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Nanobodies and their medical applications

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ABSTRACT. Nanobodies (Nbs) are mini antibodies 10 times smaller than conventional antibodies. They constitute a new class of antibodies devoid of light chains. Their paratope consists of a single variable domain "VHH" (heavy chain variable domain) formed by a single peptide chain. Nevertheless, Nbs maintain the same repertoire of diversity and affinity characteristics as conventional antibodies. Because of their high specificity, small size, and low production cost, Nbs are emerging progressively as a potential alternative to overcome the limitation of conventional antibodies in targeted therapy. Indeed, the therapeutic spectrum of Nbs has been confirmed in animal models and human clinical trials. The present review provides a summary of the technological advances in the production of Nbs and their potential targeted therapeutic biomarkers over the past 10 years.

Key words: Nanobodies; Biomarkers; Therapeutic applications; Production Techniques; Conventional antibodies

INTRODUCTION

Advances in immunotherapy and molecular biology are the basis for the development and production of therapeutic monoclonal antibodies. These conventional antibodies have gradually invaded the pharmaceutical sector and the world of targeted therapy through their use

in oncology, neurological and inflammatory diseases, among others (Pérol and Arpin, 2011). Besides, the leading pharmaceutical companies continue to be interested and committed to the development of novel monoclonal antibodies (Andersen and Krummen, 2002). However, due to their high production cost and low penetration rate into certain tissues (large size), their therapeutic use remains limited. Thus, in the case of solid cancers, which account for 85% of cancers, only three monoclonal antibodies have been accepted for clinical use (Shemi et al., 2015).

Other successful studies have demonstrated the possibility to obtain affinities from picomolar to nanomolar using only the variable domain (VHH) of some antibodies from camelids and cartilaginous fish (Hamers-Casterman et al., 1993; Greenberg et al., 1995; Dooley et al., 2003) named "Nanobodies" (Nbs). The Nbs are known as heavy chain antibodies (HCAb) (Siontorou 2013)or single domain antibodies (sdAb) (Comor et al., 2017). Given the lack of the light chain, Nbs antigen binding sites carry a single variable domain called VHH for Camelidae and vNAR for sharks (Flajnik et al., 2011; Griffiths et al., 2013). However, while monoclonal antibodies (mAbs) rely on the immortalization of the antibody producing B-cell, most Nbs are obtained by isolating the RNA from the circulating peripheral B-cells to capture the animals immune repertoire of variable heavy domains displayed on the surface of phage. From this phage library, Nbs that binds the target of interest can be isolated and provide a unique mode of binding and recognition to the antigen-epitopes. The latter are inaccessible to conventional antibodies (Desmyter et al., 1996; Lauwereys et al., 1998; Dooley et al. 2003; Streltsov et al., 2005).

The simple structure of Nbs, their easy manipulation, therapeutic efficiency and their cost-effective production have attracted biotech companies to invest further in Nbs development (Roovers et al., 2007). Indeed, several companies have specialized in the production of Nbs including the Belgian company "Ablynx" developing Nbs from Camelidae, and the American company "GenWay Biotech" from cartilaginous fish with an ongoing clinical trials (Wolfson, 2006; Siontorou, 2013). In this review, the major focus is on the potential therapeutic activities of Nbs based on their simple structures and high affinities to specific biomarkers of viruses, inflammatory and neurodegenerative diseases as well as certain types of cancers.

STRUCTURE AND PHYSICOCHEMICAL PROPERTIES OF NANOBODIES

Structure

Conventional antibodies are mainly formed by two heavy and light chains. The heavy chain contains three constant domains (CH1, CH2, CH3) as well as a variable domain (VH). The light chain contains a constant domain (CL) which interacts with the constant domain (CH1) by a disulfide bridge and a variable domain (VL) as presented in Figure 1 (Wang et al. 2016). The therapeutic use of these antibodies remains limited due to their high molecular weight (150 kda) that hinders their penetration into certain tissues and to their strong immunogenicity with a very long half-life may lead to a toxicity risk (Saerens et al., 2008; Siontorou, 2013).

In contrast to the conventional antibodies, Nbs have a simpler monomeric structure. In fact, their structure is almost identical to conventional IgG VH with four conserved FR regions surrounding the three-hypervariable CDR regions, which confers a specific recognition to the antibody (Figure 1). In CDR regions, the difference is quite remarkable and the CDR1 and CDR3 regions are moderately longer (16-18 amino acids (CDR3)) than the human VHs (9-12 amino acids). Thus, the CH1 domain was excised out of the mRNA by alternative splicing and

CH2 and CH3 domains are conserved and constitute the FC fragment like conventional antibodies. However, the VHH domain replaces the entire IgG. Hence, the VHH small variable fragments able to bind perfectly to unique conformational epitopes owing to its long CDR3 region and its monomeric structure without the need for dimer formation, as in the case of conventional antibodies. The VHH (15 kD) domain is called “Nanobody”(Transue et al., 1998).

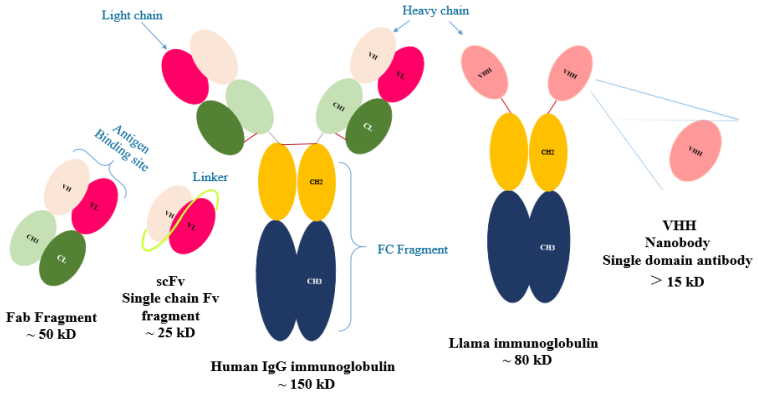

Figure 1. Conventional antibodies (Ab) carrying combination of heavy chains and light chains and camelids Ab with heavy chains and a single variable domain, VHH, Adapted from Muyldermans (2013)

Physicochemical properties and advantages of Nanobodies

The affinity of a Nb for its antigen is very strong because their interaction with their target leads to the rapid formation of a complex (Kon between 105 and 106 M-1 S-1) and a slow dissociation (Koff between 10-4 and 10-2 S-1) (Wesolowski et al., 2009; Hassanzadeh-Ghassabeh et al., 2013). Moreover, Nbs have a high solubility and stability that allow them to have a resistance to extreme physicochemical conditions due to their intrinsic properties and to have strong binding with the target antigen during the selection stage (Rahbarizadeh et al., 2011). In addition, the simple nature of Nbs heavy chain promotes a declination in bi- and tri-specific antibodies and their resemblance to the VH domains of human antibodies make them little or none immunogenic.

SELECTION AND PRODUCTION OF RECOMBINANT NANOBODIES

The production process of Nbs is well controlled and takes place in several stages. In fact, many recombinant expression systems have been established and optimized for this purpose, especially in yeasts, bacteria, fungi or mammalian cells. For example, in *Saccharomyces cerevisiae*, expression levels of Nbs were reported to 100 mg/L in a flask under stirring and 1 g/L using the fed-batch fermentation with a yield up to 1,3 kg at the scale of 15 m^3 (Frenken et al., 2000; Thomassen et al., 2002).

Nanobodies are generally non-immunogenic when injected into human, as there is a strong sequence identity with the human VH domain. In addition, their small size ensures a rapid elimination from blood, a better elimination by the renal system and a decrease in matters of toxicity (Siontorou, 2013).

VHH engineering uses advanced and robust techniques. Indeed, Nbs library should be constructed in order to select only the ones that have successfully linked to the target antigen (Figure 2). Due to several specific applications, the immune library is the most commonly used

and considered as an additional time effective option. Differently to the naive library, the immune library goes through a step of immunizing the camelid with the antigen of interest.

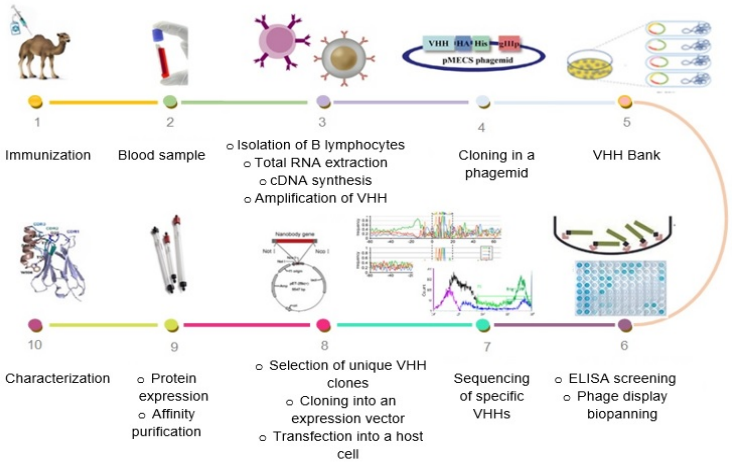

Figure 2. Traditional process of the variable domain (VHHs) production

However, the immunization step is not always possible if the injected antigen is non-immunogenic or toxic for the camelid (Yan et al., 2015). This pathway has several advantages including somatic maturation, which consists of the selection with high antigen affinity. The blood from the camelid is then collected, the peripheral blood mononuclear cells (PBMC) harvested, and the DNA extracted. To amplify sequences coding for the VHH, the cloning strategy consist of performing two successive PCRs from cDNAs, which represent the whole IgG of the B-lymphocytes from the immunized Camelid. The first PCR serves to amplify the region located between the constant domain CH2 and the leader sequence of VH and VHH variable domains while using primers hybridizing with sequences conserved among the set of Camelid IgG isotypes. The second PCR serves to generate shorter fragments corresponding to VHH sequences. This Second PCR is also used to introduce restriction sites facilitating phage-oriented cloning in order to obtain a phagemid library representing all the diversity of Nbs repertoire. Nbs specific to the target antigen will be selected and enriched (biopanning) by phage-display. Biopanning is realized on immobilized antigens by passive adsorption on a solid surface (micro titer plate), three rounds of biopanning are sufficient to enrich interesting clones. Subsequently, the ability of these Nbs to recognize the antigen is tested by ELISA, and positive clones are then sequenced to get access to the sequence of Nbs of interest. Afterward, VHH sequences will be cloned into an expression vector for the production in host cells. By including the sequence coding for the signal peptide, the protein produced can be exported to the periplasm, where oxidative conditions favor the correct folding of Nbs and the formation of disulfide bridges. After periplasmic extraction, a simple purification is generally carried out since the 6His tag is integrated into the expression vector which allows purification via an affinity column and then via a gel filtration. Beside this classic approach, Hassanzadeh-Ghassabeh et al. (2013) have described another strategy to generate potent Nbs and streamline their identification (Hassanzadeh-Ghassabeh et al., 2013). They mentioned the importance of Nbs immunization with more difficult targets such as receptor complexes, multipass membrane proteins or intrinsically disordered proteins. They have also highlight the advantage of implementing NGS as a deep sequencing approach to rapidly discover antigen-specific Nb

sequences. In another study, Fridy et al have described an approach, which can be adapted to the development of Nb reagents against diverse type of proteins targets with high affinity and specificity (Fridy et al., 2014). This approach has been effective to produce and generate large repertoires of Nbs against GFP and mCherry antigens with Kb values into the subnanomolar range. In fact, they have demonstrated the possibility to design ultrahigh-affinity dimeric Nbs with Kd values as low as 30 pM.

Phage Display

The production of Nbs requires the adoption or the development of a mastered strategy with advanced techniques with high sensitivity and specificity. Among the most powerful and used techniques in Nbs production is the "Phage Display". This technique is considered as a powerful tool for high throughput screening of protein interactions (Crameri and Kodzius, 2001). Indeed, As a screening platform, Phage Display has a great importance for pharmaceutical companies in the development of new patentable entities (Rader and Barbas III, 1997; Castel and Tordo, 2009). This technique is mainly based on the exposure of the protein of interest (peptides, antibodies or other) on the surface of a bacteriophage and on the binding affinity and specificity of the protein of interest with the NH2-terminal domain of one of bacteriophage surface proteins (Souriau et al., 1998; Castel et al., 2009).

Pishia pastoris expression system

Being a methylotrophic yeast, *Pishia pastoris* is widely used as an expression system for the production of both intracellular and extracellular proteins with a large number of expression vectors including; pPIC9, pPIC9k, pPICZα, pHIL-D2, pHIL-S1, pFLD and pFLDα, pGAPZ and pGAP (Safder et al., 2018). In fact, the *Pishia* expression system allows easy purification and culture with high cell densities allowing to obtain high yields of protein per liter (Daly and Hearn, 2005).

Pishia pastoris has two alcohol oxidase genes, AOX1 and AOX2. They have strong and inducible promoters. These two genes allow *Pishia pastoris* to assimilate methanol as a single source of carbon and energy. In fact, by using highly inducible methanol, *Pishia* is able to convert 30% to 40% of its weight into proteins (Näätsaari et al., 2012). The stability of the expression cassette in the yeast genome, its secretion capacity, the low levels of contaminating proteins in culture medium and the rapid growth of the yeast on inexpensive media and in fermentors, make *Pichia patoris* an excellent choice for the expression of heterologous proteins.

THERAPEUTIC APPLICATION OF NANOBODIES

While the use of mAbs in targeted therapy remains limited, in recent years, rapid progress has been made in the engineering and selection of VHH domains, including Nbs, for therapeutic purposes. Thus, it has been demonstrated that these Nbs have a high ability to be modified without losing their structural integrity and are compatible with a variety of high throughput screening platforms such as yeast, phages and bacteria (Ghahroudi et al., 1997; Ryckaert et al., 2010; Fleetwood et al., 2013). In addition, the robustness of Nbs, particularly those resistant to acidic pH and peptic proteolysis that exist in the gastrointestinal environment, have been explored for oral administration. Indeed, this modality decreases the constraints and considerably improving the comfort of the patients (Hussack et al., 2011). The major ongoing clinical trials using Nbs are summarized in Table 1. Their strong potential use is applied to

different sectors including diagnosis and especially therapeutic medicine against certain viral infections, inflammation, neurodegenerative diseases and cancers.

Table 1. Major nanobodies in clinical trials.

Nanobodies	Mechanism/targest	Indications	Clinical trial studies	Developer/Sponsor
ALX – 0761 [69]	Anti-IL-17A/F Nanobody	Moderate to severe psoriasis	Phase 1	Merk KgaA, Darmstadt, Germany
Caplacizumab (ALX-0681) [70]	Bivalent VHH against Vwf	Treatment of acquired thrombotic thrombocytopenic purpura (aTTP)	Approved	Ablynx
ALX-0171 [71]	Trivalent Nanobody against antigenic site II of F	treatment for acute RSV (Respiratory syncytial virus) infection	Phase 2	Ablynx
ALX-0061Vobarilizumab	Nanobody against the interleukin-6 receptor (IL-6R)	Treatment of Rheumatoid arthritis	Phase 2b	Ablynx
Ozoralizumab (ATN-103) [72]	Bispecific VHH targeting both TNF and HAS		Phase 2	Taisho
Anti-HER2 5F7 Nanobody [73]	Anti-HER2 5F7 Nanobody	Imaging of HER2 receptors expression in cancer	Phase 1	Ablynx
M6495 [74]	Anti-ADAMTS-5 nanobody	Treatments for osteoarthritis, protects against cartilage breakdown	Phase 1	Merk KgaA, Darmstadt, Germany
ALX-0962 [72]	Bispecific VHH targeting both IgE and HSA	Treatment of severe allergic Asthma	Preclini-cal trials	Ablynx
ALX-0141 [75]	Targets RANKL	Cancer-related bone diseases, osteoporosis	Phase 1	Ablynx
Dekavil [72]	scFv-IL-10 targeting Fibronectin	Treatment of inflamed tissues	Phase 2	Philogen/Pfizer
ATN-192 [72]	Trivalent bispecific VHH	Treatment of SLE (Systemic Lupus Erythematosus)	Phase 1	Ablynx
DR5Nb1 [76]	Human DR5 (death receptor)-specific Nanobody targeting death receptor signaling.	Treatment of colon and pancreatic cancers, and tumor xenografts.	Phase 1	Novartis
ALX-0651	Bivalent nanobody inhibiting CXCR4	Treatment of non-Hodgkin's lymphoma and multiple myeloma	Phase 1	Ablynx
ALX-0081 [77]	Nanobody against against Von Willebrand factor	Treatment of acute coronary syndrome and percutaneous coronary intervention	Phase 2	Ablynx

Viral infections

Nbs possess a simple structure and strong affinities with the potential to target numerous antigen-epitopes, which is perplexed for conventional antibodies. This makes Nbs useful against infectious pathogens. Indeed, Stohr et al have developed ALX-071 a trimeric Nb targeting the fusion protein of RSV (Respiratory Syncytial Virus). They have reported the successful of clinical trial phase 2 and the superior in vitro neutralization compared to pavilizumab, the conventional mAb version used to prevent the same infection (Detalle et al., 2016). Other recent study demonstrated a significant reduction of RSV with improved clinical symptoms in RSV-infected animals (Mejias et al., 2017). In the same year, Koch et al identified new Nbs neutralizing relevant HIV subtypes. Those Nbs are classified as candidates for preclinical and clinical development (Koch et al., 2017). A recent interesting study has shown Nbs contribution to antiviral treatments and prevention of DENV (Dengue virus) which is a mosquito-borne virus that infects over 50 million people worldwide annually (Morgan, 2019). Vanlandschoot et al. (2011) have described the therapeutic applications of Nbs against other viruses such as Hepatitis B virus, influenza virus, Poliovirus, Foot-and-mouth disease virus, Rotavirus and Porcine endogenous retrovirus (Vanlandschoot et al. 2011).

Alzheimer Disease

Alzheimer Disease (AD) is a chronic neurodegenerative disease characterized by specific changes that include intraneuronal and extracellular parenchymal lesion. Those changes impair social function and activities of daily living of patients (Dubois et al., 2010).

Due to their small size and the presence of hydrophilic residues in the FR2 fragment, Nbs have the capacity of crossing the blood-brain barrier and targeting the central nervous system (CNS) (Farrington et al., 2014). These characteristics make Nbs powerful therapeutic tools to consider in the fight against neurological diseases. Among these diseases, AD represents a major social issue (behavioral and neuropsychiatric changes) in which it is essential to detect biomarkers that are neurochemical indicators used to estimate the risk or the presence of this disease. Apolipoprotein E (ApoE) remains a risk factor for AD, which can lead to an excess of amyloid formation in the brain. In 2017, Xiang Ren et al developed Nbs directed against ApoE acting as immuno-detectors (Ren et al., 2017) that may have potential in clinical diagnosis and real-time monitoring of the progression of AD.

Coronary Syndrome and Rheumatoid Polyarthritis

Other Nbs have been tested in clinical trials phases I and II as therapeutic molecules. A major study is underway with two bivalent Nbs, ALX-0081 and ALX-0061. ALX-0081 is directed against Von Willebrand factor as an antithrombotic agent for patients with acute coronary syndrome and percutaneous coronary intervention. ALX-0061 is directed against the interleukin-6 receptor as an anti-inflammatory agent for patients with rheumatoid arthritis. Indeed, when it is difficult to achieve desirable levels of pharmacokinetics, Nbs can be adapted to prolong the half-life in the blood to achieve high serum levels and prolonged therapeutic efficacy (Van Bockstaele et al., 2009; Ulrichts et al., 2011).

Parkinson Disease

Parkinson's disease (PD) is a neurodegenerative disease of aging PD is characterized neurologically by tremors and uncontrolled bradykinesia due to the loss of dopamine-producing neurons in the substantia nigra (Perez et al., 2007) specifically by the misfolding and aggregation of amyloid-b proteins and alpha-synuclein (αS) (Choi and Gandhi, 2018). In autopsy, the detection of these amyloid structures in brain tissue allows an accurately diagnosis subtypes of neurodegenerative diseases (Roberts et al., 2015). Klenerman et al. (2015) has developed two Nbs; NbSyn2 and NbSyn87, which bind to the C-terminal region of αS (Roberts et al., 2015). They were able to show that both Nbs inhibit the formation of fibrS fibrils. In addition, by using single-molecule fluorescence techniques, they demonstrated that the binding of Nbs promotes a rapid conformational conversion of more stable oligomers to less stable oligomers of αS. This leads to a strong reduction of oligomers induced cellular toxicity .

Cancer therapy

Despite the successful cancer therapy of mAbs, their high production cost pushed researchers to look for more cost-effective candidates. Thus, Nbs can be the best alternative because of their low cost production and outstanding features for targeting tumors such as angiogenesis factors (VEGF family, HER-2, and EGFR) (Alibakhshi et al., 2017). They could be considered as promising tools to identify and obtain site-specific targeting. Nbs are also considered as efficient tools for cancer research as they can enable specific modulation of targets and enzymatic and non-enzymatic proteins alike (Van Audenhove and Gettemans, 2016).Indeed, Nbs can be used to block ligand-receptor interactions as it has been shown in a recent study by Salvador et al where Nb1 and Nb6 were able to block the EGF-EGFR (Guardiola et al., 2018) as well as the anti-EGFR Nbs liposome for blocking the EGFR on the tumor cell surface (Allegra et al., 2018). In addition, EGFR and HER-2 Nbs has also the potential for targeting cancer cells as presented in Tao Zou's et al study using HER-2 Nb for functionalizing PEG-b-Polycaprolactone polymersomes that could target HER-2 over expression in breast cancer (Zou et al., 2015). Other studies have tested four Nbs (Nb22, Nb23, Nb35 and, Nb42) providing their potential to inhibit endothelial cell proliferation (Kazemi-Lomedasht et al., 2015). Whereas, other multi-specific Nbs binding multiple targets with only one Nb molecule are under development (DeFrancesco, 2017). In addition, some multivalent Nbs (Table 1) are used as radionuclide vehicles in radioimmuno-therapy which is becoming more and more an interesting strategy for cancer treatment (Dekempeneer et al., 2016). Finally, Nbs have also emerged as probes for non-invasive imaging of tumor cells for breast cancer (Du et al., 2018) and colon carcinoma (Vaneycken et al., 2011).

Imaging and antibody chip

The development of Nbs for imaging purposes has not only involved the fusion with fluorescent proteins but also with coupling to radio-elements (Vaneycken et al., 2011)and fluorochromes (Wang et al., 2015). The principle is to fuse the Nb to a fluorescent protein, expressed in mammalian cells. The goal is to label antigens *in vivo* at

different subcellular compartments throughout the cell cycle (Rothbauer et al., 2006). Further, Nbs can be used for dynamic imaging at very high resolution (Albrecht et al., 2015, Platonova et al., 2015).

Fluorescent protein-fused Nbs named "chromobodies" can serve as *in vivo* tracers and versatile tools to study dynamics and cellular localization of proteins, the abundance of cellular compounds and the dynamics of their interactions with other molecules (Traenkle and Rothbauer, 2017). Additionally, the easy modification of Nbs allowed their fusion to a variety of fluorescent molecules, as well as their chemical coupling to branched gold nanoparticles that has strongly produced photo-thermal therapeutic agents which target antigens during the light irradiation (Wang et al., 2016). In other study, it was discussed that NP-based immuno-sensors provide good results with a promising sensitivity and reproducibility (Farka et al., 2017). The same study has described the role of nanopaticles in immuno-sensing as a promising approach for fast, low-cost, and accessible detection of trace prognostic biomarkers, drugs, microbial pathogens, toxins and environmental pollutants.

Since Nbs have the ability to penetrate the blood-brain barrier due to their small size, which allows them to be removed quickly from kidneys. This gives rise to images with an excellent signal/noise and allows an interpretation after a few hours only a posteriori of the injection of a marker. Unlike conventional antibodies that take between 2 and 4 days after injection to interpret the image. Moreover, the size of Nbs is much higher than the threshold of renal clearance causes their accumulation in the liver, so their half-life in the blood can go up to 10 days. In addition, the Fc domain of Nbs interacts with cell surface receptors, further increasing their retention in the bloodstream and their background noise in imaging (Huang et al., 2010; Hemmer, 2015).

Other applications of Nbs in imaging are in the cancer field. For example, in the case of HER2 positive cancers (breast, gastric and ovarian cancers), Ganesan et al. have evaluated a noninvasive imaging procedure that could assess the expression of HER2 in primary and metastatic lesions as well. This method called “Immuno-PET”could be a valuable tool for optimizing HER2-targeted therapy. The concept in this study is to use ^{18}F-labeled Nbs as probes for evaluating HER2 status by ‘Immuno- PET’. For that purpose, they have labeled the “5F7 Nb” with the ^{18}Fand they demonstrate that the ^{18}F-labeled anti-HER2 Nbs are the adequate tracers for the evaluation of HER2 expression (Vaidyanathan et al., 2016). Another study has shown that the labeled“2Rs15dNb” with ^{68}Ga enabled high contrast PET-imaging of HER2-positive breast cancer (Zhou et al., 2017). Due to their fast extravasation, good tumor penetration and quick renal clearance of excess tracer, Nbs allow sensitive imaging of target tissue within a short time after injection instead of several hours when using conventional mAbs.

Ultimately, the simple single-domain structure and the enhanced stability make Nbs potential candidates for the rapid development with high-density of antibody chips (Wang et al., 2016).

CONCLUSIONS

Nanobodies represent a new generation of mini-antibodies with particular advantages of size, structure, affinity, stability, and ease of production on a large scale. They are considered an effective therapeutic alternative to conventional mAbs that often

have limited efficacy and high production cost. Indeed, several Nbs are already in clinical trials phases I or II, especially against cardiovascular, neurodegenerative or autoimmune diseases.

These properties and advantages make Nbs powerful tools that can be used in many fields, including diagnostics, cellular and medical imaging, and finally targeted immunotherapy. Only the future can confirm if Nbs will have the potential to surpass and replace conventional mAbs currently used in many therapeutic areas.

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AUTHORS' CONTRIBUTIONS

HB and FA defined the concept of the present review. NB and YA were involved in the acquisition of the data. HB, YC and HS were involved in drafting and revision of the manuscript. All authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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