

# Nanobody-derived nanobiotechnology tool kits for diverse biomedical and biotechnology applications

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**Abstract:** Owing to peculiar properties of nanobody, including nanoscale size, robust structure, stable and soluble behaviors in aqueous solution, reversible refolding, high affinity and specificity for only one cognate target, superior cryptic cleft accessibility, and deep tissue penetration, as well as a sustainable source, it has been an ideal research tool for the development of sophisticated nanobiotechnologies. Currently, the nanobody has been evolved into versatile research and application tool kits for diverse biomedical and biotechnology applications. Various nanobody-derived formats, including the nanobody itself, the radionuclide or fluorescent-labeled nanobodies, nanobody homo- or heteromultimers, nanobody-coated nanoparticles, and nanobody-displayed bacteriophages, have been successfully demonstrated as powerful nanobiotechnological tool kits for basic biomedical research, targeting drug delivery and therapy, disease diagnosis, bioimaging, and agricultural and plant protection. These applications indicate a special advantage of these nanobody-derived technologies, already surpassing the “me-too” products of other equivalent binders, such as the full-length antibodies, single-chain variable fragments, antigen-binding fragments, targeting peptides, and DNA-based aptamers. In this review, we summarize the current state of the art in nanobody research, focusing on the nanobody structural features, nanobody production approach, nanobody-derived nanobiotechnology tool kits, and the potentially diverse applications in biomedicine and biotechnology. The future trends, challenges, and limitations of the nanobody-derived nanobiotechnology tool kits are also discussed.

**Keywords:** nanobody, VHH, V-NAR, HCAs, IgNAR, nanobiotechnology

## Introduction

Over the decades, single-domain antibodies (sdAbs) have received a progressive interest from pharmaceutical and biotechnology industries owing to their peculiar properties, including small size, robust structure, high affinity and specificity, superior cryptic cleft accessibility, and deep tissue penetration.<sup>1-3</sup> The sdAbs could be categorized into man-made sdAbs and naturally occurring counterparts,<sup>4</sup> the latter including variable domain of heavy-chain-only antibodies in camelids (VHH), a naturally occurring antigen-binding variable domain of heavy chain from heavy-chain-only antibodies (HCAs),<sup>1</sup> and variable domain of immunoglobulin new antigen receptors in sharks (V-NAR), a variable domain from immunoglobulin new antigen receptors (IgNARs).<sup>5</sup> Totally different from the man-made sdAbs, which have been pursued for the ideal ones by scientists for half a century, several naturally occurring sdAbs, including VHHs and V-NARs, already exist in nature in camelids or cartilaginous fish, sharing a surprising structural convergent evolution and performing similar biological functions.<sup>1-5</sup>

In addition to the classical antibodies containing two heavy and two light chains, all camelidae including camels (*Camelus dromedarius* and *Camelus bactrianus*), llama (*Lama glama* and *Lama guanicoe*), and vicugna (*Vicugna vicugna* and *Vicugna pacos*) have HCAs in their sera, lacking L chains and devoid of a canonical constant heavy-chain (CH) 1 domain.<sup>1</sup> Some cartilaginous fish, including nurse shark (*Ginglymostoma cirratum*), wobbegong (*Orectolobus maculatus*), and dogfish (*Squalus acanthias* and *Mustelus canis*) sharks, also remarkably produce functional heavy-chain-only immunoglobulins (Igs), named IgNARs.<sup>1,5–8</sup> Interestingly, some pathological and nonfunctional HCAs were also discovered in sera of humans or in mouse hybridoma due to a partly genetic deletion of the variable heavy-chain (VH) and CH1 regions.<sup>1</sup> In camels or sharks, these HCAs (or IgNARs) recognize the antigens via single variable domains, referred to as VHHs or V-NARs, respectively. Thus, the VHHs or V-NARs are the smallest intact antigen-binding domain derived from the HCAs or IgNARs naturally occurring in camelids or cartilaginous fish.

Nanobody is referred to the VHHs, the sdAb fragments derived from naturally occurring camelid HCAs or the counterpart domain V-NAR of IgNARs, the homodimeric antibodies devoid of light chains in sharks. Owing to the small dimensional size of 2.5 nm in diameter and 4 nm in height (~12–15 kDa), Ablynx in 2003 dubbed these sdAbs “nanobody” to emphasize their smaller dimensional sizes, compared to larger molecular sizes of single-chain variable fragments (scFvs; 27 kDa), antigen-binding fragments (Fabs; ~57kDa), and the intact conventional immunoglobulin- $\gamma$  (IgG) antibody (~150 kDa).<sup>3,5,8</sup> In this review, we summarize the current state of the art in nanobody research, focusing on the nanobody structural features, nanobody production approach, nanobody-derived nanobiotechnology tool kits, and the potential applications in biomedicine and biotechnology.

## Structure of nanobodies

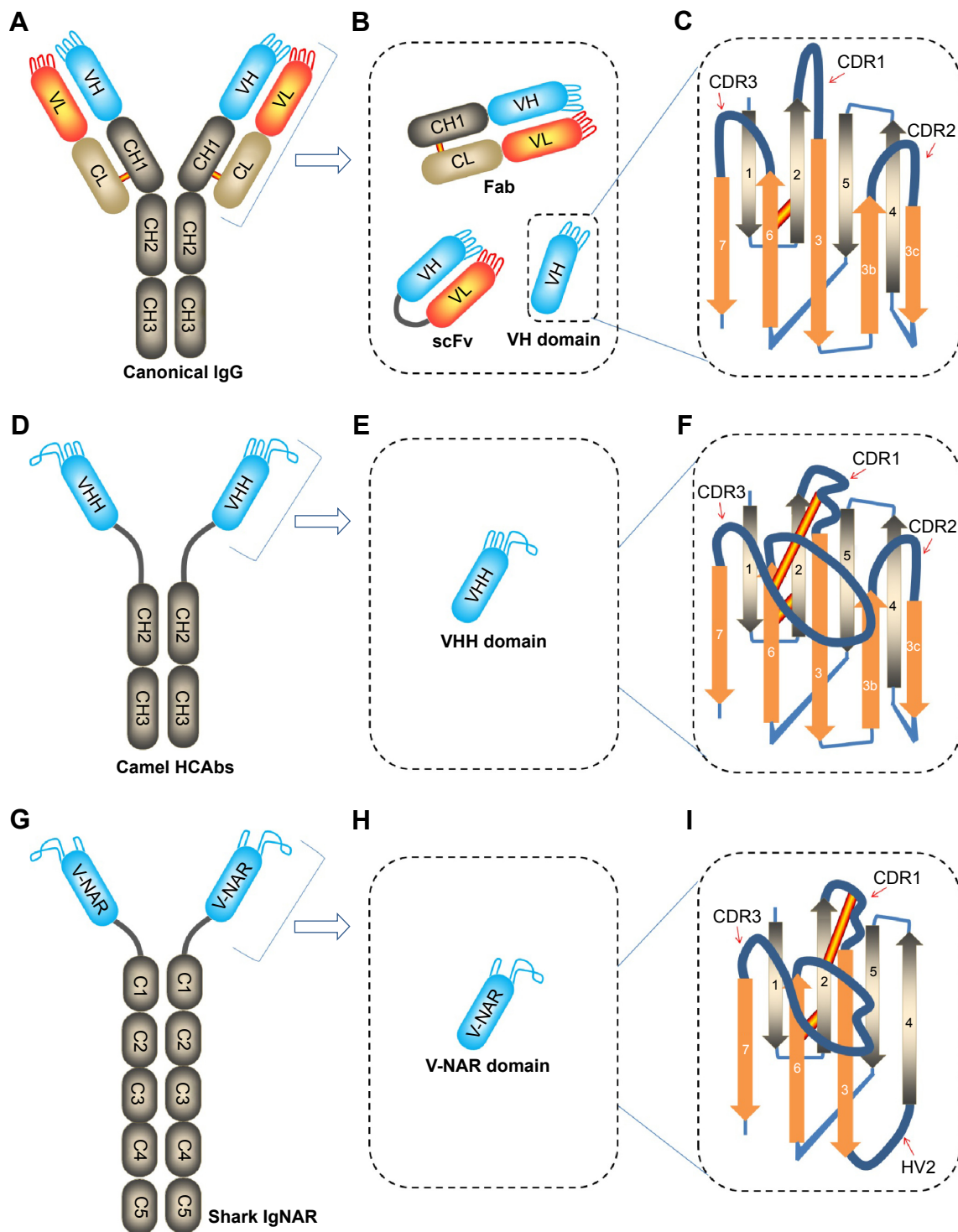
In comparison with the conventional IgG antibody assembled from two identical heavy-chain and two identical light-chain (heterotetrameric structure, Figure 1A–C), camelid HCAs are HCAs (homodimeric structure, Figure 1D–F), devoid of the light-chain polypeptide and the first constant domain (CH1) of heavy-chain polypeptide.<sup>1–3</sup> The absence of light chains in the whole antibody and lack of the CH1 domain in the heavy chain are two significant features of camelid HCAs, which provide them with a more compact

architecture and smaller dimensional size with a molecular weight of ~90 kDa rather than ~150 kDa for the canonical antibody IgG.<sup>1,3</sup> Similarly, the Ig isotype IgNAR (novel antigen receptor) discovered in the shark bloodstream is also a homodimeric structure of two heavy-chain polypeptides, each comprising a single variable domain and five constant domains (homodimeric structure, Figure 1G–I).<sup>7</sup>

The percentage of HCAs in the bloodstream of camelids varies greatly among species because of a variation in mutation rates.<sup>3</sup> It might reach relatively high level in camels, ranging from ~50% to ~80%, whereas it totals up to 10%–25% in South American camelid species.<sup>1</sup> Relative to the HCAs in camelidae, the shark IgNARs are slightly at lower level, ranging from ~0.1 mg/mL to 1 mg/mL, counting for ~5% of the total Igs in the bloodstream.<sup>5,9</sup> The HCAs and IgNARs have shown very high hypermutation, apparently in response to antigens in the immune protection of the camelid and the shark.<sup>1,5,9</sup> Therefore, to some extent, the nanobodies (VHHs or V-NARs) with a molecular weight of ~12–15 kDa in the HCAs or IgNARs are the structural and functional counterparts of the Fabs in the conventional IgGs (Figure 1B, E, and H).<sup>1,3,5,8</sup>

Similar to the VH domain in conventional IgGs, the folded VHH in camel HCAs comprises nine  $\beta$ -strands. These  $\beta$ -strands, organized in a four-stranded  $\beta$ -sheet and a five-stranded  $\beta$ -sheet, were connected by three hypervariable (HV) loops, also called complementarity-determining regions (CDRs), and by a conserved disulfide bond between Cys23 within framework region (FR) 1 and Cys94 within FR3.<sup>1</sup> The three CDRs located at the N-terminal end of the domain forms a continuous surface (paratope) in response to recognizing epitopes of antigens. This means that VHHs of the camel HCAs and VH domains of the conventional IgGs share the similar structural architecture of the FRs and loops (Figure 1C and F).<sup>1,2,10,11</sup> The alignment of the VHH amino acid sequences indicates that two major significant differences between both the VHH and VH domains exist within the FR2 and in the CDRs, especially in the CDR3.

The first notable difference between VHH and VH is within FR2.<sup>1,3</sup> The highly conserved hydrophobic amino acids found within FR2 of the conventional VH region at positions (Val37, Gly44, Leu45, and Trp47) are replaced by hydrophilic and/or smaller amino acids, mostly Phe42, Glu49, Arg50, and Gly52, respectively.<sup>1,12–15</sup> These hydrophobic amino acids normally participate in the interaction with chaperone proteins and the variable light chain (VL) domain during the assembly of heavy chains and light chains



**Figure 1** Schematic representations of intact antibodies, including canonical antibodies (IgG1) and heavy-chain antibodies in camels (HCAbs) and sharks (IgNARs), and intact antibody-derived fragments.

**Notes:** (A) Intact canonical antibodies (IgG1) comprising two light chains (VL and CL domains) and two heavy chains (comprising VH, CH1, hinge, and CH2 and CH3 domains). (B) Canonical antibody-derived fragments: Fabs, scFvs, and VH domains. (C) The structure and packing of VH domain from canonical antibodies IgG1. (D) Intact HCAbs in camelids, comprising homodimeric heavy chains (containing VHH and CH2 and CH3 domains), devoid of light chains in intact antibodies and lack of CH1 domains in heavy chains. (E) Camel HCAb-derived single-domain antibodies: VHH. (F) The structure and packing of VHH from camel HCAbs. (G) Intact IgNARs in sharks, comprising homodimeric heavy chains (containing V-NAR and C1–C5 domains), devoid of light chains in antibodies. (H) Shark IgNAR-derived single-domain antibodies: V-NAR. (I) The structure and packing of V-NAR from shark IgNARs.

**Abbreviations:** CDRs, complementarity-determining regions; CH, constant heavy chain; CL, constant light chain; Fabs, antigen-binding fragments; HCAbs, heavy-chain-only antibodies; HV, hypervariable; Ig, immunoglobulin; IgNARs, Ig new antigen receptors; scFvs, single-chain variable fragments; VH, variable heavy chain; VHH, variable domain of HCAbs in camelids; VL, variable light chain; V-NAR, variable domain of IgNARs in sharks.

of the conventional antibodies.<sup>1</sup> Thus, reshaping this VL side of the domain deprives the heavy chains of their binding sites for any VL interaction, enhancing solubility and reducing aggregation of VHH in the absence of a VL domain.<sup>1,3</sup>

The second difference between VHH and VH is found in the HV loops. There is a broader structural repertoire of loops in VHH, especially an enlarged HV region in the H1 loop (CDR1) and an extended H3 loop (CDR3) (Figure 1C and F). These uniquely extended loops provide a sufficient antigen-interacting surface, large enough to 600–800 Å<sup>2</sup> as offered by six loops from the VH–VL domain pair in the traditional IgGs.<sup>1,3</sup> It has been established that the elongated CDR3 is capable of extending into cavities on antigens, such as the active site crevice of enzymes and ligand-binding cavity of receptors.<sup>1,3</sup> However, an enlarged loop suggests broader flexibility, expectedly leading to be entropically counterproductive for binding. This problem is solved in camel VHHs by fastening the extended loop of CDR3 with an extra disulfide bond toward either the CDR1 loop (Figure 1F) or the CDR2 loop or even the FR2.<sup>1,3,16</sup> These disulfide bonds might maximally optimize the binding surface topology and facilitate the orientation of the CDR3 toward the antigens.<sup>3</sup>

As for the V-NARs, it shows homology to V $\alpha$  domain of the T-cell receptor.<sup>5,17</sup> Contrary to VHH, V-NAR only consists of seven  $\beta$ -strands instead of nine  $\beta$ -strands due to the truncation in FR2–CDR2 (Figure 1F and I). This means that V-NARs have only two CDRs, ie, CDR1 and CDR3 (Figure 1I). Similar to the camelid VHHs, the V-NAR has abnormally long CDR3 loops that considerably reflect the greatest diversity of the V-NAR in both sequence and length.<sup>7</sup> Furthermore, highly frequent somatic mutation is also found in CDR1, at the equivalent site of the deleted CDR2, where the shorten loop forms a belt-like structure at the opposite side of the CDR1 and CDR3, and in a loop that resembles HV4 in T-cell receptors.<sup>5,18</sup> Similar to the camel VHHs, the shark V-NARs also evolved to generate equivalent disulfide bridges tethering the extended antigen-binding loops.<sup>1,19,20</sup>

Although the sequences of VHHs and V-NARs are very diverse,<sup>1</sup> the camel VHHs shared the similar architecture with V-NARs. Overall, both show a rugby ball-shaped structure and a convex or protruding surface at the N-terminal end of the domains.<sup>19,21</sup> This protruding surface increases the actual interaction surface of the paratopes, extremely facilitating insertion of nanobodies in cavities on the surface of the antigens or ligand-binding sites of receptors.<sup>22</sup> In limited cases, a flat paratope surface<sup>23</sup> and occasionally a cavity for the antihapten binders<sup>24,25</sup> were also observed owing to the long loops in most nanobody structures folding over the FR2

region in VHH.<sup>1</sup> These different shapes of the paratope surfaces demonstrated the extreme flexibility and great diversity of both the sdAbs.

## Nanobody libraries

To fish out a desired nanobody with high stability and subnanomolar or picomolar affinity, preparation of nanobody libraries using different approaches was reasonably proposed.<sup>1,4,7,26,27</sup> The technologies used for the preparation of nanobody library do not significantly differ from the ones otherwise used for recovering the Fab and scFv libraries. First of all, retrieval of nanobodies from an immune library is a priority consideration because somatic maturation in lymphocytes of immunized *Camelidae* will give antibody libraries more specific and higher affinity to antigens of interest.<sup>4</sup> However, for each new antigen, we have to prepare a new immune library, which might unnecessarily spend more time and costs than other strategies proposed where else, such as large one-pot libraries without immunization of animals.<sup>4</sup> Thus, a suitable naive library using blood samples from nonimmunized animals or the semisynthetic and synthetic libraries are practically alternative choices. Despite the lack of somatic maturation, it is possible that the selection based on phage display using such one-pot large libraries (>10<sup>9</sup> clones per library) allowed to isolate such VHHs with high affinity in the subnanomolar or picomolar range that are suitable for the diverse biomedical applications.

## Immune library

Preparation of the immune nanobody library first needs an immunization of camelidae, through which antigen-specific HCAs are affinity matured. The immunization procedures are mostly involved in prime–boost strategy using various antigens of interest as immunogens. After a brief immunization, the nanobodies are generally readily obtained by cloning the V gene repertoire from peripheral blood lymphocytes and by screening through phage display or other biological carriers.<sup>1,26</sup> The entire Fab of the HCAb comprises only one VHH, and it contains ~120 amino acid residues, encoded by a gene fragment of only ~360 bp. Thus, the VHH gene is easily cloned by polymerase chain reaction (PCR) in one single amplicon. As a result, small libraries (just ~10<sup>6</sup> individual clones per library) created by using a ~50 mL of blood sample already represent the immune VHH repertoire of lymphocytes present in bloodstream of the immunized animals.<sup>1</sup>

As for the amplification and cloning of an scFv, the VH and VL exons needed to be first individually PCR amplified, which probably results in scrambled pairs of the VH and VL



domains due to their random assembly. In contrast to the scFv cloning, the PCR amplification of the VHH present in only one exon generally facilitated generation of intact and affinity-matured VHHs from peripheral B-lymphocytes. Thus, the unique specificity and high affinity of nanobodies from immune libraries are guaranteed. Kinetic  $k_{\text{on}}$  and  $k_{\text{off}}$  rate constants of the nanobodies retrieved from the immune libraries are routinely reported to be low nanomolar or even picomolar levels, ranging from  $10^5$  to  $10^6 \text{ M}^{-1} \text{ s}^{-1}$  and  $10^{-2}$ – $10^{-4} \text{ s}^{-1}$ , respectively.<sup>1</sup> Such affinity parameters are excellent properties for the most biomedical applications, including disease diagnosis, bioimaging, drug screening, and targeting therapy.

## Naive library

When toxic or nonimmunogenic antigens are potentially used as immunogens, or immunization is not available for some other reasons, naive V repertoires could be employed to replace the immune VHH libraries. This approach allows us saving the time and costs related to preparing a new library for any new antigens of interest. Due to the lack of somatic maturation stimulated *in vivo* by immunization process, such libraries theoretically need to include  $\sim 10^9$  individual clones to allow the retrieval of high-affinity binders to a given antigen in general.<sup>1</sup> Practically, the theoretical diversity of a naive library increases with increasing of the number of lymphocytes initially collected. Thus, large volume of blood samples ( $> 1 \text{ L}$ ) collected from different animals is a prerequisite for preserving the greatest genetic diversity. In addition, to avoid unnecessary diversity reduction during cloning of this type of library, all of the necessary steps should be performed with the highest care to reduce the material loss. As a practical alternative, the final library size could also be beneficially increased by mixing independently prepared collections to guarantee the diversity of the library.<sup>4</sup>

## Semisynthetic/synthetic library

Limited size and diversity is a key problem that affects even large size naive-based nanobody libraries.<sup>27</sup> Without an enormous germline diversity and the recombinatorial diversity from VH/VL pairing in traditional IgGs, affinity maturation in camelids or sharks relies to a larger extent on somatic hypermutation that precisely tunes the CDRs to recognize any given antigens.<sup>27</sup> Thus, in an effort to mimic such *in vivo* diversification to yield diverse enough libraries capable of generating nanobodies to any given antigen, another strategy, ie, semisynthetic/synthetic library, was proposed. The high-affinity nanobody could also be fished out using this type

of library without immunization of animals. The strategy is trying to conserve the FRs surrounding the CDRs, which may be crucial in conserving the structural integrity of nanobodies, and to randomly diversify the sequences of the CDRs, especially of CDR3.<sup>27</sup>

Based on naturally occurring VHH or V-NAR sequences, the semisynthetic or synthetic nanobody libraries could be created by introducing length and sequence variations in CDR3 using randomized CDR3 primers,<sup>18</sup> or error-prone PCR combined with splice-overlap extension PCR method.<sup>7,27</sup> Using small blood samples ( $< 10 \text{ mL}$ ), the complexity of diversity-enhanced semisynthetic or synthetic nanobody library is close to  $10^9$ , whereas the complexity of  $10^6$  of naive library could only be reached by using the same volume of blood samples. At least  $> 1 \text{ L}$  of blood samples might be consumed in order to obtain  $\sim 10^9$  individual clones per library for the naive library.<sup>4</sup> This means that the semisynthetic or synthetic library had better CDR3 diversity and better utility than the naturally occurring naive VHH or V-NAR libraries without immunization of animal or collection of large volume of blood samples. Thus, this may be a promising path toward obtaining a limitless source of nanobodies against a variety of antigens without immunization of animals.

Independent of the strategies to construct libraries, the established libraries further need to be displayed on different biological carries, including phages, bacteria, yeasts, and ribosomes, to facilitate screening and panning for a given antigen-specific nanobody. Retrieval of nanobodies from the libraries by phage display or any other selection protocols described earlier, including bacterial display, yeast display, intracellular two-hybrid selection, and ribosome display, has been well documented.<sup>1</sup> By a variety of standard biopanning strategies, these libraries are precious hidden treasure with great molecular diversity and could be utilized to fish out the nanobody binders with desired properties to various antigens of interest.

## Nanobody-derived nanobiotechnology tool kits

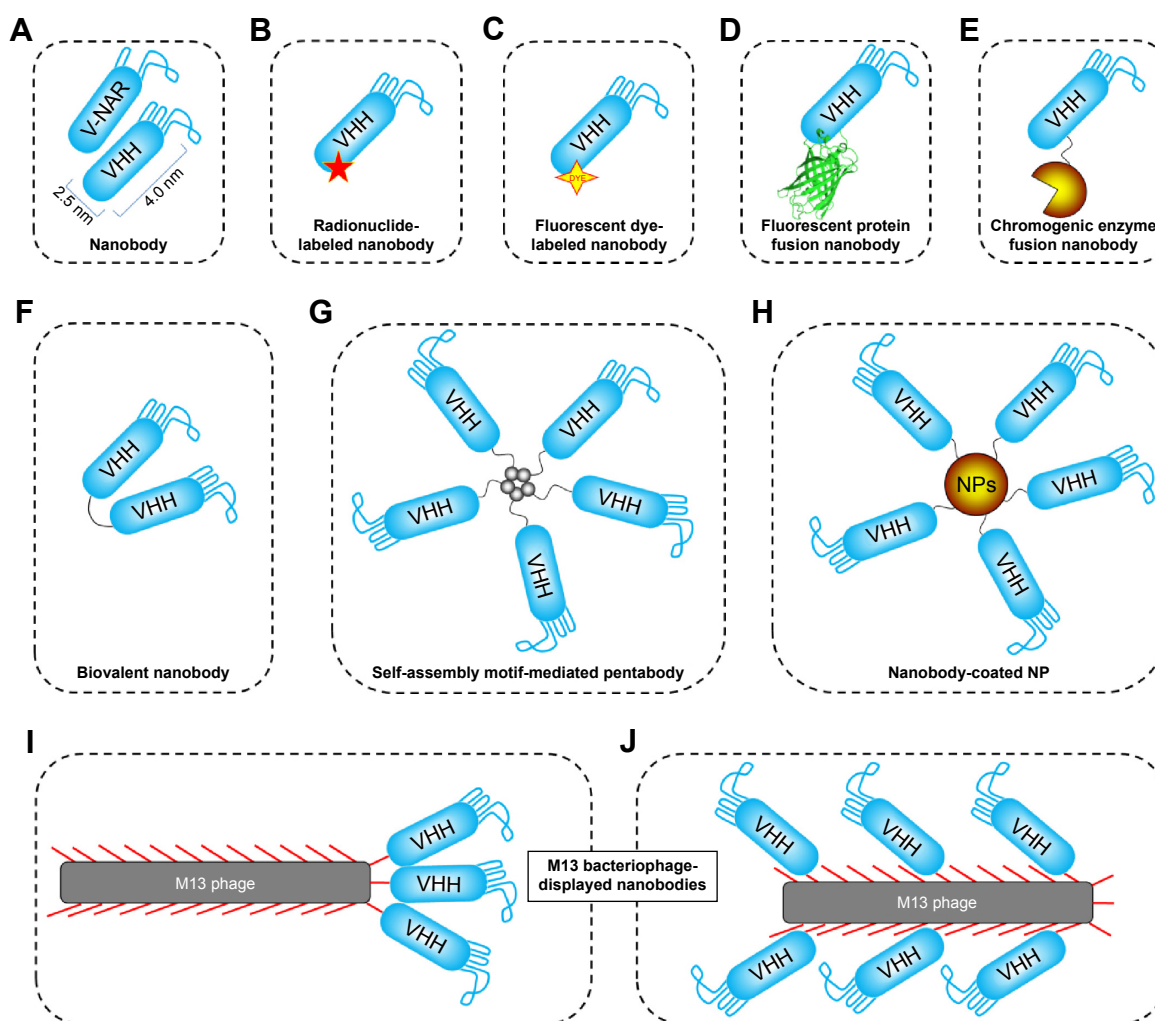
Within the new vista of nanobiotechnological applications, different nanosized biotools, nanoscaled biomacromolecules, and engineered bacteriophages have been employed as promising approaches to meet the unmet needs of biomedicine and biotechnology development for human health. Owing to the desired properties of nanobody, including nanoscaled size, stable and soluble behavior in aqueous solution, reversible refolding, humanizable sequences, and specific and high affinity for only one cognate target, as well as a

sustainable source, nanobody has been an ideal research tool for the development of sophisticated nanobiotechnologies. Currently, the nanobody has been evolved into versatile research and application tool kits for diverse nanobiotechnology applications.<sup>1–3,10,11,28</sup> A variety of nanobody-derived formats, including the nanobody itself, the radionuclide or fluorescent dye-labeled nanobodies, fluorescent protein or chromogenic enzyme fusion nanobodies, bivalent nanobodies, self-assembly motif-mediated nanobody homo- or heteromultimers, nanobody-coated nanoparticles, and nanobody-displayed bacteriophages (Figure 2), have been successfully demonstrated as powerful nanobiotechnological tool kits for diverse biomedical applications, including targeting drug delivery and therapy,<sup>1,3</sup> disease diagnosis,<sup>2,3</sup> bioimaging,<sup>11,29–31</sup> and agricultural and plant protection.<sup>32,33</sup>

These applications indicate a special advantage of these nanobody-derived technologies, already surpassing the “me-too” products of other equivalent binders, such as the full-length antibodies, scFvs, Fabs, targeting peptides, and DNA-based aptamers.

## Versatile applications of nanobody-derived nanobiotechnologies

There are various biomedical applications using the nanobody-derived nanobiotechnologies, which has been extensively covered recently elsewhere.<sup>1–3,10,11,28,34–37</sup> Here, we focus on a number of examples, wherein nanobodies provide special advantages over other equivalent binders. These applications demonstrated a promising future of the use of nanobodies in versatile environments, including basic



**Figure 2** Schematic of versatile nanobody-derived nanobiotechnological tool kits containing nanobody itself (A), radionuclide-labeled (B) or fluorescent dye-labeled nanobodies (C), fluorescent protein fusion nanobodies (D), chromogenic enzyme fusion nanobodies (E), bivalent nanobodies (F), self-assembly motif-mediated nanobody homo- or heteromultimers (G), nanobody-coated nanoparticles (H), and nanobody-displayed phages (I and J).

**Note:** These nanobiotools have been successfully applied to a variety of biomedical applications.<sup>1–3,10,11,28,34–37</sup>

**Abbreviations:** NPs, nanoparticles; VHH, variable domain of heavy-chain-only antibodies in camelids; V-NAR, variable domain of immunoglobulin new antigen receptors in sharks.

research, bioimaging, clinical diagnosis, therapeutics, and agricultural and plant protection. Compared to the conventional sdAbs, these diverse applications indicate the versatile and novel properties of nanobodies as promising sdAbs.

## Nanobodies as versatile research tools in biotechnology

With the versatile properties of the nanobodies, they have been developed into various research tools used in basic research, including affinity purification, immunoprecipitation, chaperone-assisted crystallization, protein degradation, gene activation or inactivation, protein–protein interaction, and many others (Table 1).

Owing to the intrinsic stability, monomeric nature, and easy directional immobilization to solid substrates, the nanobody is considered an ideal ligand for biomolecule purifications. Compared to full-length antibodies, nanobodies could yield a high-column regeneration capacity, produce an increased amount of paratopes per gram of support materials, and only need milder elution conditions.<sup>1–3</sup> For example, antihuman IgG VHH has been developed for IgG purification and depletion from blood, outperforming canonical protein A-based method.<sup>38</sup> Apart from concave epitopes on properly folded proteins, nanobodies could recognize small linear peptide sequences, which has been confirmed by isolating anti-EPEA VHHs. EPEA is a C-terminal tetra-amino-acid

Glu–Pro–Glu–Ala sequence that can be cloned as a tag behind any protein,<sup>39</sup> facilitating a rapid and robust affinity purification of proteins. This linear peptide sequence recognition by nanobodies has also been demonstrated by isolating VHHs against another tetra-amino-acid sequence KDEL, a C-terminal signature tag of endoplasmic reticulum-resident protein.<sup>40</sup> This anti-KDEL VHH nanobody was shown to be an excellent tool to study differences in ER-resident protein expression by recognizing the KDEL sequence at the C-terminus of proteins, irrespective of the protein context.<sup>40</sup>

The high affinity and unique specificity of nanobodies also make themselves excellent candidates for immunoprecipitation applications and for chromatin immunoprecipitation with DNA microarray (ChIP-on-chip) technology, facilitating uncovering new transcription factor-binding sites.<sup>2,41</sup> Furthermore, by the combination of nanobody and magnetosome, a VHH-coated magnetosome approach was proposed for in vitro and in vivo immunoprecipitation by magnetical recruitment of antigen partners from cell extracts or within living bacteria.<sup>42</sup> As membranous organelles present in magnetotactic bacteria, magnetosomes contain magnetite particles enabling orientation of bacteria in a magnetic field.<sup>2</sup> By expressing red fluorescent protein (RFP)-binding nanobody with a magnetosome membrane protein MamC, VHH-coated magnetosomes were generated to efficiently recognize and bind their antigens in vitro by magnetically separating

**Table 1** The typical applications of nanobody-derived nanobiotechnologies in basic biomedical research

Antigens of interest	Specific nanobodies	Potential applications	References
Human IgG	Anti-hlgG VHH	IgG purification and depletion from blood, outperforming protein-A-based method	38
C-terminal tag: Glu–Pro–Glu–Ala (EPEA)	Anti-EPEA VHH	Highly efficient affinity chromatography for any EPEA-tagged protein purification	39
ER-resident protein C-terminal tag: KDEL	Anti-KDEL VHH	In situ monitoring the expression of ER-resident protein	40
Lrp-like regulator Ss-LrpB	Anti-Ss-LrpB VHH	ChIP-on-chip	41
RFP	Anti-RFP VHH	Immunoprecipitation via VHH-coated magnetosomes in vitro and in vivo	42
β2-AR	Anti-β2-AR VHH	Crystallization chaperone for β2-adrenergic receptor	44
Anti-toxic protein MazE in <i>Escherichia coli</i>	Anti-MazE VHH	Crystallization chaperone for MazE protein	45
β2-MF	Anti-β2-MF VHH	Crystallization chaperone for β2-microglobulin fibril formation	48
Prion	Anti-prion VHH	Crystallization chaperone to inhibit prion oligomerization, understanding early prion formation	49
Epsl:EpsJ pseudopilin heterodimer	Anti-pseudopilin VHH	Crystallization chaperone for Epsl:EpsJ pseudopilin heterodimer, the bacterial type 2 secretion system	50
Secretin GspD	Anti-GspD VHH	Crystallization chaperone for periplasmic N-terminal domain of GspD; the type 2 secretion system	51
GFP	Anti-GFP VHH	Protein knockout by the deGradFP complex, ie, anti-GFP VHH fused to the F-box domain	52,53
Different epitopes of GFP	Antiepitopes of GFP VHHs	Gene activation or inactivation in GFP-expressing cells by active transcription factor complex	54

**Abbreviations:** AR, adrenergic receptor; ChIP-on-chip, chromatin immunoprecipitation with DNA microarrays; GFP, green fluorescent protein; Ig, immunoglobulin; IgG, immunoglobulin-γ; MF, microglobulin fibril; RFP, red fluorescent protein; VHH, variable domain of heavy-chain-only antibodies in camelids; hlgG, human IgG; EPEA, Glu–Pro–Glu–Ala; KDEL, Lys–Asp–Glu–Leu; ER, endoplasmic reticulum.

the VHH particle–antigen complexes from unbound protein contaminants. Such VHH particles could potentially also be used for intracellular recognition and magnetosome recruitment of RFP-tagged proteins and their interaction partners within living bacteria.

Nanobodies were also used as effective chaperones to assist crystallization process and structural determination of membrane proteins and large protein complexes for which it is difficult to determine these protein structures by X-ray crystallography. Since the convex or protruding surface at the N-terminal end of nanobodies could be targeting and recognizing clefts on the surface of antigens and these clefts usually coincide with active enzymatic sites or ligand-/receptor-binding cavities, the nanobodies are very suitable chaperones to assist crystallization and structural determination of these challenging targets.<sup>43,44</sup> It has been demonstrated that, compared to the conventionally full antibodies, such chaperones could facilitate crystal formation by maintaining highly dynamic proteins in one of the particular protein conformations and stabilize intrinsic flexible regions or detergent-solubilized membrane proteins through preventing hydrophobic surfaces from contact with solvent to facilitate the effective crystal formation (Figure 3).<sup>45–47</sup> For example,  $\beta$ 2-adrenergic receptor crystal has been elucidated in the presence of active state-specific VHH, which could stabilize the instable active state of the receptor,<sup>2,44</sup> and a VHH also plays its unique roles as a chaperone for the formation of  $\beta$ 2-microglobulin fibril by stabilizing early fibril intermediates and preventing their self-oligomerization.<sup>2,48</sup> The structural information on the disordered N-terminal prion protein region has also been elucidated by a VHH-inhibiting prion oligomerization, eventually contributing to the understanding of early prion formation.<sup>2,49</sup> Similarly, crystallization process of components of bacterial type 2 secretion system demonstrated that the VHHs could substantially facilitate well-diffracting crystal formation by merely providing additional contact surface to the target proteins.<sup>2,50,51</sup>

By an elegantly experimental design, another example of the use of nanobody is to trigger the depletion of antigen via the ubiquitin pathway. Caussinus et al<sup>52,53</sup> designed a deGradFP nanocomplex to induce the degradation of protein in vivo. A proof of concept study was conducted in fruitfly (*Drosophila melanogaster*) and zebrafish (*Danio rerio*). The anti-green fluorescent protein (GFP) nanobody was fused to the F-box domain that recruits the polyubiquitination machinery. Once a target was captured in the ubiquitinated nanobody-based nanocomplex, the proteasome-mediated degradation could be initiated.<sup>1,52</sup> This is a new protein

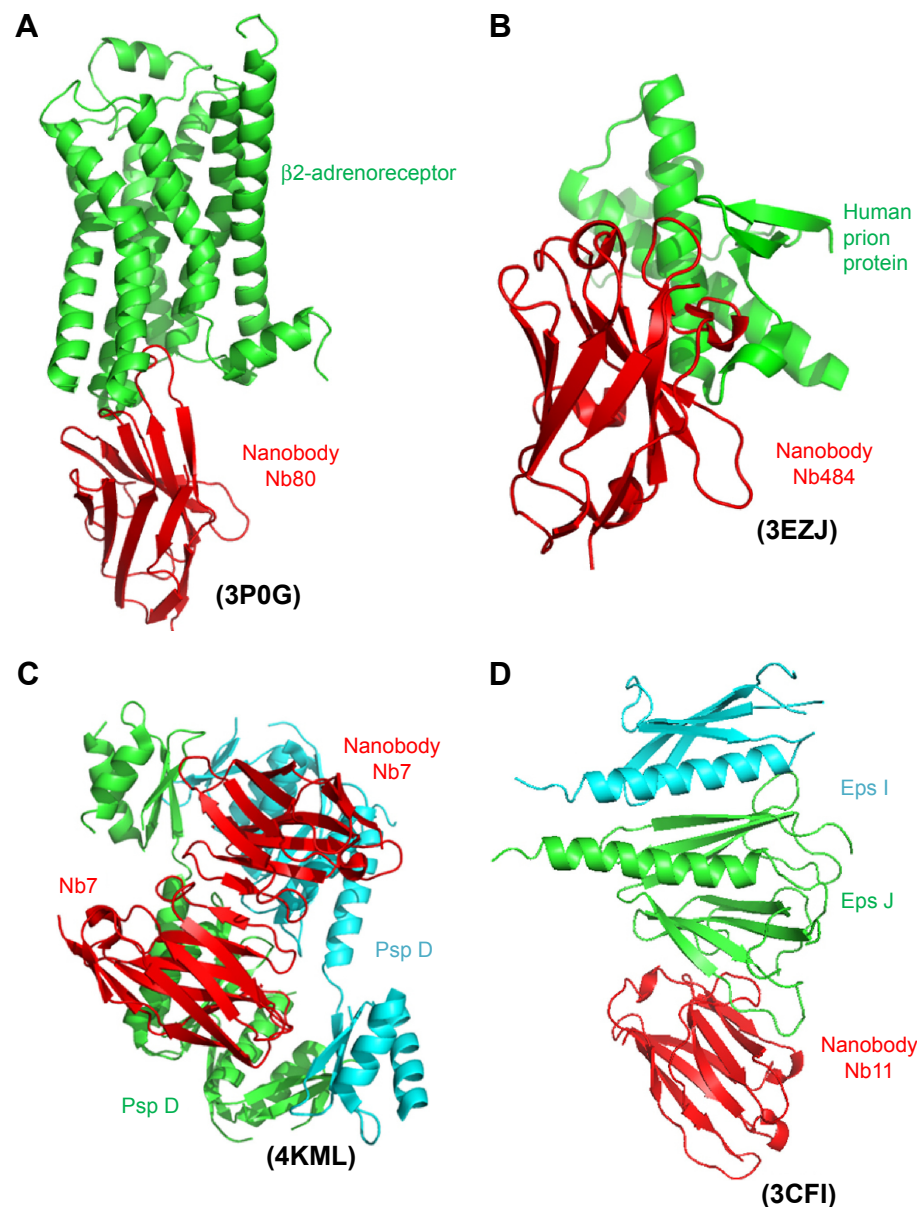
knockout tool potentially for more sophisticated applications in biomedicine and biotechnology. The resulting ubiquitinated nanobody-based nanocomplex could be effectively restricted in certain tissues, and the extent of protein degradation could be real-time monitored by just measuring GFP fluorescence.<sup>2</sup>

Recently, some other elegant works also demonstrated a nanobody-based system using fluorescent proteins as scaffolds for cell-specific gene manipulation.<sup>54</sup> Two different nanobodies that could bind different regions of GFP were fused to a transcriptional activation domain or a DNA-binding domain, respectively. As a result, the GFP expression in GFP-expressing cells could specifically lead to the formation of a nanoscaled active transcription factor.<sup>54</sup> This approach could be utilized to conveniently induce cell-specific transgene expression or gene knockdown by RNAi in a vast collection of transgenic GFP cell lines.<sup>2</sup>

## Nanobodies as powerful bioimaging reagents

Owing to small dimensional size and high affinity of the nanobodies against various targets of interest, eg, intracellular signaling molecules and cancer biomarkers, the nanobodies and their derivative formats used as versatile nanotracers have been successfully employed in this postgenomic era (Table 2).<sup>1</sup> Using fluorescent protein fusion nanobodies or anti-GFP nanobodies, some elegantly fabricated nanobody-based tracers have been developed for bioimaging in living cells. As a proof of concept, intracellular expression of the genetic fusion of a fluorescent protein with a nanobody produces useful chromobodies or fluobodies to trace in vivo intracellular target in various cellular compartments in living cells,<sup>55,56</sup> avoiding the need of genetic modification of target proteins with fluorescent tags. For example, an anti-GFP nanobody, termed GFP-binding protein (GBP), was fused to a monomeric RFP to generate a chromobody. The resulting GFP-based chromobody could specifically label the intracellular GFP fusion proteins localized in cytoplasm or nuclear.<sup>55</sup> Several of these nanobody-based tracers were also developed for high-content analysis, eg, the direct visualization of endogenous native proteins or infectious HIV virions in living cells.<sup>57,58</sup> Furthermore, the GBP is also applied in super-resolution microscopy for the visualization of GFP fusion proteins. By coupling organic dyes to GFP-binding nanobodies, the nanobody-based tracker could recognize any GFP-tagged construct, enabling single-molecule localization with super-resolution imaging techniques.<sup>1,59</sup> Similarly, a GBP-coated gold nanoparticle was also employed as a





**Figure 3** Some challenging structures elucidated using nanobodies as crystallization chaperones.

**Notes:** (A) Nanobody Nb80 (red), as a structural mimic of G $\alpha$ S, stabilizes the active-state conformation of  $\beta$ 2-adrenoreceptor (green).<sup>44,47</sup> (B) Ribbon representation of the full-length human prion protein (HuPrP, green) in complex with nanobody Nb484 (red).<sup>49</sup> (C) A periplasmic N-terminal domain of GspD (green or cyan) from the type 2 secretion system secretin in complex with nanobody Nb7 (red), forming a compact GspD:Nb7 heterotetramer.<sup>46,51</sup> (D) Nanobody Nb11 (red)-aided structure determination of EpsI (cyan):EpsJ (green) pseudopilin heterodimer, a component of the bacterial type 2 secretion system in *Vibrio vulnificus*.<sup>50</sup> Protein Data Bank accession numbers are given in parentheses.

single-molecule tracer to monitor GFP-tagged membrane proteins and is even capable of tracking intracellular proteins in living cells by internalization.<sup>2,60</sup>

Recently, the nanobody-based tracer was applied to study in vivo protein–protein interactions.<sup>2</sup> By a sophisticated experimental design, the GBP was first fused to an anchoring protein that will localize the GBP at a particular subcellular compartment. The fluorescent protein GFP or RFP was then fused to the two proteins of interest. Once interaction of the two proteins occurs, both the proteins tethered together

will lead to a strong GFP–RFP colocalization signal at the subcellular compartment defined by the GBP. In the nucleus and cytosol of human cells, it has been successfully demonstrated the effectiveness of this approach. By this way, peptide inhibitors of protein–protein interactions in these intracellular environments were analyzed and screened.<sup>2,61</sup> More importantly, intracellularly expressed nanobodies themselves remaining soluble and specific antigen recognition activities could also be used to interfere with particular protein functions by competing with normal

**Table 2** The representative applications of chromobodies-based nanobiotechnologies for intracellular bioimaging

Antigens of interest	Specific nanobodies	Potential applications	References
GFP	Anti-GFP VHHs fused to RFP	Nanobody-based tracer: specifically label cytoplasmic or nuclear localized GFP fusion proteins	55
		High-content analysis: eg, direct monitoring apoptosis assay in living cells	57
HIV-1 CA	Anti-CA VHH fused to GFP	Dynamic visualization of HIV virions in living cells	58
GFP	Anti-GFP VHH labeled with dye AF647	Super-resolution microscopy for GFP fusion proteins, reaching nanometer spatial resolution	59
GFP	Anti-GFP VHH-coated gold nanoparticle	Live cell single-molecule imaging for GFP-tagged membrane proteins and intracellular proteins	60
GFP	Anti-GFP VHH fused to localization protein	Intracellular protein–protein interactions: F3H for GFP and RFP fusion proteins	61
GFP	Multiple anti-GFP VHHs	Monitoring intracellular translocation of estrogen receptor by nuclear expression of GFP-enhancing VHH	64
GFP	Anti-GFP VHH fused to nuclear lamin I	Trapping nuclear promyelocytic leukemia protein in lamina	67
GFP	Anti-GFP VHH fused to RFP	Trapping nuclear GFP fusion proteins in the cytoplasm in plant cells	68

**Abbreviations:** CA, capsid protein; F3H, fluorescent-three-hybrid; GFP, green fluorescent protein; RFP, red fluorescent protein; VHH, variable domain of heavy-chain-only antibodies in camelids.

in situ protein–protein interactions. Thus, the identification of such bioactive nanobodies could offer an opportunity for target validation and lead molecule optimization to investigate difficult interactions or interactions considered undruggable.<sup>1,62,63</sup>

More interestingly, versatile nanotraps have been developed, by which nanobodies were exploited to report particular conformational variants of a target and even to alter target translocation and localization in different organelles of living cells.<sup>64–67</sup> For example, nanobodies could modulate the conformation and spectral properties of GFP. The tamoxifen-induced translocation of human estrogen receptor from the cytoplasm to the nucleus could be monitored in a sensitive, high-throughput manner by nuclear expression of a GFP-enhancing GBP and then by monitoring the ratio of imaging.<sup>2,64</sup> Promyelocytic leukemia protein originally dispersed throughout the nucleus, whereas GFP fusion promyelocytic leukemia protein is specifically redirected to the nuclear lamina by coexpressing GBP-lamin1 as a nuclear lamina anchor.<sup>2,67</sup> Contrary to trapping target proteins in nuclear lamina, GBP was also demonstrated to block the action of nuclear GFP fusion proteins by trapping them in the cytoplasm of plant cells.<sup>2,68</sup>

## Nanobodies for disease diagnosis

Apart from the use of nanobodies as versatile bioimaging tools in living cells, nanobodies have been used as valuable in vivo detection probes for cancer, infectious disease, atherosclerotic lesions, inflammatory response, and many other diseases in preclinical and clinical environments (Table 3).

The nanobodies could be quickly eliminated from blood-stream due to their small sizes with a molecular weight of ~12–15 kDa that is well below the renal clearance cutoff of molecular weight of ~50 kDa.<sup>1,69</sup> Thus, the small-size property of nanobodies facilitates their fast extravasation, good tumor penetration, and rapid renal clearance, which eventually lead to rapid and sensitive imaging of target tissue just within a few hours postinjection.<sup>2,29,30</sup> This is requirement exactly for a good in vivo imaging agent.<sup>1</sup> Radionuclide-labeled nanobodies have been successfully applied to noninvasively image in vivo tumors via positron emission tomography and single-photon emission computed tomography. These nanobody-derived nanoprobe demonstrated a high target specificity, high stability, good solubility, high tumor-to-background signal, fast clearance of excess tracer, and low immunogenicity.<sup>2</sup> For example, <sup>99m</sup>Tc-labeled VHHs could be used to recognize HER2, a cancer antigen for breast cancer diagnosis in a preclinical setting,<sup>29</sup> to distinguish moderate and high expression of epidermal growth factor receptor for improved prognosis of cancer therapy,<sup>30</sup> to detect the status of inflammatory responses by imaging dendritic cells,<sup>31</sup> and to target VCAM1, an antigen used to diagnose vulnerable atherosclerotic plaques.<sup>70</sup>

Besides the promising results obtained by using <sup>99m</sup>Tc-VHH with half-life time ( $t_{1/2}$ ) of ~6 hours, some short-lived nuclides were also tested for more patient-friendly diagnoses. Rapid targeting to diseased tissue and fast blood clearance of unbound nanobodies make it possible to use short-lived nuclide-labeled formats for in vivo diagnosis, such as <sup>68</sup>Ga and <sup>18</sup>F with the  $t_{1/2}$  of 68 minutes and 110 minutes, respectively. This approach makes it possible to measure

**Table 3** The representative applications of nanobody-derived nanobiotechnologies for disease diagnosis in vitro and in vivo

Antigens of interest	Specific nanobodies	Potential applications	References
HER2	<sup>99m</sup> Tc-labeled anti-HER2 VHH	SPECT for molecular diagnosis of breast cancer	29
EGFR	<sup>99m</sup> Tc-labeled anti-EGFR VHH	SPECT imaging for in vivo monitoring of EGFR expression	30
Murine bone marrow-derived dendritic cells	Multiple <sup>99m</sup> Tc-labeled VHHs	SPECT imaging for the status of inflammatory responses	31
VCAM1	<sup>99m</sup> Tc-labeled VHHs	SPECT imaging for atherosclerotic lesions	70
EGFR	<sup>68</sup> Ga-labeled anti-EGFR VHH	Immuno-PET imaging in epidermoid carcinoma A431 xenografts	71
MMR	<sup>99m</sup> Tc-labeled anti-MMR VHH	SPECT/micro-CT for in vivo imaging of tumor-associated macrophages	72
HER2	IRDye 800CW-labeled anti-HER2 VHH	NIR optical imaging of human breast tumor xenografts for image-guided surgery	73
EGFR	IRDye 800CW-labeled anti-EGFR VHH	NIR optical imaging for human tumor xenografts	74
hPSA	Anti-hPSA VHH	Surface plasmon resonance-based sensing for human prostate-specific antigen	75
Chaperonin GroEL from <i>Brucella</i>	Anti-GroEL VHH	Species-specific diagnosis of <i>Brucella</i> infections in livestock	79
Ts14 from <i>Taenia solium</i>	Anti-Ts14 VHH	Species-specific diagnosis of <i>T. solium</i> infection in pigs	80
Surface glycoprotein of <i>Trypanosoma evansi</i>	Anti- <i>T. evansi</i> VHH	Diagnostic of <i>T. evansi</i> , allowing easy species typing of prevailing parasites	81
Human glycophorin A erythrocyte (CD235a)	Anti-CD235a VHH fused to HIV-1 p24	VHH-based agglutination reagent for HIV diagnosis	82

**Abbreviations:** EGFR, epidermal growth factor receptor; HER2, Human epidermal growth factor receptor-2; hPSA, human prostate-specific antigen; MMR, macrophage mannose receptor; NIR, near-infrared; PET, positron emission tomography; RFP, red fluorescent protein; SPECT, single-photon emission computed tomography; micro-CT, micro-computed tomography; VCAM1, vascular cell adhesion molecule-1; VHH, variable domain of heavy-chain-only antibodies in camelids.

picomolar concentrations of nanoprobe within 1–3 hours postinjection by using positron emission tomography and single-photon emission computed tomography imaging techniques, thus resulting in a very low radiation burden for patients.<sup>1</sup> Using a mouse epidermoid carcinoma A431 xenograft model, the <sup>68</sup>Ga-labeled nanobodies against an epidermal growth factor receptor were recently tested for their performances, yielding a relatively high ratio of tumor to blood after 3 hours postinjection.<sup>1,71</sup>

Owing to rapid renal clearance of nanobodies, the disadvantage of using nanobodies as in vivo probes is high accumulation of unbound nanobodies in kidneys soon after the probe injection, finally leading to a high radiation dose in kidneys and a complicated analysis of nearby tissues.<sup>2,29,31,70</sup> A method has been further proposed to reduce the background level encountered with radiolabeled VHHs by preinjection of unlabeled bivalent VHHs to first occupy all extratumoral sites.<sup>72</sup> Alternatively, nonradioactive VHH-based probes with near-infrared fluorophores have also been fabricated to image in vivo tumors. These near-infrared fluorophore-labeled VHHs also showed faster imaging compared to approved monoclonal antibodies (McAbs) targeting the same antigens.<sup>2,73,74</sup>

Like a coin has two sides, the small size of nanobodies, however, might affect antigen–probe interaction when they are coated in adsorptive substrates in an in vitro enzyme linked immunosorbent assay (ELISA) for disease biomarkers in blood

or other biopsies. Compared to traditional IgGs, when these small-size nanobodies are coated on adsorptive substrates, the paratopes of nanobodies are very close to the vicinity of adsorbing surface, which might hinder antigen–probe interaction.<sup>75</sup> C-terminal peptide extension could improve the accessibility of coated VHHs in ELISA, eg, by fusion to peptide fragments, including a myc-His-tag, a llama long hinge region-His-tag,<sup>76</sup> and an Fc chain.<sup>2,75</sup> Through this way, the engineered camel sdAbs were immobilized for sensing human prostate-specific antigen, which demonstrated that a higher probe density mediates enhanced detection sensitivity in a surface plasmon resonance-based detection system.<sup>75,77</sup> This means that the use of nanobodies to generate sensitive and selective biosensors for in vitro disease diagnosis is highly feasible.<sup>2,75</sup>

More particularly, the nanobodies have also been developed to detect disease biomarkers in human biopsies by antibody-based slide and bead arrays. For an instance, a biotinylated VHH has been applied to streptavidin beads for sensitive biomarker detection in patient sera.<sup>2,78</sup> As for pathogen diagnosis, traditionally McAb-derived antibodies have been used for decades. Although these McAb-derived formats recognize pathogen antigens with high sensitivity, they often lack the required specificity, leading to unsatisfactory performances. It has been demonstrated that the performances of nanobodies far exceed traditional McAbs. For example, *Brucella* and *Yersinia* infections in livestock have been

successfully distinguished by a VHH rather than conventional antibodies.<sup>79</sup> Similarly, *Taenia solium* infection in pigs could be successfully detected by species-specific VHHs, whereas the existing genus-specific McAbs have failed to discriminate between *T. solium* and *Taenia hydatigena* infections.<sup>80</sup> Both species-specific and genus-specific VHHs have been developed for the diagnosis of *Trypanosoma* parasites without purification of antigens, leading to easy species typing of the prevailing parasites.<sup>2,81</sup> Recently, a general nanobody-based agglutination reagent, consisting of a fusion protein between a red blood cell-specific VHH and a disease antigen of interest, has been developed for diagnosing a variety of diseases when different disease-specific antigens are available. This elegantly designed system could be used for HIV diagnosis when an HIV-1 p24 antigen was fused with red blood cell-specific VHH.<sup>82</sup> Thus, agglutination mediated by anti-p24 antibodies in patient serum and the added p24-VHH fusion protein could be observed if the anti-p24 antibodies are present in HIV-positive patient sera.<sup>2</sup>

## Nanobodies as targeting therapeutics

The development of nanobodies as targeting therapeutics is still in a very early stage. Some elegant works have demonstrated

that the use of the bioactive nanobodies for antitoxin, anti-infection, anti-inflammation, or enzyme inhibition is a potentially feasible way for novel therapeutic development (Table 4). For example, nanobodies have been evaluated for passive immunization to treat envenomed victims, demonstrating extreme high-neutralization potency. By far, nanobodies for antiscorpion toxins, antibacterial toxins, and anti-snake venom are actively being investigated.<sup>83–87</sup> Owing to their small size and extended CDR3, nanobodies also showed special advantages as therapeutics for infectious disease, including the infection of viruses, bacteria, and parasites, over conventional antibodies that usually obstruct the access of hidden and essential epitopes on pathogens.<sup>88–96</sup> The added value of the nanobodies as targeting therapeutics stems from their capacity to distinguish the cognate target from closely related variants. Most of the small organic antagonists or even the conventional antibodies to a larger extent cannot reach such high specificity. Therefore, nanobodies could be used to specifically inhibit unwanted enzymatic activities related to different cellular signaling pathways. For example, ecto-ADP-ribosyl transferase ART2.2-specific VHH effectively blocked the enzymatic and cytotoxic activities of ART2.2 in lymphatic organs following intravenous injection.

**Table 4** The representative applications of nanobody-derived nanobiotechnologies for potential targeting disease therapy

Antigens of interest	Specific nanobodies	Potential applications	References
Scorpion toxin	Antiscorpion toxin VHH	Antitoxin therapy against lethal scorpion envenoming	83
Toxin A from <i>Clostridium difficile</i>	Antitoxin and VHH	Potent neutralizer against cytopathic effects of toxin A for <i>C. difficile</i> infection	84
Snake venom	Antisnake venom VHH	Antitoxin therapy for snake venom-induced pathology	85–87
RP	Anti-RP VHH	Antitoxin therapy for rotavirus-induced diarrhea	88
Antigen I/II adhesin from <i>Streptococcus mutans</i> S36	Anti-S36 VHH	Antibacterial prophylaxis against dental caries caused by <i>S. mutans</i>	89
FMDV	Anti-FMDV VHH	Passive FMD immunoprophylaxis to reduce FMD transmission between pigs	90
Env proteins from HIV-1	Anti-Env VHH	Anti-HIV-1 microbicides for neutralizing HIV-1 subtypes A, B, and C	91
HIV-1 gp120	Anti-gp120 VHH	Potent inhibitors of HIV entry for potential anti-HIV therapy	92,93
Lactococcal protein ORF18	Anti-ORF18 VHH	Prevent phage infection in <i>Lactococcus lactis</i>	94
Hemagglutinin from H5N1 influenza virus	Anti-H5N1 VHH	Antivirus therapy against H5N1 influenza virus infection	95
VSG from <i>Trypanosomes</i>	Anti-VSG VHH	Potential immunotoxins for trypanosomosis therapy	96
T-cell ecto-ADP-ribosyl transferase ART2.2	Anti-ART2.2 VHH	New antidotes against ADP-ribosylating toxins for specific blockade of ART2.2 activities	97
TNF $\alpha$	Anti-TNF $\alpha$ V-NAR	Potential therapeutics for rheumatoid arthritis treatment	98,99
IL-6R	Anti-IL-6R VHH (ALX-0061)	Potential therapeutics for rheumatoid arthritis treatment	100
vWF	Anti-vWF VHH	Potential therapeutics for acute thrombotic thrombocytopenic purpura	101
Human GFAP	Anti-GFAP VHH	Potential therapeutics for central nervous system disease	103,104
HER2	Anti-HER2 VHH-coated gold nanoparticles	Potential photothermal therapeutics targeting breast and ovarian cancers	107

**Abbreviations:** Env, envelope; FMD, foot-and-mouth disease; FMDV, FMD virus; GFAP, glial fibrillary acidic protein; IL-6R, interleukin-6 receptor; RP, rotavirus proteins; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; VHH, variable domain of heavy-chain-only antibodies in camelids; V-NAR, variable domain of immunoglobulin new antigen receptors in sharks; VSG, variant surface glycoproteins; vWF, von Willebrand factor; ADP, adenosine diphosphate.



This blockade was highly specific, ie, only targeting blockade of enzyme ART2.2 but not the related enzymes ART1 or ART2.1, which eventually leads to develop new antidotes against ADP-ribosylating toxins.<sup>97</sup> In addition, several other nanobody-derived therapeutics are already in the pipeline. For instances, anti-IL6R and anti-tumor necrosis factor  $\alpha$  nanobodies were developed to treat rheumatoid arthritis,<sup>98–100</sup> and an anti-RANKL nanobody was generated for bone loss disorder therapy.<sup>1</sup> Some therapeutic nanobodies, such as an anti-von Willebrand factor nanobody for the treatment of acute thrombotic thrombocytopenic purpura, have passed Phase I and Phase II clinical trials.<sup>101</sup> However, there is expectedly high competition from other therapeutics, including conventional McAbs and other antibody-derived small-size formats, such as scFvs and Fabs.<sup>1</sup>

On the other hand, the success of nanobodies as targeting therapeutics in clinic environments will also come from a more patient friendly administration, such as topical, oral, and inhalation or targeting delivery of cargoes to tissues that are difficult to access, and from obtaining improved biopharmaceutical parameters, including blood concentrations and prolonged residence time.<sup>1,102</sup> To this end, nanobodies have been extensively used for targeting delivery of cargoes to different targeting antigens in *in vivo* organs to improve their biopharmaceutical parameters. For example, nanobodies have been assessed in their capacity of crossing blood–brain barrier and transcytosis across epithelia, which will potentially lead to develop nanobody-based brain-targeting therapeutics.<sup>103–106</sup> Moreover, chemical conjugation of nanobodies to branched gold nanoparticles also effectively produced antigen-targeting photothermal therapeutics upon light irradiation in a near biological window.<sup>107</sup>

## Nanobodies for agricultural protection and food analysis

Besides the promising results from enzyme inhibition exerted by nanobodies in mammals, nanobodies could also be used as enzyme inhibitors to modulate plant physiology and increase starch content in plant. For example, an inhibitory VHH against starch-branching enzyme A has been generated to interfere with the function of enzymes in potato.<sup>108</sup> Because of specific enzyme inhibition by an inhibitory VHH, the content of amylose in potato tuber increases upon targeting the VHH to plastids. An increase in amylose is even higher than that of the antisense controls, demonstrating the unique performance of the nanobodies as plant enzyme inhibitors. Furthermore, nanobodies could be used as nanocarriers for more efficient and specific delivery of existing chemicals to crops and weeds. To this respect, plant-specific VHHs

were covalently coupled to engineered agrocapsules to improve the chemical retention time at target sites and reduce the chemical amounts needed.<sup>109</sup> The plant-specific VHHs have also been generated that could bind stomata, trichomes, grass leaves, and the surface area of potato.<sup>32</sup> Importantly, these plant-specific VHHs also showed good tolerance for harsh field conditions, such as temperature, variable pH, and salt concentrations. Besides the crops and weeds, nanobodies have also been applied for specific and targeting delivery of insecticides to insects.<sup>33,109</sup> To pursue a high-specific VHH, the VHH phages are selected directly against living aphids and even whole-insect ELISA has been developed to characterize these insect-specific VHHs. Interestingly, nanobodies also been applied to food analysis because of highly extreme thermostability. Several anticaffeine VHHs have been already generated for the quantification of caffeine in hot beverages. At 70°C, one of these VHHs could recognize caffeine and amazingly recover its binding functionality after an incubation step at 90°C,<sup>110</sup> demonstrating the excellent thermostability of these nanobodies.

## Conclusion and perspectives

In this postgenomic era, searching for picomolar affinity and high specific binders against different targets of interest, including proteins, peptides, DNAs, RNAs, polysaccharides, and small molecules, are overwhelming challenges. Compared to conventional antibodies, nanobodies have following excellent properties: nanoscale size, robust structure, stable and soluble behavior in aqueous solution, reversible refolding, high affinity and specificity for only one cognate target, superior cryptic cleft accessibility, and deep tissue penetration, as well as a sustainable source. It is the amazingly excellent properties of nanobodies that could meet the unmet demand for screening these novel types of binders from renewable nanobody resources, making them attractive alternatives to conventional antibodies and their single-domain formats, such as Fab and scFv. The nanobody-derived binders have already been demonstrated to not only recognize only one cognate target of interest with high affinity but also differentiate different conformations of these targets with superior specificity. Therefore, these unique properties of nanobodies offer us opportunities to develop specific nanobiotechnological tool kits for various biomedical and biotechnological applications.

Current research on applying the nanobody-derived nanobiotechnological tool kits in affinity purification, immunoprecipitation, chaperone-assisted crystallization, protein degradation, gene activation or inactivation, protein–protein interactions, cellular bioimaging, *in vivo* and *in vitro* disease

diagnosis, targeting therapeutics, agricultural and plant protection, and food analysis indicate a promising future of the use of these nanobodies and their derivative formats in biomedical and biotechnological fields. In the future, application of nanobodies as versatile molecules in different fields, including targeting therapy, targeting delivery, immunosensors, and in vivo imaging, nano-based research tools, is highly anticipated. Among these future trends, commercialization of nanobodies as a next-generation targeting therapeutics is high priority and technical challenge.<sup>111,112</sup> Although some nanobody-derived products are in the pipeline, some have passed Phase I and Phase II trials,<sup>1,101</sup> the competition from conventional McAbs or other antibody-derived small-size formats is huge. As compared to conventional McAbs, the small size of nanobodies not only confers their good tumor penetration but also enables them for a rapid renal clearance, indicating the challenge to balance improved systemic distribution with decreased plasma half-life in designing nanobody-based targeting therapeutics.<sup>111</sup> Therefore, for some intended uses, properties and pharmacokinetics should be tailored by linking nanobodies to albumin-binding moieties or by changing their hydrodynamic volume in various ways to reach a high blood concentration over a prolonged blood residence time,<sup>1,102</sup> ie, obtaining long-circulating nanobody-based therapeutics. Furthermore, several other obstacles, such as immunogenicity and functionalization,<sup>3</sup> still have to be clinically addressed before the use of nanobodies in the clinic as targeting therapeutics become feasible.

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## Disclosure

The authors report no conflicts of interest in this work.

## References

- Muyldermans S. Nanobodies: natural single-domain antibodies. *Annu Rev Biochem.* 2013;82:775–797.
- De Meyer T, Muyldermans S, Depicker A. Nanobody-based products as research and diagnostic tools. *Trends Biotechnol.* 2014;32(5):263–270.
- Siontorou CG. Nanobodies as novel agents for disease diagnosis and therapy. *Int J Nanomedicine.* 2013;8:4215–4227.
- Saerens D, Muyldermans S. *Single Domain Antibodies: Methods and Protocols*. Totowa, NJ: Humana Press; 2012.
- Zielonka S, Empting M, Grzeschik J, Konning D, Barelle CJ, Kolmar H. Structural insights and biomedical potential of IgNAR scaffolds from sharks. *MAbs.* 2015;7(1):15–25.
- Flajnik MF, Kasahara M. Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nat Rev Genet.* 2010;11(1):47–59.
- Shao CY, Secombes CJ, Porter AJ. Rapid isolation of IgNAR variable single-domain antibody fragments from a shark synthetic library. *Mol Immunol.* 2007;44(4):656–665.
- Liu JL, Anderson GP, Delehanty JB, Baumann R, Hayhurst A, Goldman ER. Selection of cholera toxin specific IgNAR single-domain antibodies from a naive shark library. *Mol Immunol.* 2007;44(7):1775–1783.
- Dooley H, Flajnik MF. Antibody repertoire development in cartilaginous fish. *Dev Comp Immunol.* 2006;30(1–2):43–56.
- Muyldermans S, Baral TN, Retamozzo VC, et al. Camelid immunoglobulins and nanobody technology. *Vet Immunol Immunopathol.* 2009;128(1–3):178–183.
- Helma J, Cardoso MC, Muyldermans S, Leonhardt H. Nanobodies and recombinant binders in cell biology. *J Cell Biol.* 2015;209(5):633–644.
- Muyldermans S, Atarhouch T, Saldanha J, Barbosa JA, Hamers R. Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. *Protein Eng.* 1994;7(9):1129–1135.
- Vu KB, Ghahroudi MA, Wyns L, Muyldermans S. Comparison of llama VH sequences from conventional and heavy chain antibodies. *Mol Immunol.* 1997;34(16–17):1121–1131.
- Harmsen MM, Ruuls RC, Nijman IJ, Niewold TA, Frenken LGJ, de Geus B. Llama heavy-chain V regions consist of at least four distinct subfamilies revealing novel sequence features. *Mol Immunol.* 2000;37(10):579–590.
- Maass DR, Sepulveda J, Pernthaner A, Shoemaker CB. Alpaca (*Lama pacos*) as a convenient source of recombinant camelid heavy chain antibodies (VHHs). *J Immunol Methods.* 2007;324(1–2):13–25.
- Govaert J, Pellis M, Deschacht N, et al. Dual beneficial effect of inter-loop disulfide bond for single domain antibody fragments. *J Biol Chem.* 2012;287(3):1970–1979.
- Criscitelli MF, Saltis M, Flajnik MF. An evolutionarily mobile antigen receptor variable region gene: doubly rearranging NAR-TcR genes in sharks. *Proc Natl Acad Sci U S A.* 2006;103(13):5036–5041.
- Liu JL, Anderson GP, Goldman ER. Isolation of anti-toxin single domain antibodies from a semi-synthetic spiny dogfish shark display library. *BMC Biotechnol.* 2007;7:78–87.
- Stanfield RL, Dooley H, Verdino P, Flajnik MF, Wilson IA. Maturation of shark single-domain (IgNAR) antibodies: evidence for induced-fit binding. *J Mol Biol.* 2007;367(2):358–372.
- Stanfield RL, Dooley H, Flajnik MF, Wilson IA. Crystal structure of a shark single-domain antibody V region in complex with lysozyme. *Science.* 2004;305(5691):1770–1773.
- Desmyter A, Transue TR, Ghahroudi MA, et al. Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme. *Nat Struct Biol.* 1996;3(9):803–811.
- De Genst E, Silence K, Decanniere K, et al. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. *Proc Natl Acad Sci U S A.* 2006;103(12):4586–4591.
- Desmyter A, Decanniere K, Muyldermans S, Wyns L. Antigen specificity and high affinity binding provided by one single loop of a camel single-domain antibody. *J Biol Chem.* 2001;276(28):26285–26290.
- Spinelli S, Tegoni M, Frenken L, van Vliet C, Cambillau C. Lateral recognition of a dye hapten by a llama VHH domain. *J Mol Biol.* 2001;311(1):123–129.
- Spinelli S, Frenken LG, Hermans P, et al. Camelid heavy-chain variable domains provide efficient combining sites to haptens. *Biochemistry.* 2000;39(6):1217–1222.
- Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. *Nat Biotechnol.* 2005;23(9):1126–1136.
- Goldman ER, Anderson GP, Liu JL, et al. Facile generation of heat-stable antiviral and antitoxin single domain antibodies from a semisynthetic llama library. *Anal Chem.* 2006;78(24):8245–8255.
- Huang L, Muyldermans S, Saerens D. Nanobodies(R): proficient tools in diagnostics. *Expert Rev Mol Diagn.* 2010;10(6):777–785.

29. Vaneycken I, Devoogdt N, Van Gassen N, et al. Preclinical screening of anti-HER2 nanobodies for molecular imaging of breast cancer. *FASEB J*. 2011;25(7):2433–2446.
30. Huang L, Gainkam LOT, Caveliers V, et al. SPECT imaging with Tc-99m-labeled EGFR-specific nanobody for in vivo monitoring of EGFR expression. *Mol Imaging Biol*. 2008;10(3):167–175.
31. De Groeve K, Deschacht N, De Koninck C, et al. Nanobodies as tools for in vivo imaging of specific immune cell types. *J Nucl Med*. 2010;51(5):782–789.
32. Jongedijk E, Verheesen P, inventor; AgroSavfe NV, assignee (Gent, BE). Specific delivery of agrochemicals. US patent 8598081. Dec 3, 2013.
33. Verheesen P, Jongedijk E, inventors; AgroSavfe NV, assignee (Gent, BE). Insect binding antibodies. US patent 20130224226. Aug 29, 2013.
34. Harmsen MM, De Haard HJ. Properties, production, and applications of camelid single-domain antibody fragments. *Appl Microbiol Biotechnol*. 2007;77(1):13–22.
35. Wesolowski J, Alzogaray V, Reyelt J, et al. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol*. 2009;198(3):157–174.
36. de Marco A. Biotechnological applications of recombinant single-domain antibody fragments. *Microb Cell Fact*. 2011;10:44.
37. Dmitriev OY, Lutsenko S, Muyldermans S. Nanobodies as probes for protein dynamics in vitro and in cells. *J Biol Chem*. 2016;291(8):3767–3775.
38. Klooster R, Maassen BTH, Stam JC, et al. Improved anti-IgG and HSA affinity ligands: clinical application of VHH antibody technology. *J Immunol Methods*. 2007;324(1–2):1–12.
39. De Genst EJ, Guillems T, Wellens J, et al. Structure and properties of a complex of alpha-synuclein and a single-domain camelid antibody. *J Mol Biol*. 2010;402(2):326–343.
40. Klooster R, Eman MR, le Duc Q, et al. Selection and characterization of KDEL-specific VHH antibody fragments and their application in the study of ER resident protein expression. *J Immunol Methods*. 2009;342(1–2):1–12.
41. Nguyen-Duc T, Peeters E, Muyldermans S, Charlier D, Hassanzadeh-Ghassabeh G. Nanobody(R)-based chromatin immunoprecipitation/micro-array analysis for genome-wide identification of transcription factor DNA binding sites. *Nucleic Acids Res*. 2013;41(5):e59.
42. Pollithy A, Romer T, Lang C, et al. Magnetosome expression of functional camelid antibody fragments (nanobodies) in *Magnetospirillum gryphiswaldense*. *Appl Environ Microbiol*. 2011;77(17):6165–6171.
43. Baranova E, Fronzes R, Garcia-Pino A, et al. SbsB structure and lattice reconstruction unveil Ca<sup>2+</sup> triggered S-layer assembly. *Nature*. 2012;487(7405):119–122.
44. Rasmussen SGF, Choi H-J, Fung JJ, et al. Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature*. 2011;469(7329):175–180.
45. Loris R, Marianovsky I, Lah J, et al. Crystal structure of the intrinsically flexible addiction antidote MazE. *J Biol Chem*. 2003;278(30):28252–28257.
46. Koide S. Engineering of recombinant crystallization chaperones. *Curr Opin Struct Biol*. 2009;19(4):449–457.
47. Pardon E, Laeremans T, Triest S, et al. A general protocol for the generation of nanobodies for structural biology. *Nat Protoc*. 2014;9(3):674–693.
48. Domanska K, Vanderhaegen S, Srinivasan V, et al. Atomic structure of a nanobody-trapped domain-swapped dimer of an amyloidogenic beta 2-microglobulin variant. *Proc Natl Acad Sci U S A*. 2011;108(4):1314–1319.
49. Abskharon RNN, Giachin G, Wohlkonig A, et al. Probing the N-terminal beta-sheet conversion in the crystal structure of the human prion protein bound to a nanobody. *JACS*. 2014;136(3):937–944.
50. Lam AY, Pardon E, Korotkov KV, Hol WGJ, Steyaert J. Nanobody-aided structure determination of the EpsI:EpsJ pseudopilin heterodimer from *Vibrio vulnificus*. *J Struct Biol*. 2009;166(1):8–15.
51. Korotkov KV, Pardon E, Steyaert J, Hol WGJ. Crystal structure of the N-terminal domain of the secretin GspD from ETEC determined with the assistance of a nanobody. *Structure*. 2009;17(2):255–265.
52. Caussinus E, Kanca O, Affolter M. Fluorescent fusion protein knockout mediated by anti-GFP nanobody. *Nat Struct Mol Biol*. 2011;19(1):117–121.
53. Caussinus E, Kanca O, Affolter M. Protein knockouts in living eukaryotes using deGradFP and green fluorescent protein fusion targets. *Curr Protoc Protein Sci*. 2013;73(73):Unit30.2.
54. Tang JCY, Szikra T, Kozorovitskiy Y, et al. A nanobody-based system using fluorescent proteins as scaffolds for cell-specific gene manipulation. *Cell*. 2013;154(4):928–939.
55. Rothbauer U, Zolghadr K, Tillib S, et al. Targeting and tracing antigens in live cells with fluorescent nanobodies. *Nat Methods*. 2006;3(11):887–889.
56. Olichon A, Surrey T. Selection of genetically encoded fluorescent single domain antibodies engineered for efficient expression in *Escherichia coli*. *J Biol Chem*. 2007;282(50):36314–36320.
57. Zolghadr K, Gregor J, Leonhardt H, Rothbauer U. Case study on live cell apoptosis-assay using lamin-chromobody cell-lines for high-content analysis. *Methods Mol Biol*. 2012;911:569–575.
58. Helma J, Schmidthals K, Lux V, et al. Direct and dynamic detection of HIV-1 in living cells. *PLoS One*. 2012;7(11):E50026.
59. Ries J, Kaplan C, Platonova E, Eghlidi H, Ewers H. A simple, versatile method for GFP-based super-resolution microscopy via nanobodies. *Nat Methods*. 2012;9(6):582–584.
60. Leduc C, Si S, Gautier J, et al. A highly specific gold nanoprobe for live-cell single-molecule imaging. *Nano Lett*. 2013;13(4):1489–1494.
61. Herce HD, Deng W, Helma J, Leonhardt H, Cardoso MC. Visualization and targeted disruption of protein interactions in living cells. *Nat Commun*. 2013;4:2660.
62. Tanaka T, Sewell H, Waters S, Phillips SEV, Rabbitts TH. Single domain intracellular antibodies from diverse libraries: emphasizing dual functions of LMO2 protein interactions using a single VH domain. *J Biol Chem*. 2011;286(5):3707–3716.
63. Perez-Martinez D, Tanaka T, Rabbitts TH. Intracellular antibodies and cancer: new technologies offer therapeutic opportunities. *Bioessays*. 2010;32(7):589–598.
64. Kirchhofer A, Helma J, Schmidthals K, et al. Modulation of protein properties in living cells using nanobodies. *Nat Struct Mol Biol*. 2010;17(1):133–138.
65. Romer T, Leonhardt H, Rothbauer U. Engineering antibodies and proteins for molecular in vivo imaging. *Curr Opin Biotechnol*. 2011;22(6):882–887.
66. Schmidthals K, Helma J, Zolghadr K, Rothbauer U, Leonhardt H. Novel antibody derivatives for proteome and high-content analysis. *Anal Bioanal Chem*. 2010;397(8):3203–3208.
67. Rothbauer U, Zolghadr K, Muyldermans S, Schepers A, Cardoso MC, Leonhardt H. A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. *Mol Cell Proteomics*. 2008;7(2):282–289.
68. Schornack S, Fuchs R, Huitema E, Rothbauer U, Lipka V, Kamoun S. Protein mislocalization in plant cells using a GFP-binding chromobody. *Plant J*. 2009;60(4):744–754.
69. Vaneycken I, D'Huyvetter M, Hernot S, et al. Immuno-imaging using nanobodies. *Curr Opin Biotechnol*. 2011;22(6):877–881.
70. Broisat A, Hernot S, Toczek J, et al. Nanobodies targeting mouse/human VCAM1 for the nuclear imaging of atherosclerotic lesions. *Circ Res*. 2012;110(7):927–937.
71. Vosjan MJWD, Perk LR, Roovers RC, et al. Facile labelling of an anti-epidermal growth factor receptor nanobody with Ga-68 via a novel bifunctional desferal chelate for immuno-PET. *Eur J Nucl Med Mol Imaging*. 2011;38(4):753–763.
72. Movahedi K, Schoonooghe S, Laoui D, et al. Nanobody-based targeting of the macrophage mannose receptor for effective in vivo imaging of tumor-associated macrophages. *Cancer Res*. 2012;72(16):4165–4177.



73. Kijanka M, Warnders F-J, El Khattabi M, et al. Rapid optical imaging of human breast tumour xenografts using anti-HER2 VHHs site-directly conjugated to IRDye 800CW for image-guided surgery. *Eur J Nucl Med Mol Imaging*. 2013;40(11):1718–1729.
74. Oliveira S, van Dongen GAMS, Stigter-van Walsum M, et al. Rapid visualization of human tumor xenografts through optical imaging with a near-infrared fluorescent anti-epidermal growth factor receptor nanobody. *Mol Imaging*. 2012;11(1):33–46.
75. Saerens D, Frederix F, Reekmans G, et al. Engineering camel single-domain antibodies and immobilization chemistry for human prostate-specific antigen sensing. *Anal Chem*. 2005;77(23):7547–7555.
76. Harmsen MM, Fijten HPD. Improved functional immobilization of llama single-domain antibody fragments to polystyrene surfaces using small peptides. *J Immunoassay Immunochem*. 2012;33(3):234–251.
77. Saerens D, Huang L, Bonroy K, Muyldermans S. Antibody fragments as probe in biosensor development. *Sensors*. 2008;8(8):4669–4686.
78. Even-Desrumeaux K, Baty D, Chames P. Strong and oriented immobilization of single domain antibodies from crude bacterial lysates for high-throughput compatible cost-effective antibody array generation. *Mol Biosyst*. 2010;6(11):2241–2248.
79. Abbady AQ, Al-Daoude A, Al-Mariri A, Zarkawi M, Muyldermans S. Chaperonin GroEL a *Brucella* immunodominant antigen identified using nanobody and MALDI-TOF-MS technologies. *Vet Immunol Immunopathol*. 2012;146(3–4):254–263.
80. Deckers N, Saerens D, Kanobana K, et al. Nanobodies, a promising tool for species-specific diagnosis of *Taenia solium* cysticercosis. *Int J Parasitol*. 2009;39(5):625–633.
81. Saerens D, Stijlemans B, Baral TN, et al. Parallel selection of multiple anti-infectome nanobodies without access to purified antigens. *J Immunol Methods*. 2008;329(1–2):138–150.
82. Habib I, Smolarek D, Hattab C, et al. VHH (nanobody) directed against human glycophorin A: a tool for autologous red cell agglutination assays. *Anal Biochem*. 2013;438(1):82–89.
83. Hmila I, Saerens D, Ben Abderrazek R, et al. A bispecific nanobody to provide full protection against lethal scorpion envenoming. *FASEB J*. 2010;24(9):3479–3489.
84. Hussack G, Arbabi-Ghahroudi M, van Faassen H, et al. Neutralization of *Clostridium difficile* toxin A with single-domain antibodies targeting the cell receptor binding domain. *J Biol Chem*. 2011;286(11):8961–8976.
85. Stewart CS, MacKenzie CR, Hall JC. Isolation, characterization and pentamerization of alpha-cobrotoxin specific single-domain antibodies from a naive phage display library: preliminary findings for antivenom development. *Toxicon*. 2007;49(5):699–709.
86. Cook DAN, Samarasekera CL, Wagstaff SC, Kinne J, Wernery U, Harrison RA. Analysis of camelid IgG for antivenom development: immunoreactivity and preclinical neutralisation of venom-induced pathology by IgG subclasses, and the effect of heat treatment. *Toxicon*. 2010;56(4):596–603.
87. Harrison RA, Hasson SS, Harmsen M, Laing GD, Conrath K, Theakston RDG. Neutralisation of venom-induced haemorrhage by IgG from camels and llamas immunised with viper venom and also by endogenous, non-IgG components in camelid sera. *Toxicon*. 2006;47(3):364–368.
88. Pant N, Marcotte H, Hermans P, et al. *Lactobacilli* producing bispecific llama-derived anti-rotavirus proteins in vivo for rotavirus-induced diarrhea. *Future Microbiol*. 2011;6(5):583–593.
89. Kruger C, Hultberg A, Marcotte H, et al. Therapeutic effect of llama derived VHH fragments against *Streptococcus mutans* on the development of dental caries. *Appl Microbiol Biotechnol*. 2006;72(4):732–737.
90. Harmsen MM, Fijten HPD, Engel B, Dekker A, Eble PL. Passive immunization with llama single-domain antibody fragments reduces foot-and-mouth disease transmission between pigs. *Vaccine*. 2009;27(13):1904–1911.
91. Strokappe N, Szynol A, Aasa-Chapman M, et al. Llama antibody fragments recognizing various epitopes of the CD4bs neutralize a broad range of HIV-1 subtypes A, B and C. *PLoS One*. 2012;7(3):e33298.
92. Koh WWL, Steffensen S, Gonzalez-Pajuelo M, et al. Generation of a family-specific phage library of llama single chain antibody fragments that neutralize HIV-1. *J Biol Chem*. 2010;285(25):19116–19124.
93. Hinz A, Hulsik DL, Forsman A, et al. Crystal structure of the neutralizing llama V-HH D7 and its mode of HIV-1 gp120 interaction. *PLoS One*. 2010;5(5):e10482.
94. De Haard HJW, Bezemer S, Ledebor AM, et al. Llama antibodies against a lactococcal protein located at the tip of the phage tail prevent phage infection. *J Bacteriol*. 2005;187(13):4531–4541.
95. Ibanez LI, De Filette M, Hultberg A, et al. Nanobodies with in vitro neutralizing activity protect mice against H5N1 influenza virus infection. *J Infect Dis*. 2011;203(8):1063–1072.
96. Stijlemans B, Conrath K, Cortez-Retamozo V, et al. Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies – African trypanosomes as paradigm. *J Biol Chem*. 2004;279(2):1256–1261.
97. Koch-Nolte F, Reyelt J, Schossow B, et al. Single domain antibodies from llama effectively and specifically block T cell ecto-ADP-ribosyltransferase ART2.2 in vivo. *FASEB J*. 2007;21(13):3490–3498.
98. Camacho-Villegas T, Mata-Gonzalez T, Paniagua-Solis J, Sanchez E, Licea A. Human TNF cytokine neutralization with a vNAR from *Heterodontus francisci* shark: a potential therapeutic use. *MAbs*. 2013;5(1):80–85.
99. Bojalil R, Mata-Gonzalez MT, Sanchez-Munoz F, et al. Anti-tumor necrosis factor (V)NAR single domains reduce lethality and regulate underlying inflammatory response in a murine model of endotoxic shock. *BMC Immunol*. 2013;14:17.
100. Van Roy M, Ververken C, Beirnaert E, et al. The preclinical pharmacology of the high affinity anti-IL-6R Nanobody® ALX-0061 supports its clinical development in rheumatoid arthritis. *Arthritis Res Ther*. 2015;17:135.
101. Holz J-B. The TITAN trial – assessing the efficacy and safety of an anti-von Willebrand factor nanobody in patients with acquired thrombotic thrombocytopenic purpura. *Trans Apher Sci*. 2012;46(3):343–346.
102. Kontermann RE. Strategies for extended serum half-life of protein therapeutics. *Curr Opin Biotechnol*. 2011;22(6):868–876.
103. Li T, Bourgeois J-P, Celli S, et al. Cell-penetrating anti-GFAP VHH and corresponding fluorescent fusion protein VHH-GFP spontaneously cross the blood-brain barrier and specifically recognize astrocytes: application to brain imaging. *FASEB J*. 2012;26(10):3969–3979.
104. Perruchini C, Pecorari F, Bourgeois J-P, Duyckaerts C, Rougeon F, Lafaye P. Llama VHH antibody fragments against GFAP: better diffusion in fixed tissues than classical monoclonal antibodies. *Acta Neuropathol*. 2009;118(5):685–695.
105. Iqbal U, Trojahn U, Albaghdadi H, et al. Kinetic analysis of novel mono- and multivalent VHH-fragments and their application for molecular imaging of brain tumours. *Br J Pharmacol*. 2010;160(4):1016–1028.
106. Emmerson CD, van der Vlist EJ, Braam MR, et al. Enhancement of polymeric immunoglobulin receptor transcytosis by biparatopic VHH. *PLoS One*. 2011;6(10):e26299.
107. Van de Broek B, Devoogdt N, D'Hollander A, et al. Specific cell targeting with nanobody conjugated branched gold nanoparticles for photothermal therapy. *ACS Nano*. 2011;5(6):4319–4328.
108. Jobling SA, Jarman C, Teh MM, Holmberg N, Blake C, Verhoeven ME. Immunomodulation of enzyme function in plants by single-domain antibody fragments. *Nat Biotechnol*. 2003;21(1):77–80.
109. Verheesen P, De Jonghe C, inventor; AgroSavfe NV, assignee (Gent, BE). Manufacturing of specifically targeting microcapsules. US patent 20150087517. Mar 26, 2015.
110. Ladenson RC, Crimmins DL, Landt Y, Ladenson JH. Isolation and characterization of a thermally stable recombinant anti-caffeine heavy-chain antibody fragment. *Anal Chem*. 2006;78(13):4501–4508.
111. Turner KB, Alves NJ, Medintz IL, Walper SA. Improving the targeting of therapeutics with single-domain antibodies. *Expert Opin Drug Del*. 2016;13(4):561–570.
112. Kijanka M, Dorresteijn B, Oliveira S, Henegouwen P. Nanobody-based cancer therapy of solid tumors. *Nanomedicine (Lond)*. 2015;10(1):161–174.



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