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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-specifity 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 (*Orectolobus 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 cerebromicrovascular endothelial cells; BBB, blood–brain barrier

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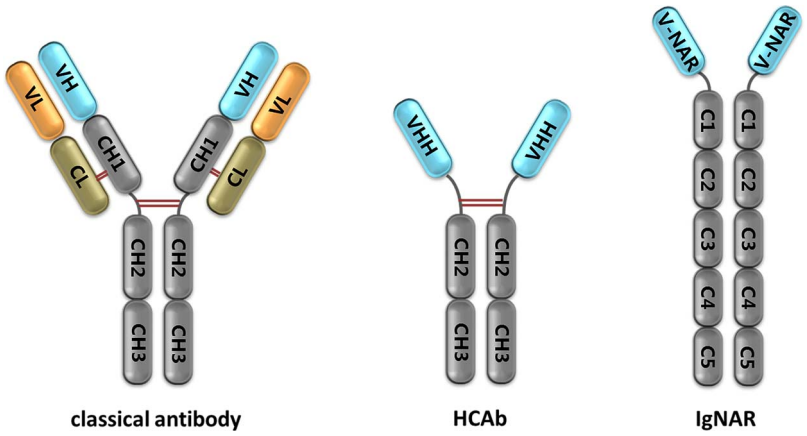

Fig. 1. Schematics of the conventional antibody, HCAb, and IgNAR.

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 HCAbs 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 HCAbs 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