Nanobodies

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1. Introduction

In addition to conventional antibodies, camelids, such as llamas and alpacas, have unique heavy-chain-only antibodies [1]. These antibodies are unique in that the variable regions are encompassed by a single domain (VHH) instead of two separate domains (VH and VL) as seen in conventional antibodies [2]. The variable domains of the camelid heavy-chain-only antibodies have found widespread applications in biomedical research.

Nanobodies are highly water-soluble and stable, have high specificity, and can bind to their targets with high affinity, often in the low nanomolar range [3]. VHHs are stable as single-domain antibodies because of several mutations on their surface that allow them to be water soluble [3]. In particular, several residues that would be at the VH–VL interface in conventional antibodies are mutated for hydrophobic to hydrophilic residues (G44E, L45R, and W47G) (Figure 1), enhancing their stability and solubility as a single domain. In addition, there is a solubility enhancing mutation, most commonly found in camel VHHs, at the VH–CH1 interface (L11S) (Figure 1A,C).

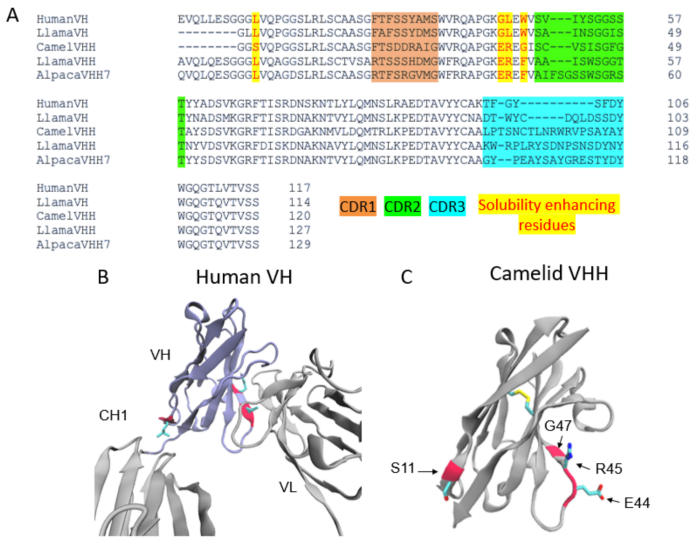

Figure 1. The architecture of VHHs promotes their solubility and stability. (**A**) Multiple sequence alignment of the Human VH, Llama VH, Llama VHH, Camel VHH, and Alpaca VHH domains. CDR regions are highlighted orange (CDR1), green (CDR2), and blue (CDR3). The VHH domains shown have a larger CDR3 region than the VH CDR3s. Highlighted in yellow and written in red are residues mutated to enhance solubility in VHH domains. Camel VHH bears four mutations (L11S, G44E, L45R, and W47G). Llama and alpaca VHHs bear G44E, L45R, as well as a W47F mutation. (**B**) Crystal structure (PDB ID 1IGY) of a human antibody with the VH domain colored in purple. Residues mutated in the camelid VHH domains are colored red. L11 makes contact with the CH1 domain while L45 and W47 form contacts with the VL domain. (**C**) Crystal structure of a camelid (camel) VHH (PDB ID 6U14). E44, R45, and G47 in the hypothetical VL binding site promote stability and solubility of the VHH as a single domain.

The factor contributing to the high affinity of these nanobodies is that their frameworks have three complementarity-determining regions (CDRs). These CDRs are analogous to those found in human antibody VH and VL domains and are subject to somatic hypermutation in the course of affinity maturation. The CDR3 of VHHs is especially long in comparison to the human counterpart [4]. The length and flexibility of VHH CDR3s enable the nanobody to access a variety of conformations. In some cases, VHH CDR3s are able to fold back and make contact with the nanobody framework [4]. Taken together, these factors compensate for the lack of sequence variability incurred by the loss of VL CDRs, allowing VHHs to bind to their targets with high specificity and affinity (Figure 1A).

Methods of generating nanobodies against an antigen of interest have already been well established [2]. In brief, a llama or alpaca (among a variety of other camelids) is immunized against the antigen(s) of interest [2]. Administration of the protein antigen is typically accompanied by an immune adjuvant that serves to enhance the overall immune response [5]. Several weeks later, blood is harvested from the immunized animal and peripheral blood mononuclear cells (PBMCs) are purified. This purification is then followed by total RNA extraction, VHH amplification, and finally, the construction of a phage display library. Phage display libraries are among the most common methods of preparing nanobody libraries, but other methods, such as *E. coli* or yeast display, could alternatively be used [2][6][7]. Finally, the lead VHHs are identified and expressed as soluble proteins using reliable approaches, such as magnetic-activated cell sorting (MACS), fluorescence-activated cell sorting (FACS), or panning against immobilized antigens (Figure 2) [8][9][10].

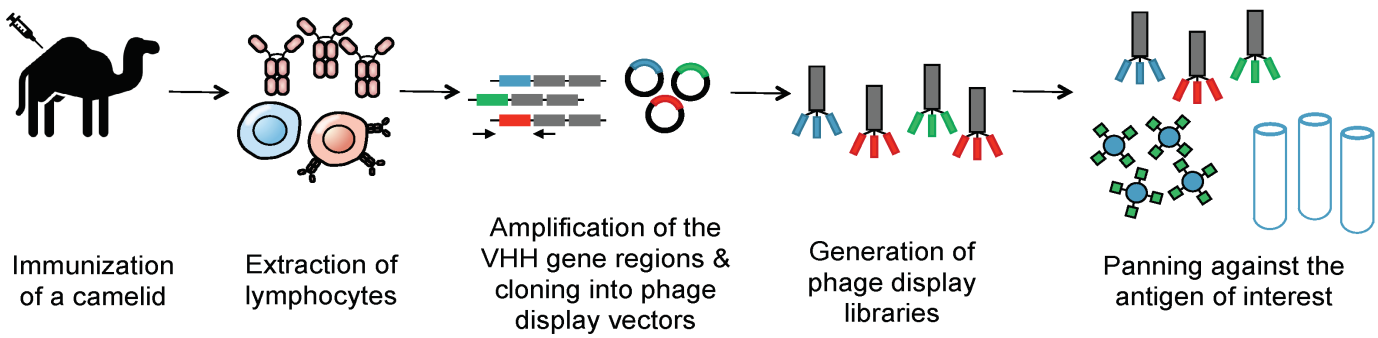

Figure 2. Generation of a nanobody library. To create an immune library, camelids are immunized against a molecule of interest. mRNA of the camelids' peripheral blood mononuclear cells is then converted into cDNA. PCR is then employed to amplify the VHH genes. These immune VHH genes will then be cloned into a phage display vector. Phages are then generated using *E. coli* strains such as TG1. Phage libraries are then panned against immobilized antigens to select for nanobodies that selectively bind the antigen with high affinity. The panned libraries are then used for reinfection of *E. coli* to obtain specific clones.

The short circulatory half-life of nanobodies have allowed the use of a range of isotopes with short half-lives for imaging, such as Galium-68 (^{68}Ga, $t_{1/2}$ = 67.71 min) and ^{18}F ($t_{1/2}$ = 109.7 min), as well as longer-lived isotopes, such as Technetium-99m (^{99m}Tc $t_{1/2}$ = 6.0 h), Copper-64 (^{64}Cu $t_{1/2}$ = 12.7 h), Indium-111 (^{111}In $t_{1/2}$ = 67.2 h), Zirconium-89 (^{89}Zr $t_{1/2}$ = 78.41 h), and Lutetium-177 (^{177}Lu $t_{1/2}$ = 6.7 days). Similar to other antibody fragments, nanobodies are commonly labeled nonspecifically via their side-chain lysine residues using chelators or radioisotopes that are functionalized with amine-reactive groups such as *N*-hydroxysuccinimide (NHS) or isothiocyanatobenzyl (pSCN) groups. While this strategy is robust and reproducible, it is not site-specific, which may damage antigen-binding sites [11]. To address this issue and to ensure the binding capacity is not compromised, a variety of site-specific labeling approaches, such as the use of sortase technology, have been developed [12]. Another common approach is using a His6 tag to install ^{99m}Tc, a commonly used SPECT isotope [13].

2. Nanobodies for Medical Imaging

Biopsies will likely remain the gold standard of cancer diagnostics for the foreseeable future; however, biopsies can sometimes be unrepresentative of the greater TME or targeted organ. Non-invasive immuno-PET imaging, as an adjunct to biopsies, can provide a holistic view of the TME and offer a complete insight into both primary and metastatic tumors. Information revealed via imaging can help to make informed treatment decisions. Imaging is also beneficial in understanding the progression and pathogenesis of a variety of diseases, such as fibrosis, cardiovascular complications, arthritis, and neurological diseases. Therefore, immuno-PET imaging is a potentially revolutionary addition to disease management and treatment.

The use of radiolabeled nanobodies as imaging probes overcomes many of the weaknesses of using full-size antibodies and larger antibody fragments. Nanobodies have excellent tissue penetration, rapid blood clearance profile, high specificity, low nanomolar to picomolar affinity for the target, high stability and water solubility, ease of production, and a pharmacokinetic profile compatible with short-lived radioisotopes. One major drawback of nanobody-PET is the high renal retention and toxicities, similar to other radiotherapeutics and imaging agents [14][15]; however, techniques such as PEGylation can help decrease kidney retention [12]. Other strategies to decrease renal toxicity involve the co-infusion of basic amino acids such as lysines, or the use of gelofusine, which is a gelatin-based plasma expander [16][17]. Gelofusine mediates a decrease in kidney uptake through the interference of its plasma expander with the tubular reabsorption of nanobodies. Immunogenicity has also been reported for some nanobodies, though it may be idiotypic and specific only to the variable regions. Once again, techniques such as PEGylation may help to decrease immunogenicity in addition to undesirable kidney retention [1][18]. A

recent study assessed the immunogenicity risk profiles of two nanobodies — anti-HER2 and anti-CD206 (MMR) — that have advanced into Phase II clinical trials for PET imaging. Strikingly, only 1 patient out of 20 showed a minimum amount of pre-existing anti-VHH antibodies, which was only marginally increased several months post-injection of the nanobody. Assessing the in vitro immunogenicity of the nanobodies using human dendritic cells did not induce T cell activation, further suggesting a low immunogenicity profile of nanobodies [19].

These issues of kidney retention and immunogenicity require better understanding and further investigation, as overcoming them would contribute greatly to clinical success. Similar to other antibody-based imaging approaches, nanobody-based imaging agents are the most effective when selected epitopes demonstrate a few common characteristics. These include antigen recognition through expression on the extracellular surface of the plasma membrane, availability of the epitope for similar recognition, high expression of the antigen on the cell surface, and little to no expression in normal tissues.

Radiolabeled nanobodies can provide valuable information about the biological processes taking place inside living organisms, giving researchers and clinicians the appropriate data that are needed to improve patient care. For example, nanobodies can be used to understand the dynamic of immune responses, helping to gain mechanistic insight into how the tumor immune landscape is shaped and responds to treatment. Understanding the response mechanisms, in turn, may lead to the identification of new targets and avenues to pursue for developing new therapeutics or biomarkers. Several studies have been performed on imaging lymphocytes, checkpoint molecules, and cancer markers; the recent more in-depth understanding of the tumor immune landscape suggests myeloid cells play a central role in shaping the TME. Therefore, pursuing the development of novel nanobodies for imaging specific subsets of myeloid cells can turn out to be both important and advantageous. Similarly, as cytokines and chemokines are key players in the pathogenesis of the disease, imaging their level of presence and movement may help to gain insight into understudied disease pathogeneses and progression models. Taken together, understanding the behavior of immune cells and immune-modulating molecules before, during, and after treatment will help to decide the best course of treatment for patients and allow for a dynamic and adaptable inpatient care experience. Harnessing the powerful imaging potential of nanobodies would be an ideal strategy to tackle these issues and ultimately achieve such ideal outcomes.

To ensure maximal effectiveness and minimal nonspecific binding, it could be worthwhile to continue searching for other targetable markers associated with the diseases mentioned in this review, but the major priority in coming years may better be focused to expand the number of different diseases that can be imaged and characterized by radiolabeled nanobodies, such as inflammation markers to diagnose fever of unknown origin, neurodegenerative diseases such as Alzheimer, and cytokines, chemokines, and their receptors that are key in pathogenesis and progression of the disease. By helping scientists better understand and visualize the driving forces behind disease progression, expanding the library of nanobody-based imaging agents will become an even more promising tool in guiding the development of novel, effective treatment plans for patients in the future.

The generation of nanobodies is now a well-established procedure [2][20]. The increasing availability of commercial sources for immunization and identification of lead candidates, along with advancements in the development of

synthetic libraries will continue to help provide easier access to new nanobodies against antigens of interest. While we have focused on the imaging applications of nanobodies, they also can be used as therapeutics, as a molecular biology tool for mechanistic studies, and to investigate biological processes. With the recent FDA approval of a nanobody-based treatment (Caplacizumab, a bivalent nanobody) and the clinical translation of several nanobodies, the repertoire of available nanobodies is only expected to grow in the years to come (Table 1).

Table 1. Nanobodies developed for noninvasive immuno-PET/SPECT imaging.

Target	Agent	Reactivity	Clinical Trials: Stage and Status (If Applicable)	References
EGFR	^{99m}Tc-8B6	Human	Preclinical	[7]
	^{99m}Tc-7C12	Human	Preclinical	[21]
HER2	^{177}Lu-2Rs15dHIS	Human	Preclinical	[22]
	^{18}F-FB-2Rs15d	Murine	Preclinical	[23]
	^{18}F-RL-I-5F7	Murine	Preclinical	[24]
	^{68}Ga-2Rs15d	Human	Clinical	[23][25]
HER3	^{89}Zr-MSB0010853	Murine	Preclinical	[26]
CEA	^{99m}Tc-NbCEA5	Human	Preclinical	[27]
PSMA	^{111}In-JVZ007	Human	Preclinical	[28]
HGF	^{89}Zr-1E2, ^{89}Zr-6E10	Human	Preclinical	[29]
CD20	^{68}Ga-9079	Human	Preclinical	[30]
CD38	^{68}Ga-NOTA-Nb1053	Murine	Preclinical	[31]
Mesothelin	^{99m}Tc-A1, ^{99m}Tc-C6	Human	Preclinical	[32]
MMR	^{99m}Tc-d a-MMR Nb cl1	Murine	Preclinical	[33][34]
	^{18} F-FB-anti-MMR 3.49	Human, Murine	Preclinical	[35]
	^{68}Ga-NOTA-Anti-MMR-VHH2	Human	Clinical, NCT04168528 (Active)	[36]
MHC II	[^{18}F]FDG -VHH7	Murine	Preclinical	[37]
	^{64}Cu- VHH4	Human	Preclinical	[38]

Target	Agent	Reactivity	Clinical Trials: Stage and Status (If Applicable)	References
CD11b	^{89}Zr-VHHDC13 (PEGylated)	Murine	Preclinical	[39]
	^{18}F-VHHDC13	Human	Preclinical	[40]
CD8	^{89}Zr-VHH-X118 (PEGylated)	Murine	Preclinical	[12]
	^{68}Ga-NOTA-SNA006	Human	Preclinical	[41]
Mouse Dendritic Cells	^{99m}Tc-Nb-DC2.1	Murine	Preclinical	[42]
	^{99m}Tc-Nb-DC1.8	Murine	Preclinical	[42]
PD-L1	^{18}F-B3, ^{18}F-A12, ^{64}Cu-B3	Murine	Preclinical	[43]
	^{99m}Tc-C3, ^{99m}Tc-C7, ^{99m}Tc-E2, ^{99m}Tc-E4, ^{99m}Tc-K2	Murine	Preclinical	[44][45][46][47]
	^{68}Ga-NOTA-Nb109	Human	Preclinical	[48]
	^{99m}Tc-NM-01	Human	Clinical, NCT02978196 (Concluded)	[49]
	^{89}Zr-envafolimab (Fc fusion)	Human	Clinical, NCT03638804 (Active)	[50][51]
CTLA-4	^{18}F-H11, ^{89}Zr-H11	Murine	Preclinical	[51][52]
LAG-3	^{99m}Tc-anti-moLAG-3 3206, ^{99m}Tc-anti-moLAG-3 3208, ^{99m}Tc-anti-moLAG-3 3132, ^{99m}Tc-anti-moLAG-3 3141	Murine	Preclinical	[53][54]
VCAM-1	^{99m} Tc-cAbVCAM1-5	Human, Murine	Preclinical	[33][55][56][57]
FN-EIIIB (ECM)	^{64} Cu-NJB2	Human, Murine	Preclinical	[58]
αSyn	NbSyn2, NbSyn87 (fused to fluorescent proteins for imaging)	Human	Preclinical	[59][60]
DPP6	^{99m} Tc-4hD29	Human	Preclinical	[61]
Vsig4	^{99m} Tc-NbV4	Murine	Preclinical	[62][63]

Target	Agent	Reactivity	Clinical Trials: Stage and Status (If Applicable)	References
Clec4F (KC)	^{99m}Tc-NbC4	Murine	Preclinical	[62]

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