

Review

Recent advances in the selection and identification of antigen-specific nanobodies

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ABSTRACT

Nanobodies represent the next-generation antibody-derived biologics with significant advances over conventional antibodies. Several rapid and robust techniques for isolating highly specific nanobodies have been developed. Antigen specific nanobodies are selected from constructed nanobody libraries, which can be classified into 3 main types: immune library, naïve library, and semisynthetic/synthetic library. The immune library is the most widely used strategy for nanobody screening. Target specific nanobodies are highly enriched in immune libraries than in non-immune libraries; however, it is largely limited by the natural antigenicity of antigens. The naïve library is thus developed. Despite the lack of somatic maturation, protein engineering can be employed to significantly increase the affinities of selected binders. However, a substantial amount of blood samples collected from a large number of individual animals is a prerequisite to ensure the diversity of the naïve library. With this issue considered, the semisynthetic/synthetic library may be a promising path toward obtaining a limitless source of nanobodies against a variety of antigens without the need of animals. In this review, we summarize the state-of-the-art screening technologies with different libraries. The approaches presented here can further boost the diverse applications of nanobodies in biomedicine and biotechnology.

1. Introduction

High-affinity and high-specificity antibodies have to be obtained to meet various requirements for research, diagnostics, and therapeutics. Over the decades, single-domain antibodies, more frequently referred to as nanobodies, have emerged as a credible next-generation antibody-derived biologics for pharmaceutical and biotechnology industries owing to their peculiar properties, including nanoscale size, robust behaviors, high affinity and specificity, deep tissue penetration, as well as a sustainable source (Wang et al., 2016).

Nanobodies are derived from heavy-chain antibodies (HCAbs), which are naturally present in all camelidae. In addition to the conventional antibodies containing 2 heavy and 2 light chains, all camelidae remarkably produce HCAbs in their serum, lacking light chains and a canonical constant heavy chain 1 (CH1) domain in the heavy

chain (Hamers-Casterman et al., 1993). Some species of cartilaginous fish, including nurse shark (*Ginglymostoma cirratum*), wobbegong (*Orrectolobus maculatus*), and dogfish sharks (*Squalus acanthias* and *Mustelus canis*), also remarkably produce functional heavy-chain-only immunoglobulins (Igs), named IgNARs (Greenberg et al., 1995; Liu et al., 2007a; Shao et al., 2007; Zielonka et al., 2015) (Fig. 1). Interestingly, some pathological and nonfunctional HCAbs were also discovered in human serum or in mouse hybridoma owing partly to the genetic deletion of significant parts of variable heavy-chain (VH) and CH1 regions (Alexander et al., 1982; Cogne et al., 1989; Morrison, 1978). In camels, these HCAbs have an antigen recognition part composed of single variable domains, referred to as the variable domain of the heavy chain of heavy-chain antibody (VHH). Owing to a small diameter of 2.5 nm and a height of 4 nm, these single variable domains are accordingly named as nanobodies by its original Belgian developer Ablynx[®], the

Abbreviations: HCAbs, heavy-chain antibodies; CH1, constant heavy-chain 1; VH, variable heavy-chain; Igs, immunoglobulins; VHH, the variable domain of the heavy chain of heavy-chain antibody; V-NAR, the variable domain of the heavy chain of NAR; PCR, polymerase chain reaction; PEG, polyethylene glycol; ELISA, enzyme-linked immune sorbent assay; PBMCs, peripheral blood mononuclear cells; LLME, Leu–Leu–OMe; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; MS, mass spectrometric; LC–MS, liquid chromatography–mass spectrometry; MALDI–TOF–MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS–PAGE, PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BMDC, bone marrow-derived dendritic cells; BtNb, biotinylated nanobodies; CDRs, complementarity-determining regions; FRs, framework regions; HCEC, human cerebrobromicovascular endothelial cells; BBB, blood–brain barrier

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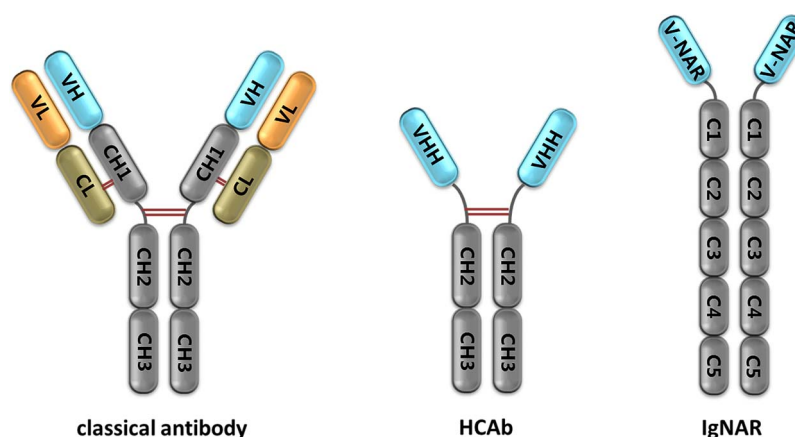


Fig. 1. Schematics of the conventional antibody, HCAb, and IgNAR.

leading biopharmaceutical company engaged in the development of VHH-related products. Although the variable domains from IgNARs (V-NARs) and VHHs have similar properties, the term Nanobody is a trademark used only for recombinant VHH from camelid. With a molecular weight of approximately 12–15 kDa, nanobodies are reported to be the smallest intact antigen-binding single polypeptide chains found in any natural antibody (Arbabi Ghahroudi et al., 1997; Harmsen and De Haard, 2007; Muyldermans, 2013).

Unlike conventional monoclonal antibodies, nanobodies can be produced in large amounts by using standard microbial expression systems (Baghban et al., 2016; Ta et al., 2015; Thomassen et al., 2002; Zarschler et al., 2013). Nanobodies are usually extremely robust, resistant to denaturation/thermal degradation, easy to manipulate with aqueous solubility, with superior cryptic cleft accessibility, and can bind antigens with affinities in the nM or sub-nM levels (Dumoulin et al., 2002; Lauwereys et al., 1998; Muyldermans et al., 2009). Nanobodies also exhibit superior tissue penetration, body distribution, and blood clearance (Huang et al., 2008; Muruganandam et al., 2001; Vaneycken et al., 2011). Notably, the conserved framework regions of nanobodies show a high sequential and structural homology with human VH domains of family III (VH3). Therefore, nanobodies have comparable immunogenicity as human VH (Bartunek et al., 2013; Holz et al., 2013), drawing interest for their potential clinical and therapeutic applications. In addition, the location of the antigen-binding site opposite the C-terminus allows nanobodies to provide opportunities for terminal modification, rendering them as versatile tools in research and biomedical applications (Campuzano et al., 2014; Ma et al., 2014). These advantages of nanobodies offer a promising alternative to the conventional antibodies in disease diagnosis, targeting drug delivery and therapy, bioimaging, basic biomedical research, and agricultural and plant protection (Wang et al., 2016). Thus, rapid and robust techniques for isolating extensive repertoires of high-affinity nanobodies are important tools for the widespread use of these molecules.

Three kinds of nanobody libraries—immune libraries, naïve libraries, and semisynthetic/synthetic libraries—have been constructed for the selection of nanobodies with high stability, specificity, and affinity. In this review, we systematically summarized the current state of the art in nanobody discovery, focusing on the screening techniques with these three different nanobody libraries.

2. Screen nanobodies from immune libraries

Preparation of immune libraries first needs the immunization of animals through which antigen-specific HCAs undergo affinity maturation (De Genst et al., 2004). Nanobodies are generally readily obtained by cloning the V-gene repertoire from peripheral blood lymphocytes and by screening through phage display or other techniques. Small libraries ($\sim 10^6$ individual transformants) constructed from a

blood sample of ~ 50 mL already represent the immune VHH repertoire of lymphocytes present in the bloodstream of the immunized animals. The unique specificity and high affinity of nanobodies retrieved from the immune libraries are ensured because of somatic maturation. In general, the affinities of these nanobodies are reported to be low-nM or pM levels (Muyldermans, 2013). However, it is unfeasible when antigens are of high pathogenicity (infectious agents) and toxicity or represent non-immunogenic small molecules (Goldman et al., 2006). In addition, maintaining camelidae animals have to be at the risk of illness and death of animals or natural differences in immunity among individuals, further complicating the process.

2.1. Phage display

Phage display was first described in 1985 by Smith (1985). This technology is based on the fusion expression of foreign peptides or proteins with coat proteins on the phage surface. Owing to its simplicity and efficacy, phage display has proven to be a powerful and versatile tool for studying specific interactions between different protein/peptide molecules (Chen and Dreskin, 2017). Since 1990s, phage display is the most widespread and standard selection technique used to isolate target-specific nanobodies (Arbabi Ghahroudi et al., 1997; Li et al., 2017; Xu et al., 2018).

Antigen-specific HCAs are generally affinity-matured after a series of subcutaneous immunization of individual camelidae with 0.1–1 mg purified antigens at regular intervals (Arezumand et al., 2016; Baharlou et al., 2017; Wan et al., 2018). Arbabi Ghahroudi et al. isolated the first nanobody sequence by phage display in 1997 (Arbabi Ghahroudi et al., 1997). In this approach, a dromedary is immunized with antigens according to standard immunization protocols. mRNA is then isolated from peripheral blood lymphocytes, and cDNA is synthesized by reverse transcription. Encoded by a gene fragment of only ~ 360 bp, VHHs can be easily amplified by polymerase chain reaction (PCR) and ligated into a cloning vector. The ligated DNA material representing the immune VHH repertoire of B cells is subsequently transformed into *E. coli* for camel VHH library construction. To generate a display of VHHs, the library is infected with M13K07 helper phage after the cells grow to mid-logarithmic phase. Phages are prepared by polyethylene glycol (PEG) precipitation. The following antigen-specific nanobody screening is called biopanning. To select phages-displaying nanobodies that specifically recognize antigens, the antigens are first immobilized in wells of microtiter plates by passive adsorption or on streptavidin-coated solid supports when the antigen is biotinylated (Hoogenboom, 2005). Normally, 2–3 rounds of panning are sufficient to enrich positive clones. Individual clones can be screened for the production of antigen-specific nanobodies in standard enzyme-linked immune sorbent assay (ELISA). Nucleotide sequences of ELISA-positive clones are sequenced to deduce the amino acids of the nanobodies (Fig. 2). The antigen

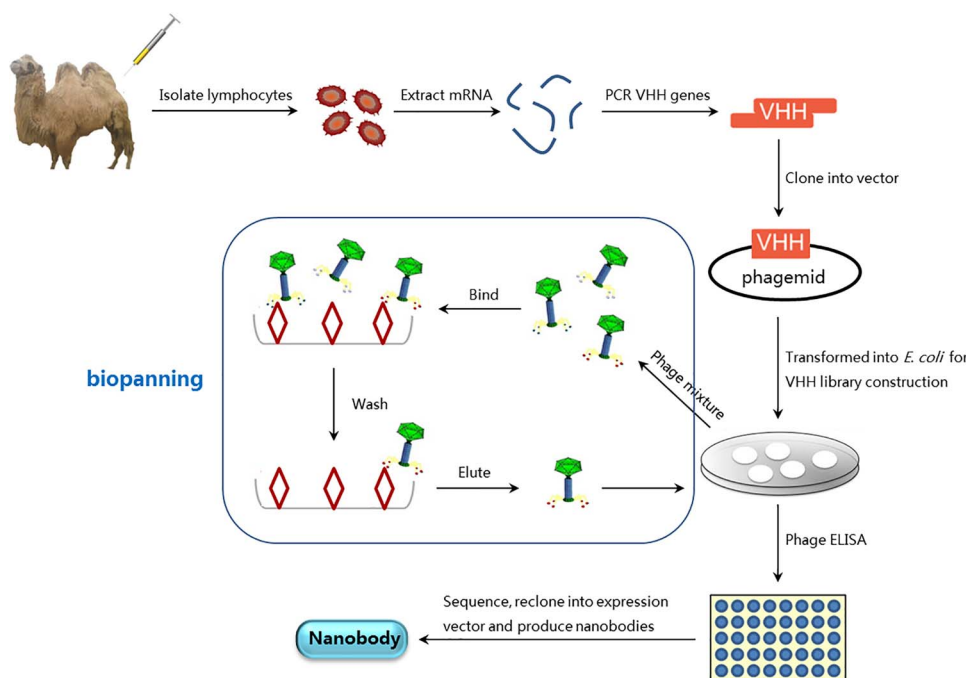


Fig. 2. Overview of nanobody identification and production with biopanning for a phage display library. A camelid is immunized with antigens at regular intervals. After mRNA isolation from peripheral blood lymphocytes, VHs are amplified by PCR and ligated into a cloning vector. The ligated DNA material is subsequently transformed into *E. coli* for VHH library construction. After 2–3 consecutive rounds of biopanning, antigen-specific binders are retrieved in phage ELISA. Nucleotides of ELISA-positive clones are sequenced to deduce the amino acids of the nanobodies. Hollow red diamonds in the figure denote antigens. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

specificity and affinity of nanobodies from immune libraries are generally sufficient for most biomedical applications, including disease diagnosis, bioimaging, drug screening, and targeting therapy. Nevertheless, affinity maturation has been successfully introduced in the critical amino acids of nanobodies to improve the stability and/or the affinity to their cognate antigens (Koide et al., 2007; Yau et al., 2005).

Antigen-induced *in vitro* production of antibodies was considered a promising alternative method to generate antibodies (Comor et al., 2017; Giacomini et al., 1999; Hooigeboom et al., 2015; Kato et al., 2012). Studies by Comor et al. described a powerful nanobody discovery technique with minimal use of animals (Comor et al., 2017). Peripheral blood mononuclear cells (PBMCs) were immediately isolated from fresh heparinized blood collected from healthy alpaca. After incubation with antigens for 72 h *in vitro*, PBMCs were collected to isolate total RNA. Nanobodies were selected by phage display as previously described. Notably, during *in vitro* immunization, 2 crucial components of Leu–Leu–OME (LLME) and interleukins are required. Lysosome-rich cytolytic T cell subpopulations, which inhibit antigen-specific B cell activation, should first be removed from PBMC culture. These subpopulations are sensitive to LLME and are thus killed prior to *in vitro* immunization (Borrebaeck et al., 1988). Meanwhile, species-specific interleukins (mainly IL-2 and IL-4) are essential for B cell activation and differentiation (Mosmann et al., 1987). Despite considerable advantages over conventional methods of antibody production, *in vitro* immunization may present several inadequacies, such as the relatively low (μM range) affinity of the antibodies obtained; in addition, this method tends to produce IgM isotypes instead of IgGs. However, studies by Kato et al. indicated that culture conditions, including cell density, type of stimulants, and initial cell preparation, are important factors for inducing IgG response in mouse spleen cells. Antigen-specific IgG-producing cells can be obtained within a period as short as 6 d by careful regulation of 3 steps: 1) initial antigen stimulation, 2) cell expansion with a cocktail of cytokines, and 3) second antigen stimulation (Kato et al., 2012). This may be a good guidance for nanobody discovery by *in vitro* immunization. Nanobodies with relatively low affinities have also been demonstrated to be useful as versatile biomedical and biotechnology tools. The nanobody-derived *in vivo* imaging agents and ligands for affinity purification are specifically required to exhibit relatively low affinities for rapid *in vivo* clearance and elution (Wang et al., 2016). *In vitro* immunization is thus regarded as an important

advancement for generation nanobodies and has wide potential applications.

2.2. Cell surface display

Display of heterologous proteins on the surface of cells is a powerful method applied in microbiology and molecular biology (Ueda, 2016). The technique is based on mechanisms of localizing proteins/peptides to the cell surface of yeast or bacteria (Fleetwood et al., 2013; Ryckaert et al., 2010). Cell surface display using different hosts and anchoring mechanisms have been developed and investigated to isolate high-affinity nanobodies from immune libraries. Unlike phages, cells are sufficiently large to be detected in a flow cytometer, enabling fluorescence-based analysis and sorting of displayed libraries (Fig. 3) (Boder and Wittrup, 1997; Daugherty et al., 1998). This approach provides means to quantify the relative affinity of each cell-displayed protein variant for the target, which is the most important parameter on which cell sorting is generally based. Compared with other existing selection techniques, this approach also provides excellent control and a straightforward method of overseeing the selection profile. Moreover, by using fluorescence-activated cell sorting (FACS), antibody affinity can be determined directly on the surface of the cells, obviating the need for subcloning, expression, and purification of various antibody candidates, which are time-consuming and laborious (Chao et al., 2006).

In the study by Ryckaert et al., a library derived from a llama immunized with green fluorescent protein (GFP) was displayed on the surface of *Pichia pastoris* via fusion to the C-terminal domain of alpha-agglutinin, which anchors the nanobodies to the cell surface and is used to functionally display nanobodies. After enrichment by FACS, GFP-specific nanobodies with K_D in the nM range were isolated (Ryckaert et al., 2010). A group also isolated G protein-coupled receptor (GPCR)-targeting nanobodies by conformational selection using yeast surface display. The nanobodies were used as important research tools to lock GPCRs in particular conformational states to obtain crystal structures (Kruse et al., 2013; Manglik et al., 2017; Ring et al., 2013). Display and engineering of antibodies on the surface of yeast has significant advantages, including a secretory pathway similar to that of higher eukaryotes. Proteins are folded in the endoplasmic reticulum, where chaperones, foldases, and a series of elaborate quality control

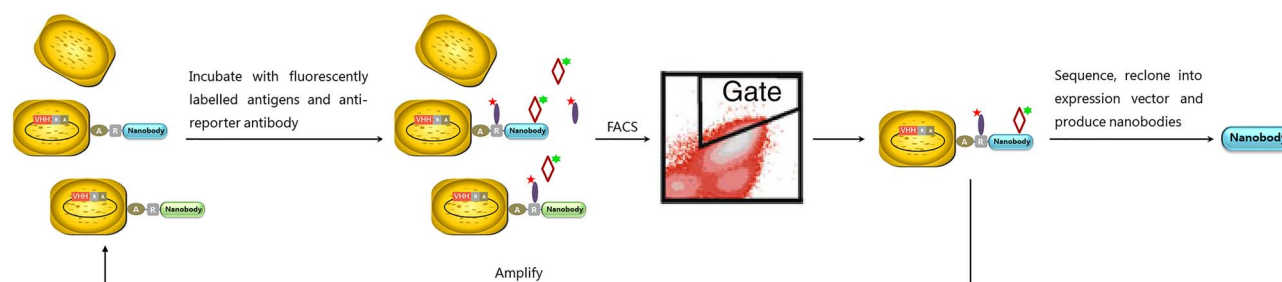


Fig. 3. Schematics of the cell surface display for nanobody screening. A nanobody library is displayed on the cell surface of yeast or bacteria *via* fusion to the anchoring domain (A) and then incubated with fluorescently labelled antigens (hollow red diamond) and an anti-reporter (R) antibody (solid purple oval). Cells are sorted using FACS. Positive cells are amplified for additional sorting rounds or isolated for sequencing the nucleotides of nanobodies, which are further analyzed with specificities and affinities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mechanisms ensure that only properly folded proteins are secreted (Ryckaert et al., 2010), particularly for these nanobody sequences that are difficult to express in prokaryotic cells.

Additionally, Gram-positive bacteria are suitable for recombinant display and combinatorial protein engineering applications. The thick layer of peptidoglycan surrounding the cell membrane protects the cells in the harsh environment of the flow cytometer, leading to excellent cell viability after cell sorting (Kronqvist et al., 2010). Fleetwood et al. developed a bacterial display system using Gram-positive bacterium *Staphylococcus carnosus* and the cell-wall anchoring domain from staphylococcal protein A. A number of sub-nM affinity binders for GFP were successfully isolated by FACS, in which the affinities were significantly higher on average compared with the nanobodies derived in parallel from phage display (Fleetwood et al., 2013).

2.3. mRNA/cDNA display

Cell-based display methods are partly limited by the transfection/transformation efficiencies of the host organisms, and these methods are not apply to stringent selection conditions. By contrast, mRNA/cDNA display employs a covalent bond to link an encoding nucleic acid fragment to its encoded antibody, allowing the maintenance of stringent, tailored antigen-binding conditions that are particularly useful in challenging targets, such as integral membrane proteins (Roberts and Szostak, 1997; Wang and Liu, 2011). The micro-scale mRNA/cDNA display method is highly integrated, streamlined, and amenable to high-throughput automation. This technique led to the discovery of high-affinity antibodies against full-length human integral membrane protein targets, which has been a serious challenge to conventional methods (Hino et al., 2013; Seddon et al., 2004).

By using DNA splint-assisted RNA–DNA ligation, mRNA can be modified at its 3′ end with an oligo DNA tail containing a polyA sequence and a puromycin group that mimics the aminoacyl moiety of tRNA. This tandem complex then enters the ribosomes during translation and accepts the growing polypeptide chain *via* a peptide bond, resulting in the C-terminus of the encoded/translated protein covalently bound to the 3′ end of the nucleic acid as a “fusion” (Roberts and Szostak, 1997). After purification by oligo(dT)-affinity chromatography, the fusions were reverse-transcribed and exposed to the histidine-tagged target antigen that was pre-immobilized on a Ni-NTA affinity solid phase. Following washes to remove the unbound nanobodies, fusion-target complexes were eluted from the solid phase, and full-length nanobody-encoding genes were regenerated using PCR. Sequences were analyzed after TA cloning (Fig. 4).

The aforementioned method was validated to discover the first reported nanobody against the human full-length glucose transporter, GLUT-1, by using a commercially available library of nanobodies. Only 10 µg of purified target antigen (1–3 µg/round of display), 20 µg of mRNA (5 µg/round of display), and commercial readily-available materials are required in nanobody discovery. The final screened

nanobodies were characterized to have a mean K_D of 22.3 ± 11.5 nM (Doshi et al., 2014).

In summary, mRNA/cDNA display can be conducted to obtain the desired antibody within 2–3 weeks for 1 specific antigen. In addition, it can be easily multiplexed for several antigens in parallel. This method has the potential to become fully automated as the entire reaction is completely *in vitro*, and mechanical procedures—accurate aspiration, centrifugation, incubation, separation, and temperature cycling—can be easily automated.

2.4. High-throughput DNA sequencing and mass spectrometric identification

Fridy et al. reported on a highly optimized strategy that allows the rapid production of large repertoires of high-affinity nanobodies against selected proteins (Fridy et al., 2014). The approach focuses on high-throughput DNA sequencing of a marrow lymphocyte VHH cDNA library from an immunized llama, combined with mass spectrometric (MS) identification of affinity-purified VHH regions derived from serum of the same animal. In this method, VHH-containing HCABs were serially fractionated in serum after llama immunization on the basis of the different affinities of Protein A and Protein G for HCAB versus conventional antibodies (Hamers-Casterman et al., 1993). To further separate the antigen-bound VHH fragments, HCABs were affinity-purified over antigen-coupled resin and digested with papain on resin to cleave away the constant regions. The desired minimal fragments of the VHH region were retained. The VHH fragments were eluted after the highest-stringency washes to recover the highest-affinity VHHs and then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel-purified bands were trypsin-digested and analyzed by liquid chromatography–mass spectrometry (LC–MS) and tandem MS. To create an animal-specific antibody sequence database, lymphocyte RNA was obtained from individual immunized llamas to generate cDNA. Nested PCR was conducted to specifically amplify sequences that encode the VHH regions for high-throughput sequencing. The sequences were translated, filtered, and trypsin-digested *in silico* to create a searchable peptide database for MS analysis. High-probability hits for nanobodies against antigens were expressed in *E. coli*. The *E. coli* cell lysates were passed over antigen-coupled resin to identify nanobodies that were robustly expressed and with high and specific affinity. The affinity of the nanobodies was further assessed by either surface plasmon resonance or other technologies, with K_D values into the sub-nM range with monomeric nanobodies or as low as ~30 pM with ultrahigh affinity dimeric version (Fig. 5).

In this method, only standard techniques are required, including IgG purification, cDNA generation, PCR, high-throughput sequencing, MS, gene synthesis, and final cloning and screening. The entire process can be completed in 4–6 weeks after the samples are collected from llamas. Moreover, the high success rate of this single screening step (57%–75%) is favorable, compared with the final panning and selection steps of phage display in which up to 10^7 clones are screened initially to

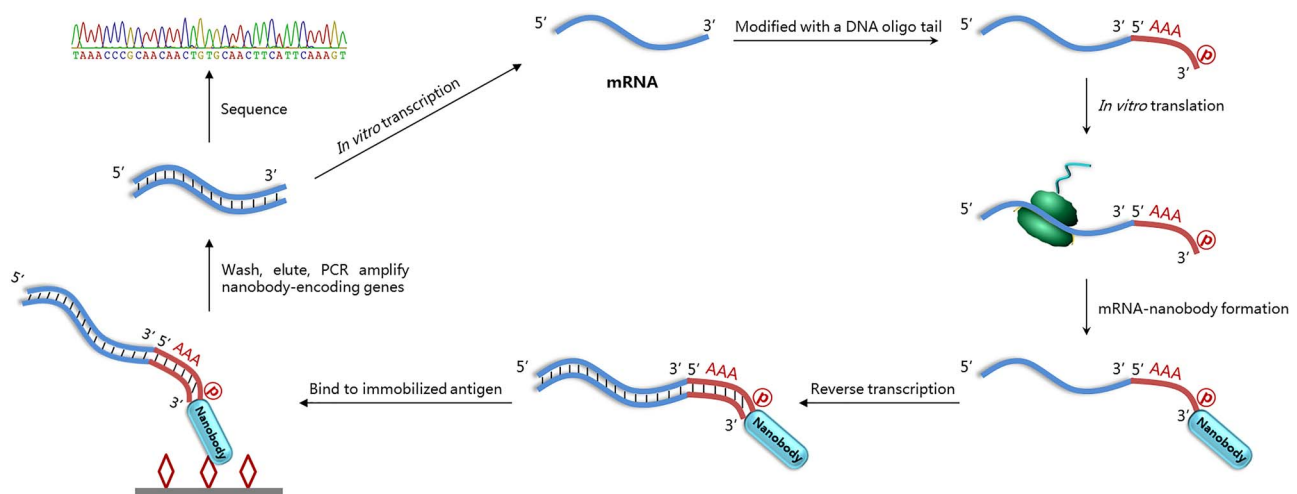


Fig. 4. Schematic of the mRNA/cDNA display. mRNA is modified at its 3' end with a polyA sequence and a puromycin group (P). Following *in vitro* translation and mRNA-nanobody fusion formation, reverse transcription is performed to synthesize cDNA. The complexes are exposed to the antigens (hollow red diamond) of interest, which are immobilized on a solid-phase affinity matrix. Following washing and elution, PCR is performed to retrieve full-length nanobody-encoding genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identify even a small number of positive clones (Arbabi Ghahroudi et al., 1997; Conrath et al., 2001).

2.5. Biotinylated nanobody screening

Although a wide variety of nanobodies have been isolated for all types of macromolecule antigens and haptens (Muyldermans, 2013; Tabares-da Rosa et al., 2011), it is more challenging when the antibody target is part of a complex antigen, such as receptors on the cell surface. Antigen recognition is highly sensitive to conformational changes in the target itself; thus, a conventional approach of using recombinant membrane proteins for immunization and selection can often lead to a deficiency in cross-recognition to the native cell receptor if the recombinant protein failed to replicate its native conformation (Pardon et al., 2014). In 2015, Rossotti et al. proposed a method for the production of biotinylated nanobodies, which is amenable for high-throughput screening, to address these limitations (Rossotti et al., 2015). A llama was immunized subcutaneously with bone marrow-derived dendritic cells (BMDC), and a phage display library was constructed as described previously. For panning, the library was first pre-adsorbed on fibroblast cells to eliminate ubiquitous cell receptor binders. Unbound fraction was then selected on BMDC by 3 rounds of panning. The VHs from the third output were cloned *en masse* into a pINQ-BtH6 vector that bears a biotin acceptor peptide to construct a sub-library. This sub-library of VHs was then co-transformed in *E. coli* together with pCY216 for overexpression of the biotin ligase BirA to directly biotinylate the VHs inside the cells. Flow cytometry was then employed to simultaneously screen a large number of biotinylated nanobodies (BtNb). After screening, soluble cell extracts containing BtNbs from individual clones were prepared to label BMDCs. The soluble extracts of positive clones were then directly loaded into streptavidin-agarose, which was then used for the immunoprecipitation experiments to isolate the VHH target receptor from BMDC extracts. Finally, the immunoprecipitated proteins were analyzed by SDS-PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Fig. 6). The potential of this strategy was demonstrated by the selection and characterization of nanobodies to Mac-1 (CD11b/CD18), MHC II, and the mouse Ly-5 leukocyte common antigen (CD45) receptors. This strategy streamlines the discovery of nanobodies without purification of individual clones in a high-throughput manner to cell membrane receptors, which comprise the largest group of drug and analytical targets.

2.6. Bacterial-two-hybrid system

The *ex vivo* selection method yields antigen binders that function properly under oxidizing conditions as encountered in an extracellular environment. However, these binders often fail to maintain their antigen-binding competence when expressed and employed in the cytoplasm with a reducing environment (Ewert et al., 2004). To avoid this problem, a two-hybrid system has been introduced. The yeast-two-hybrid system is thus far the most widely used selection technique for identifying protein-protein interactions. Unfortunately, this approach is inadequate for considerably large and diverse libraries ($> 10^7$ potential binders), which are commonly required for the efficient selection of proper target binders. Therefore, a step to narrow down the library size is needed for further selection by the yeast-two-hybrid system (Visintin et al., 2004). This method is also presented with the problem of having to re-clone the enriched library to yeast-two-hybrid vectors. Moreover, the yeast-two-hybrid system is limited to the slow growth of yeast cells. Fortunately, these shortcomings can be remedied by hosting the two-hybrid system in fast-growing bacteria instead of yeast.

In 2012, Pellis et al. demonstrated single-step isolation of intracellular functional camelid nanobodies from immune libraries by using a bacterial two-hybrid approach. To construct the system, the DNA encoding the antigen is cloned into the pTRG vector to encode a fusion protein of the antigen with the α -subunit of RNA polymerase, and the VHH library is ligated into the pBTL vector to encode the nanobodies fused to λ cI. The *E. coli* BM11 reporter cells, where the *His3* and *aadA* genes (scorable genotypes) are under control of the phage λ operator, are then co-transformed with both plasmids. Nanobodies fused to λ cI (that can bind to the phage λ operator) interact with the antigen fused to the α -subunit of RNA polymerase to recruit the transcription machinery for the specific expression of the reporter genes (*His3* and *aadA*) from the λ operator, allowing the survival of the transformed cells on a selective medium (i.e., medium containing 3-AT and streptomycin) (Fig. 7) (Pellis et al., 2012). This approach led to the successful isolation of nanobodies against HIV-1 integrase, GFP, and *Trypanosoma vivax* nucleoside hydrolase with sub- μ M affinity. Moreover, the sequence diversity, intrinsic domain stability, antigen-specificity and affinity of these nanobodies compare favorably with those that were retrieved in parallel by phage display panning (Pellis et al., 2012).

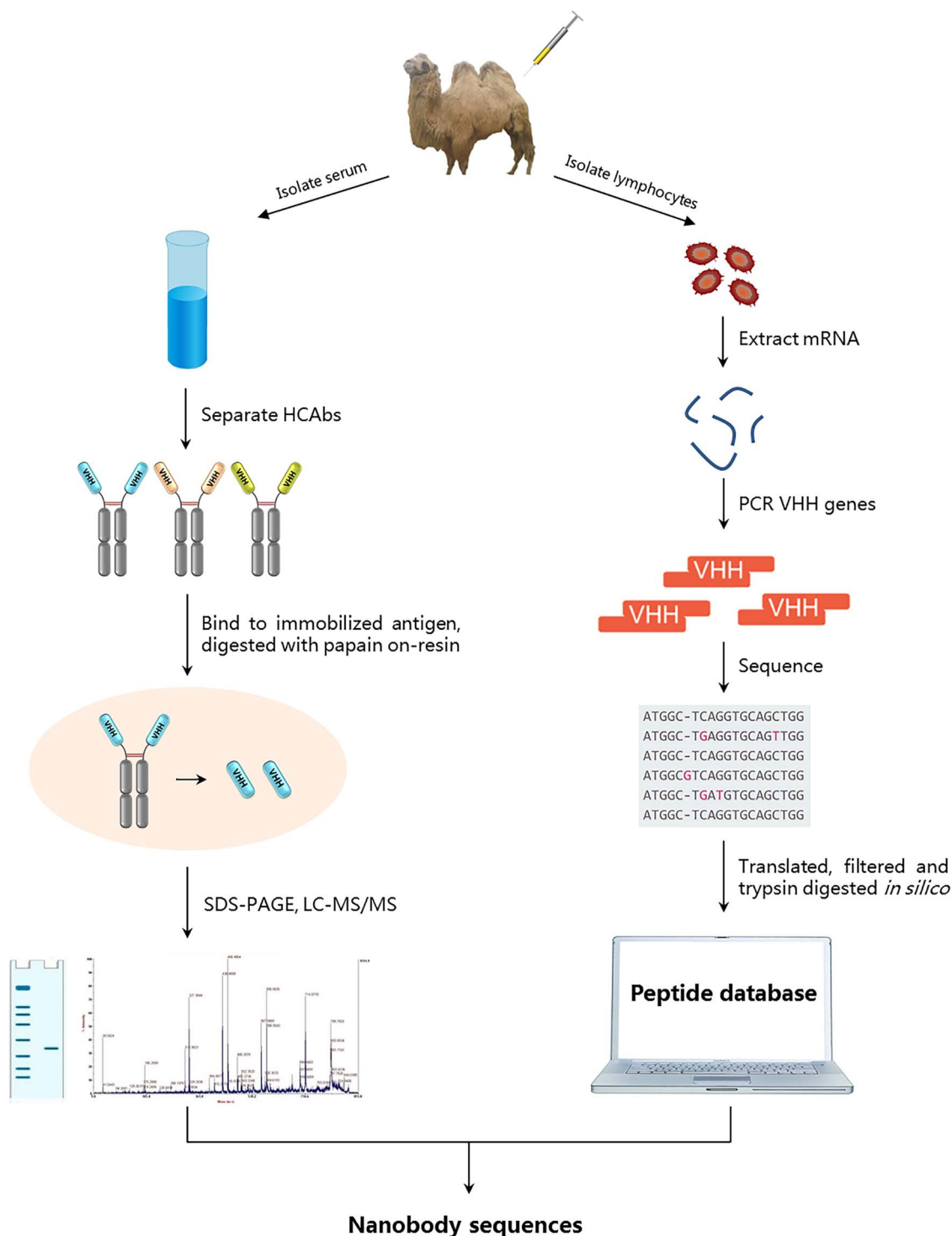


Fig. 5. Overview of nanobodies produced by high-throughput DNA sequencing and mass spectrometric identification.

3. Screen nanobodies from naïve libraries

Antibodies have been successfully raised by animal immunization; regardless, the approach is limited by animal usage, bloodstream stability, antigenicity and toxicity of the target, and overall practical feasibility of scale-up. By contrast, naïve libraries allow for the identification of a wider diversity of binders for any potential antigen (Monegal et al., 2009; Yan et al., 2015). Owing to the lack of somatic maturation stimulated *in vivo* by immunization, high specificity and affinity can be achieved only when selecting from large-diversity libraries ($\sim 10^9$

individual clones) (Muyldermans, 2013). In addition, *in vitro* maturation techniques, such as DNA shuffling, error-prone PCR, and randomized primers can be employed to increase the diversity of complementarity-determining regions (CDRs) as well as the specificity and affinity of nanobodies (Farasat et al., 2017; Fromant et al., 1995; Hoseinpoor et al., 2014; Stemmer, 1994). To the best of our knowledge, phage display and ribosome display are the only 2 main strategies that have been attempted to screen nanobodies from naïve libraries.

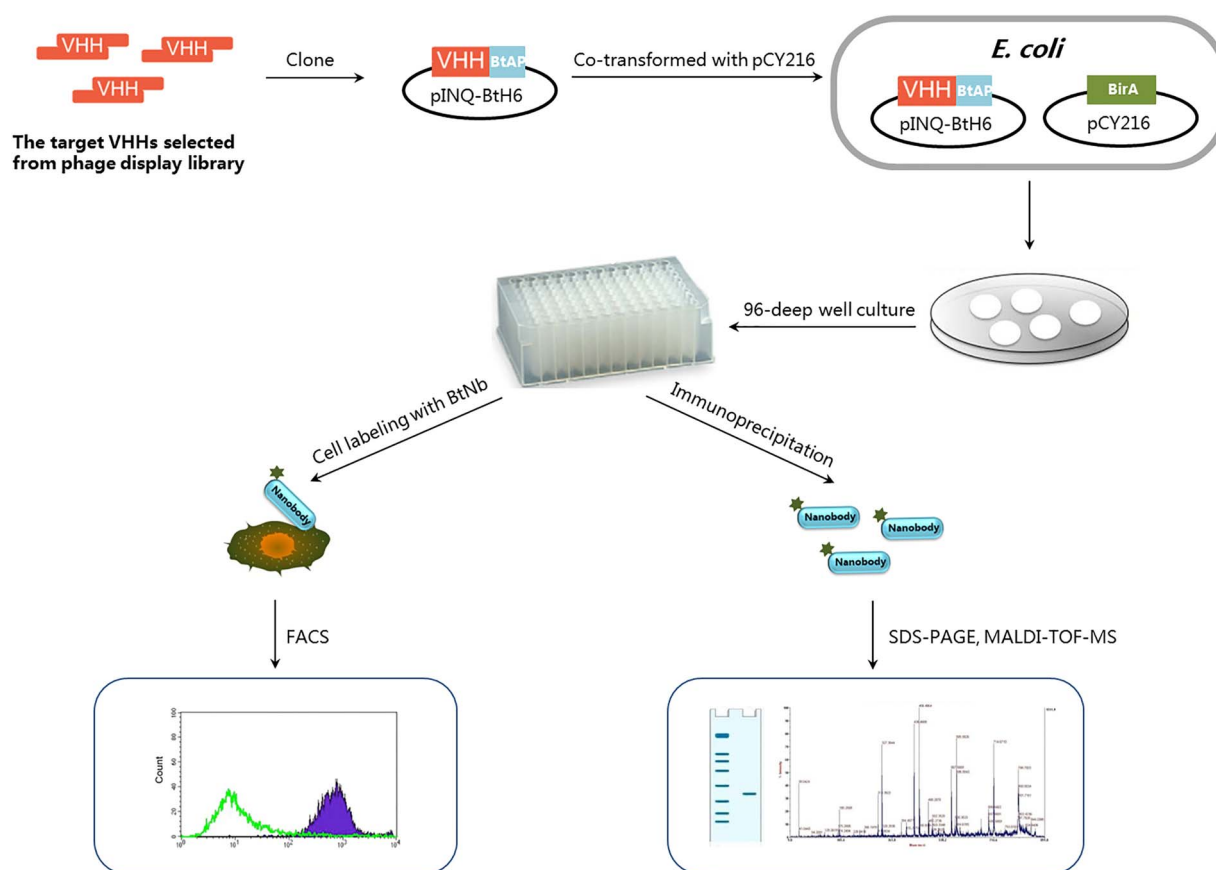


Fig. 6. Schematic of the method for the isolation of biotinylated nanobodies to cell receptors. After panning on cells, the VHH output is cloned *en masse* in the expression vector pINQ-BtH6, which adds the biotin acceptor peptide. This sub-library of VHH is then co-transformed in *E. coli*, together with pCY216, for overexpression of the biotin ligase BirA. Individual clones are then cultured in 96-well deep well plates, and the soluble cell extract containing BtNb can be characterized by FACS and MALDI-TOF-MS.

3.1. Phage display

To construct a naïve VHH library, peripheral blood lymphocytes collected from the blood of non-immunized llamas were used for mRNA extraction. The theoretical diversity of a naïve library increases with an increasing number of lymphocytes initially collected. Thus, a large volume of blood samples (usually more than 1 L) collected from different individual animals is a prerequisite for preserving the highest genetic diversity. In addition, to avoid unnecessary diversity reduction during cloning of the library, all necessary steps should be performed with maximum care to reduce material loss. Alternatively, the final library size could also be increased by mixing blood samples collected from different individual animals to ensure the diversity of the library (Wang et al., 2016). The first described llama naïve VHH phage-display library has successfully provided useful binders for human cerebrovascular endothelial cells (HCEC) in 2001, although it was

initially constructed from only 10^7 lymphocytes of heparinized blood from a male llama (Muruganandam et al., 2001). These nanobodies demonstrated effective abilities in transmuting across an *in vitro* human blood–brain barrier (BBB) model and found more applications to facilitate drug transport across the BBB. In 2006, Verheesen et al. isolated VHHs for 4 muscle disease-related proteins: emerin, actin, tropomyosin-1, and nuclear poly(A)-binding protein from blood samples collected from 8 non-immunized llamas. The affinities are reported to be in the nM levels (Verheesen et al., 2006). Another study showed that the llama naïve library, constructed from 10^9 lymphocytes that were isolated from 11 non-immunized llamas, can be used to recover nanobodies against human fibroblast growth factor receptor 1 with nM affinity having a wide range of application in only few weeks (Monegal et al., 2009).

Shark heavy-chain antibody IgNAR is secreted from splenocytes. The sequences in the variable domains of IgNAR seem highly diversified

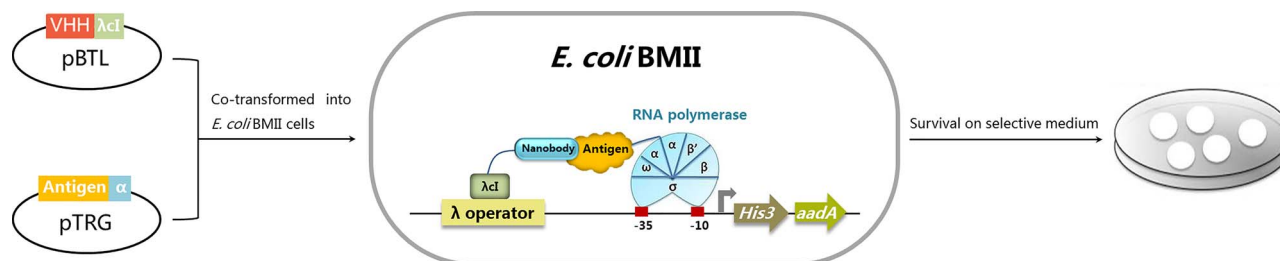


Fig. 7. Schematic of the bacterial-two-hybrid system. The DNA encoding the antigen is cloned into the pTRG vector, and the VHH library is ligated into the pBTL vector. The *E. coli* BMII reporter cells are then co-transformed with both plasmids. Nanobodies fused to λ CI (that can bind to the phage λ operator) interact with the antigen fused to the α -subunit of RNA polymerase to recruit the transcription machinery for specific expression of the reporter genes (*His3* and *aadA*). This process allows the survival of the transformed cells on selective medium.

because of frequent somatic mutations (Diaz et al., 1998; Greenberg et al., 1995). Naturally-occurring V-NAR can be obtained either from immunized sharks or naïve (non-immunized) sharks (Dooley et al., 2003; Liu et al., 2007a; Nuttall et al., 2001). Given that sharks are difficult to maintain, naïve shark libraries have been constructed only by several groups, and panning using different toxins and proteases has been performed to obtain V-NARs with therapeutic value (Liu et al., 2007a; Nuttall et al., 2002; Nuttall et al., 2001). Although these binders are of relatively low affinity, protein engineering can be employed to improve this affinity.

3.2. Ribosome display

Similar to the mRNA/cDNA display mentioned previously, ribosome display is based on mRNA linked to its encoded protein via the ribosome (Hanes et al., 2000; Hanes et al., 1998). The correctly folded proteins in the complex are affinity-selected on an immobilized ligand (antigen) at the selection step (biopanning). Unbound complexes are removed by stringent washing, and mRNA molecules linked to the selected complexes are released for reverse transcription into cDNA, which can be isolated for cloning or mutated for affinity maturation (Jermutus et al., 2001; Yan and Xu, 2006). The library size is not limited by the transformation of DNA into cells; thus, the upper limit of the size of a ribosome-displayed antibody library (up to 10^{15} copies) is 3 orders of magnitude higher than that for the phage library (Yau et al., 2003). With the phage display system, the affinity of a binder against large antigens generally ranges from mid μM to high nM, whereas the affinity is 10- to 100-fold lower for small haptens (M.W. < 500 Da). Interestingly, by employing ribosome display, the binders against small haptens were isolated with μM affinity, indicating that this technology may be particularly useful in the production of an antibody against small molecules (i.e., M.W. < 500 Da) (Yau et al., 2003). Thus, this technology should more efficiently obtain desired antibodies without animal immunization to be able to detect small haptens in agriculture and the food industry, such as pesticides (Hall et al., 1993), environmental contaminants, or toxic substances (McElhiney et al., 2002).

In 2003, the first nanobody isolated by ribosome display was reported by Yau et al. In this study, a VHH cDNA library was constructed from 10^7 lymphocytes of a non-immunized llama and then engineered to allow transcription and translation *in vitro*. With no stop codons present on the transcripts, trimeric complexes of mRNAs, ribosomes, and nascent peptides were generated. After several cycles of panning with picloram (a systemic herbicide), nanobodies with K_D in the high μM range were isolated (Yau et al., 2003). By contrast, panning of a non-immunized llama VHH-phage display library against picloram failed to yield any binders (Tanha et al., 2002). Regardless, high-affinity binders against hen egg lysozyme with K_D in the pM range were isolated from a shark library by ribosome display combined with error-prone mutagenesis (Fennell et al., 2010).

Despite the numerous advantages of *in vitro* ribosome display, the large-scale production of an mRNA-ribosome-antibody complex is extremely costly, and the protein yield is relatively low. To address this problem, Contreras-Martinez and DeLisá developed a streamlined method of producing ribosome complexes *in vivo*, which costs considerably less than producing ribosome complexes *in vitro* (Contreras-Martinez and DeLisá, 2007). However, *in vivo* expression is usually considered time-consuming, and the toxicity of the target protein can present a significant challenge. Considering the advantages and disadvantages of each expression system, Bencurova et al. constructed a universal cassette for the synthesis of mRNA-ribosome-VHH complexes both *in vitro* and *in vivo* (Bencurova et al., 2015). With a double-overlap extension PCR, VHH was fused with the F1 fragment (contained T7 promoter, species-independent translation sequence, and ATG initiation codon) and the F2 fragment (contained mCherry, Myc-tag, tether, SecM arrest sequence, and 3' stem loop) to generate a full-length DNA cassette. mCherry was included to simplify the detection of the synthesis

and proper folding of the nascent chain. Myc-tag was incorporated to capture the ribosome complexes on anti-Myc-affinity beads to purify the library efficiently. The tether sequences served as spacers that tethered the protein to the ribosome and maintained proper folding. The SecM sequence terminated the translation with the formation of a stable ribosome complex *in vivo* in *E. coli*, whereas the 3'-loop sequence protected mRNA from exonucleases. The synthesized expression cassette was incubated directly with cell-free lysates (*E. coli*, *Leishmania tarentolae* or rabbit *reticulocyte*) or ligated in pQE-30 vector and then transformed into *E. coli* to produce ribosome complexes *in vitro* and *in vivo*. Whole-cell antigen derived from 3 different *Borrelia* and *Francisella* species confirmed the ligand-binding ability of the VHH complex (Bencurova et al., 2015). This rapid and universal technique can be effectively used in antibody ribosome display and VHH production.

4. Screen nanobodies from semisynthetic/synthetic libraries

Limited size and diversity are key concerns affecting the efficiency of antibody screening from immune libraries and even large-size naïve libraries. Without a wide range of diversity from the variable heavy/variable light pairing in conventional IgGs, affinity maturation in camelids relies to a larger extent on somatic hypermutation; this process precisely tunes the CDRs to recognize any given antigens (De Genst et al., 2006; Nguyen et al., 2000). Thus, semisynthetic/synthetic libraries were proposed, which mimic such *in vivo* diversification to yield sufficiently diverse libraries capable of generating nanobodies against any specific antigen. Each nanobody contains CDRs that correspond to hypervariable sequences, which determine antigen specificity, and conserved framework regions (FRs), which may be crucial in conserving the structural integrity of nanobodies. The approach attempts to conserve the FRs and to randomly diversify the lengths and sequences of the CDRs, particularly those of elongated CDR3, which is known to increase the antigen-binding surface and to contribute significantly to antigen-binding selectivity (Muyldermans, 2013).

In 2016, Moutel et al. created a non-immune recombinant nanobody library of high diversity on the basis of a robust nanobody scaffold hs2dAb. Synthetic diversity was introduced in the 3 CDRs by rationally controlling each position of CDR1 and CDR2, based on the statistical analysis of the diversity found in more than 200 natural VHs, while reducing the presence of the most hydrophobic residues to prevent aggregation propensity. The CDR3 region was introduced with a random amino acid (except cysteine) in each position. For CDR1 and CDR2, the size was fixed at 7 amino acids, whereas for CDR3, 4 sizes were chosen (9, 12, 15, and 18 amino acids) to cover this spectrum. Large-scale PCR was then conducted, ensuring that at least 10^{10} different DNA molecules were used as a matrix. The synthetic library was inserted into a modified pHEN2 phagemid vector and transformed into *E. coli* TG1 cells to construct a high-diversity phage display library of 10^9 independent clones. The universal synthetic library was successfully screened to identify highly functional binders directed against highly diverse targets (fluorescent proteins, actin, tubulin, p53, and HP1). The affinities of selective binders were in the order of 10 nM and up to 50 pM, which are rather good and usually rarely observed for monovalent binders obtained without *in vivo* immunization or *in vitro* affinity maturation (Moutel et al., 2016).

Shark V-NARs contain 4 FRs and 2 CDRs, lacking a conventional CDR2 (Diaz et al., 1998; Stanfield et al., 2004; Stanfield et al., 2007). Similar to camelid VHH antibodies, the V-NAR region has abnormally long CDR3 loops with the highest diversity in both length and sequence (Diaz et al., 1998; Diaz et al., 2002; Nuttall et al., 2001). CDR3 is known to contribute significantly to antigen-binding selectivity. On the basis of naturally occurring V-NAR sequences, several studies constructed semisynthetic/synthetic shark nanobody libraries by introducing size and sequence variations in CDR3, using randomized CDR3 primers (Liu et al., 2007a) or error-prone PCR combined with splicing-overlap extension PCR (Goldman et al., 2006; Shao et al., 2007). According to the

V-NAR regions deposited in the Genbank database, the length of CDR3 ranges from 11 amino acids to 25 amino acids with a sample mean of 18.3 amino acids (Shao et al., 2007). Synthetic CDR3 regions 13–22 residues in length are used to construct semisynthetic/synthetic shark nanobody libraries. Binders against the outer membrane translocase receptor from human mitochondria (Tom70) (Nuttall et al., 2003), toxins (Staphylococcal enterotoxin B, ricin, and botulinum toxin A complex toxoid) (Liu et al., 2007b), Gingipain K protease from *Porphyromonas gingivalis* (Nuttall et al., 2001), lysozyme, and leptin (Shao et al., 2007) were isolated from panning the display library. In general, the affinities of the selected binders were in the nM range.

The diversity of semisynthetic/synthetic nanobody library is close to 10^9 , and at least 1 L of blood samples might need to be consumed to obtain $\sim 10^9$ individual clones per library for the naïve library. Thus, the semisynthetic/synthetic library has higher CDR3 diversity and utility than the naturally occurring naïve VHH or V-NAR libraries without immunization or collection of large volume of blood samples. In addition, experimental conditions can be tightly controlled with relatively easy operation requirements because all steps involved in the screening of antibodies from semisynthetic/synthetic library are conducted *in vitro*. Thus, a semisynthetic/synthetic nanobody library may be a promising path toward obtaining a limitless source of nanobodies against a variety of antigens without the need for animal immunization or collection of a large volume of blood samples.

5. Conclusions

Owing to their superior properties, nanobodies exhibit broad potential for application in various fields, including *in vivo* and *in vitro* disease diagnosis, targeting therapy, targeting drug delivery, developing immunobiosensors, *in vivo* imaging, and nano-based research. This review systematically summarizes and evaluates the state-of-the-art technologies developed thus far to quickly select and improve nanobodies from highly diverse libraries (Table 1). First, retrieval of nanobodies from an immune library remains a first choice because target specific nanobodies are highly enriched in immune libraries. However, this method is largely limited by the natural antigenicity of antigens. Moreover, new immune libraries must be prepared accordingly for every new antigen, starting from animal immunization. To address these concerns, naïve libraries and semisynthetic/synthetic libraries have been developed. Non-immune libraries enable the rapid selection of diverse and highly functional binders. However, because of lacking somatic maturation, high-specificity and high-affinity binders can only be obtained when selecting from vast functional libraries. A substantial amount of blood samples (usually more than 1 L) collected from different individual animals is a prerequisite to ensure the diversity of the naïve library. In this consideration, semisynthetic/synthetic libraries may be a more practical alternative by avoiding both animal immunization and collection of blood samples. Independent of the approaches to constructing libraries, numerous selection techniques, including phage display, yeast display, bacterial display, mRNA/cDNA display, intracellular two-hybrid system, and ribosome display, have been well documented to facilitate screening and panning for a given antigen-specific nanobody. Most of these methods or display platforms can be applied to any library type. By combining the library with selection protocols, together with affinity maturation techniques, more nanobodies may be efficiently produced for various applications.

Conflict of interest disclosure

The authors declare no commercial or financial conflict of interest.

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Table 1
Available technologies for nanobody screening.

Libraries	Screening technologies	Affinity	Advantages	Disadvantages
Immune libraries	Phage display	nM to pM	1. Target specific nanobodies are highly enriched. 2. Most common and standard selection technique (2–4 months for 1 specific antigen).	Largely limited by the antigenicity of antigens.
	Cell surface display	nM	No subcloning, expression, and purification of various antibody candidates.	Partly limited by the transfection/transformation efficiencies of the host organism.
	mRNA/cDNA display	nM	1. Only a handful of materials (10 µg antigen and 20 µg mRNA) is required. 2. Short time (takes 2–3 weeks after animal immunization for 1 specific antigen).	1. Costly. 2. Limited by the translation efficiency <i>in vitro</i> .
	High-throughput DNA sequencing and mass spectrometric identification	nM	1. Short time (takes 4–6 weeks after animal immunization for 1 specific antigen). 2. High success rate (57%–75%).	Requires a peptide database.
	Biotinylated nanobody screening	Not reported	1. Screening nanobodies that recognize conformational proteins. 2. Requires no purification of individual clones.	Limited by the transformation efficiency of the host organism.
	Bacterial-two-hybrid system	µM	Maintains antigen-binding competence in reducing environment (cytoplasm). 1. No animal immunization.	Lack protein modification.
Naïve libraries	Phage display	nM	2. Capable of generating nanobodies against any specific antigen.	Need substantial amount of blood samples collected from different individual animals.
	Ribosome display	µM to pM	1. No animal immunization. 2. Capable of generating nanobodies against any specific antigen, particularly useful for small antigens (<i>i.e.</i> , M.W. < 500 Da). 3. Larger library size (10^{15}).	Need a substantial amount of blood samples collected from different individual animals.
Semisynthetic/synthetic libraries		nM to pM	1. No animal immunization. 2. Capable of generating nanobodies against any specific antigen. 3. High diversity.	Need design large and diverse semisynthetic/synthetic libraries.

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