers the foreign DNA (Nanobody gene) into the cell. Another crucial component in recombinant protein development is the expression of a plasmid. Its importance stems from its unique ability to self-replicate and choose a specific genotype following ligation with foreign DNA. To synthesize the nanobody within the bacterial system, one must first create the appropriate plasmid, in which cloning of nanobody-synthesis gene of interest. For generating nanobodies inside bacterial systems, various plasmid systems are used. The nanobody gene is introduced into the vector *via* the MCS site after restriction digestion of both the vector and the recombinant gene with specialized restriction enzymes in the standard technique of plasmid build for nanobody manufacturing (such as Eco911 and Pst.1.The vector is subsequently transformed into the appropriate host cell *(E. coli* WK6 cells). The ampicillin resistance gene (ampR) promoted selective growth in nutrient broth (LB ampicillin media). The overexpression of the nanobody gene downstream of the LacZ promoter was allowed by inducing the LacZ promoter with -D1-thiogalactopyranoside (IPTG) [5]. The expressed protein is then transported to the periplasmic region for proper folding into its functional form. The correct folding of a protein within the cytoplasm of a bacterial cell is difficult due to the crowded environment. As a result, most recombinant proteins have a secretion signal that allows for appropriate folding of the

generated protein, allowing for overexpression and preventing inclusion bodies (insoluble masses of aggregated proteins) created the plasmid to test the ability of cDNA immunotherapy to generate nanobodies against membrane proteins in their original state. In which they used that the cDNA expression vector has a bacterial antibiotic resistance gene, a potent universal promoter and an Open Reading Frame (ORF) expressing the desired membrane protein. Epitope tags, an intron and a mammalian antibiotic resistance gene are optional traits. A potent ubiquitous promoter increases the membrane protein's open reading frame transcription in immunized camelid cells [6].

Bacterial sources

VHH can be quickly produced in gram-negative bacteria like *E.coli*, gram-positive bacteria like *Brevibacillus choshinensis*, some LAB species, and Bifidbacterium species due to their small size, hydrophilic nature and singledomain nature. Bacterial systems benefit from being relatively easy to alter and more economically viable as production systems. *E.coli* is the most common microbiological host for VHH production. In the case of nanobody production, the tendency is toward more straightforward but dependable (bacterial) technologies that can replace more time-consuming eukaryotic-based processes. This bacterial system is easy to culture, grows quickly, has been thoroughly characterized and provides an ever-growing variety of cloning vectors and mutant host strains. Overexpression strategies in *E. coli* have also been successfully researched and described. They aid transcription by either raising the number of copies of the gene or boosting the binding strength of the promoter region. Bacterial cells have several advantages, including rapid growth, well-established genetics and low cultivation costs. Various bacteria, including *E.coli*, *Corynebacterium glutamicum, Pseudomonas putida* and *Bacillus megaterium*, were used to produce recombinant proteins, producing the specific nanobodies. Mammalian cells have been used predominantly for the expression of nanobodies are helpful because they can introduce post-translational changes similar to those found in human cells. However, a few drawbacks to using a mammalian expression system have prompted manufacturers to look for other host systems. Predicting the manufacturing behaviour of cell lines at an early stage and selecting clones with acceptable growth properties are two of the most challenging issues. Improvements to processes have traditionally been costly and time-consuming. Mammalian systems also have several limitations, such as medium complexity, serum needs and shear sensitivity [7].

Display techniques used for screening and purification of nanobodies

Protein function is frequently selected using display methods.

Although it has successfully expressed proteins on the surface of bacteria, the display of peptides and polypeptides on the surface of filamentous phages (phage display) is still the most extensively utilized display technology (Table 1).

Table 1. Cellular and acellular display methods.

Cellular display	Acellular display
Bacterial display	Ribosome display
Phage display	mRNA display
Yeast cell surface display	Aptamers
	In vitro compartmentalization (IVC)
	Cis-Activity-Based (CIS) display
	Covalent Antibody Display (CAD)

As stated in the table, there are two types of display systems: Cellular and acellular. Acellular methods do not necessitate the existence of cells [8].

Phage display

Phage display is a laboratory approach for studying protein-protein, protein-peptide and protein–DNA interactions that connect proteins to the genetic information that encodes those using bacteriophages (viruses that infect bacteria). A gene for a protein of interest is introduced into a phage coat protein gene, leading the page to "display" the protein on the surface while containing the gene for the protein on the inside, resulting in a genotype-phenotype link. These displaying phages can then be screened against other proteins, peptides or DNA sequences to see if they show protein interacts with those other molecules. *In vitro* selection, which mimics natural selection can screen and expand large protein libraries in this way. M13 and fd filamentous phage are the most commonly utilized bacteriophages in phage display; however, T4, T7 and phage have also been used. Bacteriophages infect bacteria and are commonly used to create proteins and peptides [9].

Cell surface display

The expression of nanobodies on the surface of living cells or their organelles by fusing them to functional components of cells exposed to the environment is known as a cell-surface display. Different surface proteins of cells can be used as anchoring motifs and nanobodies can be used as passenger proteins. Selections from cell displayed libraries are typically performed using Fluorescence-Activated Cell Sorting (FACS) by labelling the antigen with a fluorophore and then incubating it with the cell displayed nanobody library in solution, unlike phage and ribosome display, which use a capture and elution procedure. The ability to quantify affinity during selection is a crucial characteristic of cell-based display systems, making the procedure more like micro-well plate screening than bio panning in phage and ribosome display.

CONCLUSION

Since the nanobodies have a good expression in microbial systems and beneficial biochemical properties (good solubility, good stability in harsh conditions, high affinity and specificities for the antigens), they are ideal tools for research purposes. Bacterial cloning holds many promising future types of research for nanobody production. Furthermore, there is an increasing awareness that nanobodies are more differentiated in their structure than initially thought. The discovery of microbial expression systems capable of providing non-immunogenic projects while, maintaining antigen specificity should open the way for more cost-effective nanobody manufacture. Nanobodies must be immobilized in many applications of conventional and biogenic Magnetic Nanoparticles (MNPs), including diagnostics, immunomagnetic separations and magnetic cell labelling.

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