Human Antibodies -1 (2019) 1–23 I
DOI 10.3233/HAB-190390
IOS Press

Nanobodies: The "magic bullets" in therapeutics, drug delivery and diagnostics

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Abstract. Antibodies represent a well-established class of clinical diagnostics for medical applications as well as essential research and biotechnological tools. Although both polyclonal and monoclonal antibodies are indispensable reagents in basic research and diagnostics but both of them have their limitations. Hence, there is urgent need to develop strategies aimed at production of alternative scaffolds and recombinant antibodies of smaller dimensions that could be easily produced, selected and manipulated. Unlike conventional antibodies, members of Camelidae and sharks produce antibodies composed only of heavy chains with small size, high solubility, thermal stability, refolding capacity and good tissue penetration *in vivo*. The discovery of these naturally occurring antibodies having only heavy-chain in Camelidae family and their further development into small recombinant nanobodies represents an attractive alternative in drug delivery, diagnostics and imaging. Nanobody derivatives are soluble, stable, versatile, have unique refolding capacities, reduced aggregation tendencies and high-target binding capabilities. They can be genetically customized to target enzymes, transmembrane proteins or molecular interactions. Their ability to recognize recessed antigenic sites has been attributed to their smaller size and the ability of the extended CDR3 loop to quickly penetrate into such epitopes. With the advent of molecular engineering and phage display technology, they can be of potential use in molecular imaging, drug delivery and therapeutics for several major diseases. In this review we present the recent advances in nanobodies for modulating immune functions, for targeting cancers, viruses, toxins and microbes as well as their utility as diagnostic and biosensor tools.

Keywords: Recombinant antibodies, nanobody, drug delivery, cancer, diagnostics, chimeric, humanized antibodies, immunoaffinity chromatography, site directed mutagenesis

1. Introduction

Immune system is one of the nature's most fascinating inventions, protecting us with ease against millions of bacteria, viruses and other pathogens. Immune system has evolved under a terrific selective pressure imposed by these pathogens [1]. As a result, the body has developed the ability to recognize the invading microbes and to eliminate them efficiently without causing damage to self [2]. The vertebrate immune system generates billions of different antibody molecules and this enormous repertoire contains highly specific binding partners for practically every existing biological and chemical entity [3]. Following immunization, B-cells that express a suitable antibody expand a million-fold by clonal proliferation in lymphatic organs [4]. Well-orchestrated mutation and selection mechanisms ensure preferential expansion of variants that express antibodies with higher affinity to the immunogen [5]. Repeated immunization thus, generally yields specific antibodies with higher quantities and affinities [6]. Hybridoma and genetic engineering technologies can be used to harness and reformat individual antibodies obtained from immunized animals and even to reconstruct recombinant molecules semi-synthetically [7]. Both these polyclonal and monoclonal antibodies have their limitations of solubility, stability and penetrability [8]. But camels, llamas and sharks also produce antibodies composed only of heavy chains [9]. The antigen-binding site of these unusual heavy chain anti-

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bodies (hcAbs) is formed by only a single domain, designated as VHH in camelid hcAbs and VNAR in shark hcAbs [10]. VHH and VNAR are easily produced as recombinant proteins, designated single domain antibodies (sdAbs), nanoantibodies or nanobodies [11]. The complementarity determining regions (CDRs) of these sdAbs possess the extraordinary capacity to form long finger like extensions that can extend into cavities on antigens, e.g., the active site crevice of enzymes [12]. Other advantageous features of nanoantibodies (nanobodies) include their small size, high solubility, thermal stability, refolding capacity and good tissue penetration *in vivo* [13]. Here in this review, we present the results of several recent proof-of-principle studies that open the exciting perspective of using nanobodies for modulating immune functions and for targeting cancers, toxins and microbes [14].

1.1. Polyclonal and monoclonal antibodies

An antigen is the substance that the body is trying to "fight off" (eliminate or reduce) by mounting an immune response [15]. An antibody or immunoglobulin on the other hand is a glycoprotein molecule that is produced by the body in response to an "invading" (foreign) substance called an antigen [16,17]. The most common one is immunoglobulin G (IgG). IgG is a protein comprised of two main structural and functional regions (Fig. 1). Antibody therapeutics are one of the fastest growing classes of pharmaceuticals, with an annual US market over $20 billion, developed to treat a variety of diseases including cancer, auto-immune and infectious diseases [18]. Antibody reagents are developed from either polyclonal or monoclonal antibodies (Fig. 2) [19–21]. Monoclonal antibodies differ from polyclonal antibodies in that they are highly specific for a single epitope on a multivalent antigen (Fig. 3). They are produced from a single cell line using hybridoma technology [22].

1.2. Chimeric, humanized and human antibodies

In the early 1990s with the advancements in molecular biology tools and techniques it was possible to clone the genes of IgG mAbs in eukaryotic expression vectors [23,24]. Cloning antibody genes was the first step towards the modification of antibodies by random or site directed mutagenesis, sub cloning and molecular evolution procedures, which made it possible to optimize recombinant antibodies at will and steered the age of antibody engineering [25]. With the result,

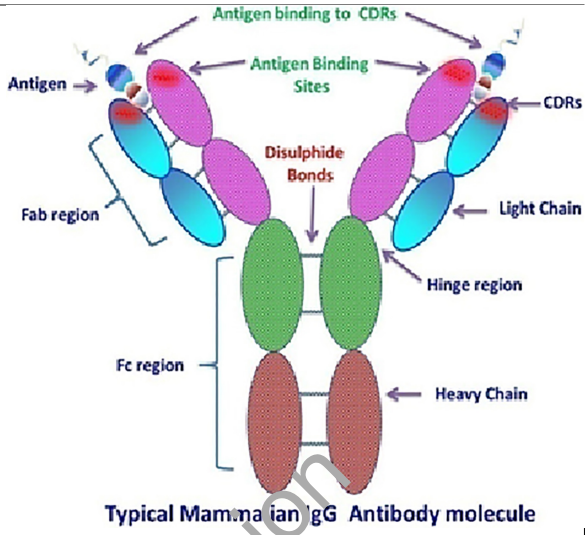

Fig. 1. Antibody structure and functional sites. Fab region: Contains the antigen (Ag) binding site that varies between different antibodies. Fc region: Region of constant structure within an antibody class. CDR: Complimentary determining region, Hinge region.

recombinant versions of any mAb could be obtained from diverse cell lines in a reproducible fashions and this solved the production issues caused by the instability of many hybridoma lines [26].

The ability to create chimeric antibodies was a major application of antibody engineering techniques. As we know, the binding activity of IgG Ab molecules is generated by the variable domains of the heavy and light chains in the Fab region. As antibodies are well conserved through evolution, it was possible to create chimeras by fusing murine variable domains responsible for the binding activity with human constant domains, leading to the development of a new generation of therapeutic candidates [27]. Chimeric antibodies produced in this way are 70% human and possess a fully human Fc portion, which allows them to interact with human effector cells and the complement cascade, which makes them considerably less immunogenic in humans [28].

By using complementarity-determining region (CDR) grafting, it became possible to decrease the murine part of mAbs even further by replacing the hypervariable loops of a fully human antibody with the hypervariable loops of the murine antibody of interest [28]. These antibodies called 'humanized', are 85–90% human and are even less immunogenic than chimeric antibodies (Fig. 4). However, CDR grafting is more technically demanding than a mere fusion and directed mutagenesis approaches are often needed to restore the affinity present in the murine parental anti-

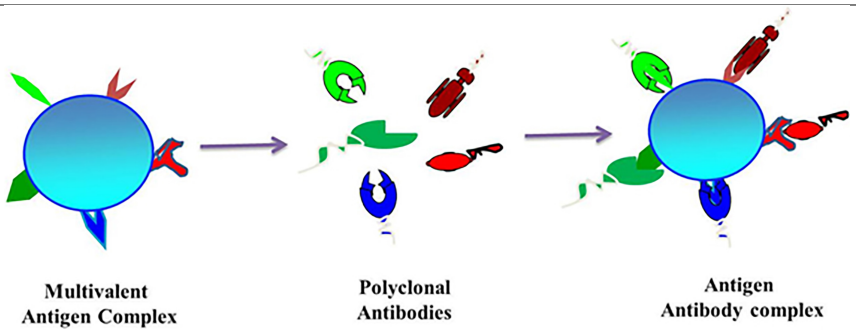

Fig. 2. Multiple antigen specificities of polyclonal antibodies.

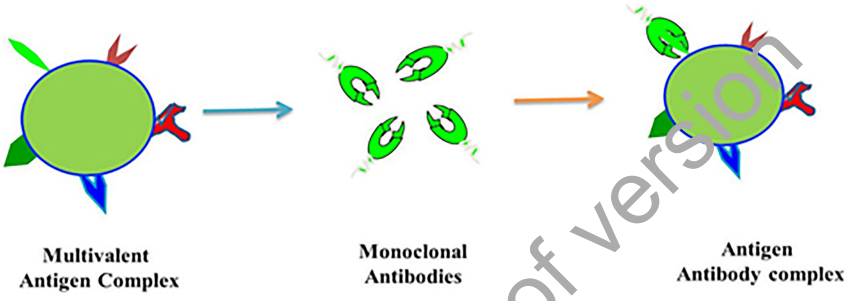

Fig. 3. Uniform specificities of monoclonal antibodies.

body. Currently most of the approved mAbs by FDA used are either chimeric or humanized (Table 1) [29].

1.3. Why nanobodies

Over the last decade, monoclonal antibodies (mAbs) have been gaining momentum in disease-specific therapy. Recently, the U.S. Food and Drug Administration has approved 27 mAbs for therapy, mostly for the systemic treatment of cancer [29]. Notable among them are for the treatment of solid tumours like cetuximab [Erbitux directed to epidermal growth factor receptor (EGFR), HER1], trastuzumab (Herceptin to HER2) and bevacizumab (Avastin to vascular endothelial growth factor) (Table 2) [30–32]. These mAbs are aimed to completely neutralize/block growth factors or growth factor receptors, the key drivers of tumor growth and survival for a prolonged period.

Despite clinical enthusiasm, however, it is fair to state that the efficacy of current mAbs is still quite limited, benefitting just a portion of patients while costs of monoclonal antibody (mAb) therapy are excessive [33]. This conclusion comes with very recent insights that selective targeting of just one single tumor target might be insufficient for fully effective cancer treatment, a reason why also mixtures of mAbs are

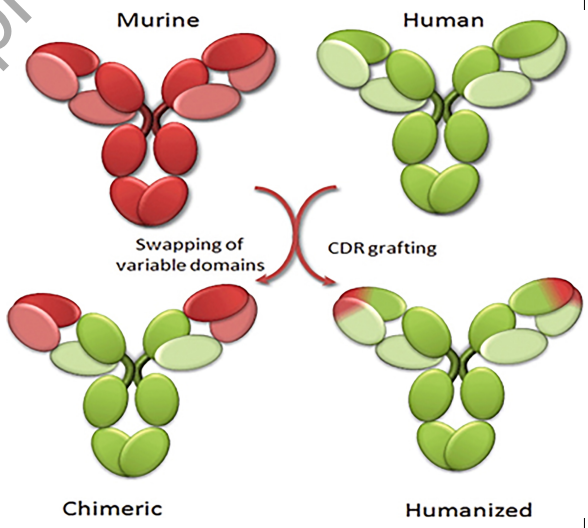

Fig. 4. Chimeric and humanized antibodies: Swapping of variable domains between murine and human antibodies gives rise to a chimeric antibody, while grafting of complimentary determining regions (CDR) between murine and human antibodies gives rise to a humanized antibody. Murine sequences are depicted in red and human sequences in green, using light colors for light chains and dark colors for heavy chains.

exploited in multitarget anticancer therapies [34,35]. The rationale for this approach is that cancer cells have the inherent ability to use several growth factor (recep-

Table 1
The FDA-approved 27 therapeutic monoclonal antibodies from 2015 to 2017 and the number has been increasing year by year [29]

Name of antibody	Approved year	Target antigen	Target disease
Dinutuximab	2015	GD2	Pediatric high-risk neuroblastoma
Idarucizumab	2015	Dabigatran	Emergency reversal of anticoagulant dabigatran
Mepolizumab	2015	IL-5	Severe asthma
Elotuzumab	2015	SLAMF7	Multiple myeloma
Nivolumab	2015	PD-1	Metastatic melanoma
Secukinumab	2015	IL-17A	Plaque psoriasis
Evolocumab	2015	PCSK9	Heterozygous familial hypercholesterolemia, refractory hypercholesterolemia
Alirocumab	2015	PCSK9	Heterozygous familial hypercholesterolemia, refractory hypercholesterolemia
Necitumumab	2015	EGFR	Metastatic squamous non-small-cell lung carcinoma
Daratumumab	2015	CD38	Multiple myeloma
Obiltoxaximab	2016	*Bacillus anthracis toxin*	Inhalational anthrax
Ixekizumab	2016	IL-17A	Plaque psoriasis
Reslizumab	2016	IL-5	Severe asthma
Atezolizumab	2016	PD-L1	Urothelial carcinoma
Olaratumab	2016	PDGFR	Soft tissue sarcoma
Bezlotoxumab	2016	*Clostridium difficile toxin B*	Prevent recurrence of *C. difficile* infection
Gemtuzumab ozogamicin	2017	CD33	Acute myeloid leukemia
Ocrelizumab	2017	CD20	Multiple sclerosis
Inotuzumab ozogamicin	2017	CD22	Precursor B-cell acute lymphoblastic leukemia
Emicizumab	2017	FIXa, FX	Hemophilia A (congenital Factor VIII deficiency) with Factor VIII inhibitors
Benralizumab	2017	IL-5R α subunit	Severe asthma, eosinophilic phenotype
Brodalumab	2017	IL17R	Plaque psoriasis
Avelumab	2017	PD-L1	Metastatic merkel cell carcinoma
Durvalumab	2017	PD-L1	Urothelial carcinoma
Dupilumab	2017	IL-4Rα	Atopic dermatitis
Guselkumab	2017	IL-23	Plaque psoriasis
Sarilumab	2017	IL-6R α subunit	Rheumatoid arthritis

tor) systems for growth advantage and survival [36]. All mAbs except one that have been approved until now are intact mAbs. This is remarkable, because these large-sized molecules (150 kDa) are considered to have limited capacity for tumor penetration [37]. For this reason, much effort has been put in the evaluation of monoclonal antibody (mAb) fragments but without convincing therapeutic success to date [38].

2. Nanobodies

Nanobodies or single-domain antibodies represent a class of next-generation antibodies that have some unique features like small size (in nanoscale), high stability in hard situations, high penetration in various tissues and easy production process in microbial systems. In fact, the nanobodies represent the smallest fragment of antibody with high binding ability [16]. Presence of unique features and characteristics in nanobodies makes them an appropriate candidate for further evaluation as the development of novel antibody-based therapeutics. Consequently, many researchers and biopharmaceutical companies are interested in nanobodies or single-domain antibodies for diagnostic and therapeutic applications [39]. Currently various nanobodies are being developed and several other are evaluated in different clinical trials (Table 3). Because of many advantages of single-domain antibodies over other formats of antibodies, they could be good replacement for other formats of antibodies in near future [39].

2.1. General features of nanobodies

Nanobodies (Nbs) are derived from a unique antibody format that is present in species from the family of camelidae including llama, camel and dromedary. These animals contain, next to their conventional antibody repertory, an antibody class consisting of heavy chains only [40]. Consequently, the variable region of the heavy chain from these "heavy-chain-only" antibodies (VHH) represents the complete binding unit of the antibody. Considering the small size of the VHH fragments (15 kDa), this binding unit is also called nanobody (Fig. 5).

Nanobodies are antibody fragments consisting of a single monomeric variable domain. These nanobodies

Table 2
Antibodies and immune-conjugates approved for the treatment of different cancers

Generic name	Trade name (specificity)	Approved use by FDA
Unconjugated antibodies		
Alemtuzumab	Campath (humanized anti-CD52)	Second-line treatment of chronic B-lymphocytic leukemia who failed fludarabine.
Bevacizumab	Avastin (humanized anti-VEGF)	Combined with intravenous 5-FU-based chemotherapy for first- or second-line therapy of colorectal cancers. Combined with carboplatin and paclitaxel for first line unresectable, locally advanced, recurrent or metastatic non-squamous, non-small cell lung cancer.
Cetuximab	Erbitux (chimeric anti-EGFR IgG)	Combined with irinotecan for metastatic colon cancer or alone in patients unable to tolerate irinotecan. Combined with radiation therapy in head and neck cancer (locally or regionally advanced) or as a single agent in patients with recurrent, or metastatic squamous carcinoma of head and neck who failed platinum-based therapy.
Panitumumab	Vectibix (human anti-EGFR IgG)	Second-line in patients who failed FOLFOX or FOLFIRI chemotherapy regimens.
Rituximab	Rituxan (chimeric anti-CD20 IgG)	Relapsed low-grade or follicular non-Hodgkin's lymphoma (NHL) first-line treatment follicular NHL in combination with CVP chemotherapy first-line treatment of diffuse large B-cell NHL in combination with CHOP or other anthracyclines-based chemotherapy; low-grade NHL with stable disease or who achieve a partial or complete response following CVP chemotherapy.
Trastuzumab	Herceptin (humanized anti-HER2 IgG)	Single agent in patients with metastatic breast cancer that overexpress HER2 and who have received one or more chemotherapy regimens; combined with doxorubicin, cyclophosphamide, and paclitaxel in an adjuvant setting for node positive breast cancer; combined with paclitaxel in patients with metastatic breast who have not received treatment for their metastatic disease.
Radiolabeled antibodies		
Ibritumomab tiuxetan	Zevalin (murine anti-CD20 IgG-DTPA conjugate; radiolabeled with ^{90}Y or ^{111}In; rituximab pre-dose)	Treatment of relapsed or refractory low-grade, follicular or transformed B-cell NHL.
Tositumomab	Bexxar (murine anti-CD20 IgG; radiolabeled with ^{131}I; tositumomab pre-dose)	Treatment of patients with refractory, low-grade, follicular, or transformed NHL, including patients with rituximab-refractory NHL.
Antibody-drug conjugate		
Gemtuzumab ozogamicin	Mylotarg (humanized anti-CD33 IgG colecheamicin)	Acute myeloid leukemia in first relapse and patients $\geqslant$ 60-year-old who are not candidates for other cytotoxic therapy.

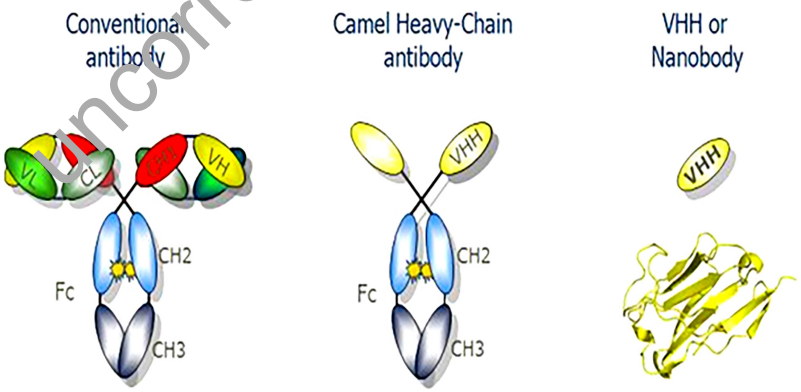

Fig. 5. Conventional antibody, camel heavy-chain antibody and nanobody.

have several advantages over the conventional antibodies (Table 4). They are derived from heavy chain antibodies from camelids, some of which only consist of two antibody heavy chains. With a molecular weight of only 12–15 kDa, nanobodies are much smaller than traditional IgG antibodies (150–160 kDa) which are conglomerates of two heavy protein chains and two light chains, intricately folded and garnished with elaborate sugars [40].

Nanobodies are having some unique advantages

Table 3
Nanobodies in trials (Data collected from clinical trials (https://clinicaltrials.gov/))

S.no	Clinical trials studies	Trail number	Phase
1	A phase II study to evaluate safety and efficacy of ALX-0061 in subjects with systemic lupus erythematosus.	NCT02437890	Phase 2
2	ALX-0171 phase I study, evaluating single ascending dose and multiple dose in healthy male volunteers.	NCT01483911	Phase 1
3	Study comparing the tolerability of three excipient formulations administered subcutaneously to healthy subjects.	NCT01401764	Phase 1
4	A single dose study of the safety and investigational product level measurement in healthy subjects.	NCT01284036	Phase 1
5	ALX-0061 phase I bioavailability study in healthy volunteers.	NCT02101073	Phase 1
6	ALX-0171 safety study in adults with hyperresponsive airways.	NCT01909843	Phase 1
7	ALX-0171 phase I pharmacokinetic study in healthy male volunteers.	NCT01875926	Phase 1
8	Study evaluating multiple ascending doses of ATN-103 In Japanese subjects with rheumatoid arthritis.	NCT01007175	Phase 1
9	Study evaluating multiple doses of ATN-103 in subjects with active rheumatoid arthritis.	NCT00959036	Phase 1
10	First in man study of ALX-0651, a nanobody inhibiting CXCR4.	NCT01374503	Phase 1
11	Study of single dose of ATN-103 administered to healthy Japanese male subjects.	NCT00916110	Phase 1
12	Comparative study of ALX-0081 versus GPIIb/IIIa inhibitor in high risk percutaneous coronary intervention (PCI) patients.	NCT01020383	Phase 2
13	Study evaluating long-term safety of ATN-103 in subjects with rheumatoid arthritis.	NCT01063803	Phase 2
14	Phase III trial with Caplacizumab in patients with acquired thrombotic thrombocytopenic purpura.	NCT02553317	Phase 3
15	Bioequivalence of liquid and reconstituted lyophilized subcutaneous formulations of Caplacizumab.	NCT02189733	Phase 1
16	A Phase IIb study for ALX-0061 monotherapy in subjects with rheumatoid arthritis.	NCT02287922	Phase 2
17	Study to assess efficacy and safety of anti-von Willebrand factor nanobody in patients with acquired thrombotic thrombocytopenic purpura (TTP).	NCT01151423	Phase 2
18	Follow-up study for patients who completed study ALX0681-C301 (post-HERCULES).	NCT02878603	Phase 3
19	A multicenter study in otherwise healthy infants and toddlers hospitalized for and diagnosed with RSV lower respiratory tract infection to evaluate the safety, tolerability and clinical activity of ALX-0171.	NCT02309320	Phase 1
20	A dose-range finding study for ALX-0061 combination therapy in subjects with rheumatoid arthritis.	NCT02309359	Phase 2
21	An extension study assessing the long-term efficacy and safety of subcutaneous ALX-0061 in subjects with rheumatoid arthritis.	NCT02518620	Phase 2
22	Caplacizumab single and multiple dose study in healthy Japanese and White subjects.	NCT03172208	Phase 1
23	Dose ranging study of ALX-0171 in infants hospitalized for respiratory syncytial virus lower respiratory tract infection.	NCT02979431	Phase 2
24	Study to assess safety and efficacy of anti-interleukin 6-receptor (IL6R) nanobody in rheumatoid arthritis (RA) patients.	NCT01284569	Phase 1

compared to conventional Abs that are important since they combine desirable features of mAbs with some of the beneficial properties of small molecule drugs.

1. *High affinity.* Nbs in nanomolar or even picomolar range show affinities and can bind a broad range of epitopes. This shows that the affinities of Nbs are superior or comparable to those of conventional mAbs, reaching picomolar affinity constant range [41,42]. Nbs have evolved to show high affinity and specificity by presenting only three CDRs instead of six, occurring in conventional Abs. Nbs antigen-binding loops display a larger structural repertoire as reported by crystallographic studies. In addition, the CDR3 regions are usually longer than the mAb VH domains [43].
2. *Possibility of formatting or multimerization.* The small size and monomeric nature of Nbs, they can be engineered genetically to obtain modular blocks that can be fused to form a new construct which will enable multi-specificity and versatility. The bivalent and bispecific Nbs are very attractive formats for therapeutic purposes. The presence of bivalency allows Abs to bind to multimeric Ags with great avidity [44] and bispecificity enables Nbs to simultaneously bind to two different types of Ags [45,46]. With the help of molecular engineering, these (Nbs) types of immunoreagents can be produced in most cases, rendering chimeric Abs. This unique option is particularly attractive for therapeutic applications and to extend the half-life of these immunoreagents.
3. *High stability and solubility.* Nanobodies show optimal biochemical and biophysical properties including thermal tolerance, solubility and proteolytic resistance. High solubility in aqueous solutions and lack of aggregation of Nbs is provided by the folding of CDR3 loop and hydrophilic

Table 4
Properties, advantages and applications of nanobodies

Nanobody properties	Advantages	Application areas
Small size	Rapid blood clearance and fast tissue penetration.	Selective tumor targeting. Selective *in vivo* imaging. Toxin neutralization.
Increased hydrophilicity	Allows for high expression yields. High solubility. Proteolytic stability.	Easy production. Intrabody production. Oral immunotherapy.
Single-domain nature	Absence of domain mispairing that allows for increased functional size of immune libraries.	Single-cell oligoclonal production.
	Allows for flexible linker design and simultaneous binding of multivalent agents.	Easy production of bispecific or trivalent forms.
	Production in microbial systems, taking advantage of simple fermentation conditions, and offering short development times.	Cost-effective large-scale production.
	Facile genetic manipulation to yield fused moieties with high solubility and low aggregation potential (decreased immunogenicity).	Selective tumor targeting. Selective *in vivo* imaging.
High sequence stability	Allows for a variety of treatments, storage and formulations.	Harsh engineering.
	Low immunogenic potential that allows for repeated or high-dose treatments.	Harsh engineering. Optimized drug manufacturing.
Extended CDR3 loops	Efficient refolding after chemical or thermal denaturation that gives high thermodynamic stability.	Harsh engineering. Optimized drug manufacturing. Oral immunotherapy.
	Recognition of recessed antigenic sites that allow for enzyme inhibition and recognition of cryptic epitopes.	Fast *in vivo* or *in vitro* diagnosis of infection. Research on biochemical mechanisms.
Variable N-terminal of CDR1	Transmigration of blood-brain barrier.	Treatment of neurological disorders.

Abbreviation: CDR, complementarity determining region.

content of frame work-2 region [47]. In addition, these properties also impart high conformational and thermal stability to Nbs. As a result, Nbs keep full binding capacities after 1 week at 37°C [48] and thermal unfolding behavior often has been described as completely reversible after long incubation periods at 90°C [49]. Nbs also show stability in the presence of proteases and chaotropic agents as well as at extreme pH [50]. Moreover, they can be customized to circumvent intracellular proteolysis. This property has led to development of intrabodies which are recombinant Ab fragments or Nbs which have been intracellularly expressed to target intracellular proteins [51]. The stability of Nbs under extreme conditions and their proteolytic resistance makes them suitable for use in alternative routes of administration (i.e., oral administration). Regarding the use of Nbs for diagnostic purposes, the high stability of Nbs at high organic solvent concentrations or at extremes of pH or temperatures opens a wide range of applications for the detection of small molecules or proteins [52].

4. *Deep, fast tissue penetration and rapid blood clearance.* The feature of fast tissue penetrability of Nbs can be very useful for targeting tumors, given that Nbs can be designed to modify or neutralize oncogenic molecules, redirecting them to a specific cellular compartment or expressing them on the tumor cell surface to trigger an immune response [53]. Several methods have been developed to increase the half-life of hcAbs as therapy usually requires high doses and frequent administration of the specific therapeutic agent. These include the addition of polyethylene glycol, conjugation to albumin or fusion to an Fc fragment of a conventional Ab [54]. Nevertheless, it is worth mentioning that for some specific applications this rapid renal clearance that characterizes Nbs may have its advantages, avoiding toxicity effects. Moreover, great expectation has been placed in the capability of Nbs to cross the blood-brain barrier (BBB) owing to their small size.
5. *Recognition of hidden epitopes.* In most cases Ag-binding surface of conventional Abs is flat or concave as revealed by Crystallographic studies [55]. Conversely, Nbs often bind into clefts and cavities [56]. The lack of variable light chain (VL) is balanced with a VHH region that shows an extended CDR1 and a more exposed CDR3. These structural changes allow standard binding architectures such as planar surfaces and cavities but also include the possibility of binding pro-

truding loops or clefts [57]. Therefore, this feature of Nbs and their smaller size promote the interaction with novel epitopes that are inaccessible to conventional VH–VL pairs, and also explain the ability of Nbs to bind and neutralize targets that are notoriously difficult to hit with conventional Abs.

6. *Immunogenicity.* The VHH domains of camelidae show great degree of homology with the VH domains of humans as a result of which they have not shown any unexpected immunogenicity reactions, which can be a potential concern for clinical applications [16]. Nevertheless, and just in case of long therapeutic treatments, it is a safe option to first humanize the framework-2 region to have available an amino acid sequence showing high degree of homology compared to human VHs. By straightforward site-directed mutagenesis the humanization of Nbs can be performed, provided that the gene is of approximately 380 base pairs [58].
7. *Production costs.* The problem of production costs of mAbs can be solved by taking nanobodies as an alternative. In microbial systems (such as bacteria, yeasts, fungi) Nbs can be easily expressed. Since immune-specificity is fully restored in Nbs and are thus rapidly selected from display libraries [59]. Moreover, they are particularly manageable for high-throughput screenings by using sequencing technologies, for example. All of these production and selection advantages result in lower manufacturing prices [60].

2.2. Generation of nanobodies

The field of recombinant antibody technology has rapidly progressed during the last two decades, mainly because of the interest in their human therapeutic use. The ability to select specific human antibodies by display technologies and to improve their affinity, stability and expression level by molecular evolution has further boosted the field. The antibodies are complex molecules that consist of heavy and light chains [61,62]. They contain an N-linked oligosaccharide attached to the second heavy-chain constant domain (CH2) that is essential for antibody effector functions such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytolysis (CDC) and for retaining a long serum half-life. Although isolated antibody heavy and light chains can retain antigen-binding specificity, their affinity and solubility is often reduced [63]. However, the paired N-terminal variable domains of heavy (VH) and light (VL) chains are sufficient for antigen binding [64]. Such antibody fragments can be produced as monovalent antibody fragment (Fab) or as single-chain Fv (scFv) where the VH and VL domains are joined by a polypeptide linker. Figure 6 describes the different fragments of an antibody and their role in therapeutics.

Heavy chain antibodies which are devoid of light chains can easily be produced in animals of the Camelidae family, among which camels are the most appropriate choice since they exhibit better ratio of heavy chain antibodies to regular antibodies. In reality, llamas are also often used for this purpose as they are easier to raise and breed due to smaller size [65,66]. Generally, an animal is immunized with appropriate quantity of antigen once a week for several weeks to raise an H2-type antibody response. The VHH gene fragments from lymphocytes are amplified by standard molecular biology techniques such as reverse transcription followed by polymerase chain reaction (RT-PCR), ligated in phage display vectors and transformed in bacteria to construct an immune VHH library [67–69]. Sometimes it is essential to generate VHHs that are cross-reactive for both human and rodent analogs of the target antigen in order to facilitate pre-clinical testing prior to clinical translation [65]. When a library of appropriate size is obtained, antigen-binding VHH fragments are selected from the library. Selection of nanobodies involves enrichment of antigen-specific binders from immune or naive libraries by phage display, bacterial two-hybrid or bacterial surface display and subsequent screening for individual colonies from enriched phage populations by enzyme-linked immune sorbent assays (ELISA) [67]. The detailed procedures for these processes have been well-documented in the literature [65,67,70]. In the following sections, we will discuss the innovative applications of nanobodies as potential next-generation molecular imaging agents.

Their production in microbial cells is often cumbersome, especially when producing multivalent formats, because of the requirement for domain association. The discovery that camelids (Bactrian camels, dromedaries and llamas) produce functional antibodies devoid of light chains, formed a further breakthrough because their single N-terminal domain (VHH, also referred to as Nanobody) binds antigen without requiring domain pairing [71]. These heavy-chain antibodies also lack the CH1 domain, which in a conventional antibody associates with the light chain and to a lesser degree interacts with the VH domain. Al-

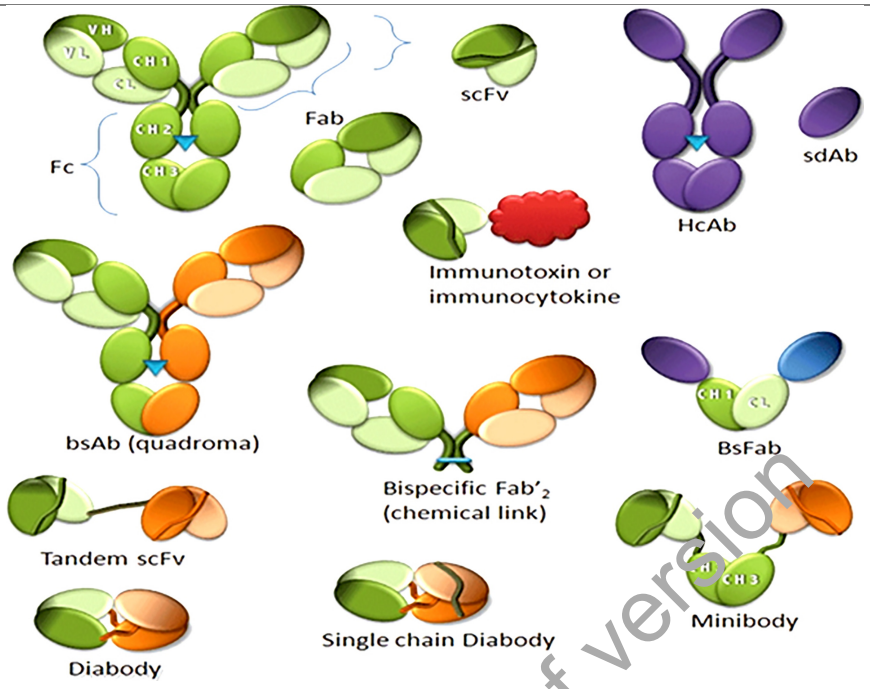

Fig. 6. Antibody fragments with therapeutic potential. A conventional antibody is depicted in green (light for light chain, dark for heavy chain, blue triangle indicates the glycosylation site) and the derived fragments (shaded areas represent the binding sites). The orange color symbolizes a different specificity. HcAb from camelids and their fragments (sdAb for single-domain antibodies) are depicted in mauve or blue. The red molecule represents a cytokine or a toxin. bsAb, bispecific antibodies; bsFab, bispecific Fab fragment; HcAb, heavy chain only antibodies.

though single-domain antibodies later were also identified in particular cartilaginous fish [72], most research on the biotechnological application of single-domain antibodies was done using camelids because of their ease of handling including immunization. Methods to isolate antigen-specific VHHs from immune [48,73], nonimmune [74–76] or semisynthetic libraries using phage, yeast or ribosome display are now well established [77].

Most antibodies are composed of two heavy and two light chains. Both chains contribute to the two identical antigen-binding sites which are usually flat or concave. In addition to these conventional antibodies, camelids and sharks also produce unusual antibodies composed only of heavy chains [50,78]. These peculiar heavy chain antibodies (hcAbs) lack light chains (in the case of camelid antibodies, also the CH1-domain). Therefore, the antigen-binding site of hcAbs is formed only by a single domain that is linked directly via a hinge region to the Fc-domain. The variable domain is designated VHH for camelid hcAbs and VNAR for shark hcAbs and more generally nanobody or single domain antibody (sdAb) [79]. The CDR3 region of these hcAbs possesses the extraordinary capacity to form long finger like extensions that can extend into cavities on antigens, e.g., the active site crevice of enzymes. The CDR3 of these sdAbs can form convex extensions that occupy the cleft for the substrate. In contrast, the Fab-fragment derived from a conventional mouse monoclonal antibody binds with a relatively flat interaction face, well outside the active site [80].

Indeed, the immune system of camelids seems to possess an inherent propensity for forming enzyme-blocking hcAbs [81]. The CDR3 of VHHs and VNARs is often much longer than that of conventional VH domains, e.g., 24 and 18 residues in the case of the illustrated anti-lysozyme sdAbs versus 7 residues in the mouse VH [70,82]. The extended CDR3 is often, but not always stabilized by an additional disulphide bond connecting the CDR3 to the adjacent CDR1 loop (common in camel and shark sdAbs) or to the CDR2 loop (common in llama sdAbs). The interface of conventional VH and VL domains is stabilized by hydrophobic interactions, the corresponding residues in llama antibodies are replaced by hydrophilic residues. In shark sdAbs, the CDR2 loop is replaced by two shorter variable loops. Molecular cloning and reformatting of camelid and shark sdAbs are usually generated by PCR cloning of the V-domain repertoire from blood, lymph node, or spleen cDNA obtained from immunized ani-

mals into a phage display vector, such as pHEN2 [70]. Antigen-specific sdAbs are commonly selected by panning phage libraries on immobilized antigen, e.g., antigen coated onto the plastic surface of a test tube, biotinylated antigens immobilized on Streptavidin beads, or membrane proteins expressed on the surface of cells. Several labs have also constructed semi-synthetic libraries by cassette-mutagenesis of the CDR regions. The latter offers the advantage of selecting antibodies against toxic or difficult to express antigens. However, sdAbs derived from such non-immune libraries often show lower affinities for their antigen than sdAbs derived from animals that have received several immunizations [70,83,84]. The high affinity of sdAbs from immune libraries is attributed to the natural selection of variant sdAbs during clonal expansion of B-cells in the lymphoid organs of the immunized animals. The affinity of sdAbs from non-immune libraries can often be improved by mimicking this strategy *in vitro* i.e., by site directed mutagenesis of the CDR regions and further rounds of panning on immobilized antigen under conditions of increased stringency (higher temperature, high or low salt concentration, high or low pH and low antigen concentrations). SdAbs derived from camelid and shark hcAbs are readily expressed in and purified from the *E. coli* periplasm at much higher expression levels than the corresponding domains of conventional antibodies. SdAbs generally display high solubility and stability and can also be readily produced in yeast, plant and mammalian cells [70].

Recombinantly expressed sdAbs display several advantages as compared to conventional antibodies and the single chain variable fragments (scFv) derived from the V-domains of conventional antibodies. Their high thermal stability, high refolding capacity and good tissue penetration *in vivo* make nanobodies ideally suited for various biotechnological and therapeutic applications [78,85,86]. Moreover, sdAbs can be readily cloned into various formats by fusion to other proteins or peptides, thereby tailoring their utility for certain diagnostic and/or therapeutic applications. For example, fusion to a fluorescent protein yields a fluorescent probe (also designated chromobody or fluobody) suitable for tracking the target antigen in cells [87,88]. Tandem cloning of two identical sdAbs connected by a linker peptide yields a bivalent reagent with higher avidity for the antigen [45]. Tandem cloning to an sdAb with a distinct specificity, e.g., for serum albumin, can help target the reagent to a particular compartment and/or help to increase the *in vivo* half-life of the reagent [89]. A bivalent hcAb can be reconstituted by genetic fusion to the Fc-domain of any conventional antibody e.g., mouse or human IgG1. Fusion to a cell surface protein e.g., of lactobacillus, a commensal gut bacterium yields a reagent designated lactobody with a massively increased binding surface for the antigen of interest e.g., rotavirus, the etiological agent of severe diarrhoea in children [90]. Owing to their high stability and excellent folding capacity under different environmental conditions, sdAbs have also been targeted successfully to the cytosol by removing the leader peptide [91] and to intracellular vesicles, such as chloroplasts [92] or the endoplasmic reticulum by genetic fusion to suitable targeting sequences [93].

2.3. General applications of nanoantibodies

The distinguishing physicochemical and pharmacological properties of camelid sdAb (nanobodies) fragments make them as new-generation therapeutic agents for the treatment of several diseases [94]. Nanobodies can bind into cavities on protein surfaces efficiently [95]. Moreover, nanobodies are able to express in intracellular spaces as "intrabodies" to target intracellular antigens [96,97]. The unique properties of nanobodies including the structural stability in harsh conditions for the therapy of gastrointestinal diseases using oral administration, easy engineering of multivalent or multi-specific formats of sdAb and transmigration across the blood-brain barrier make them useful instrument for developing selective and efficient therapeutic agents [98–100].

Researchers have recently developed and characterized several sdAbs targeting various antigens [101–107]. In a study, Vuchelen et al. studied the NMR projects of a new Nb, NbSyn2, which recognizes the C-terminus of human alpha-synuclein (aS) that its abnormal self-association is related in Parkinson's disease [95]. SdAb fragments (Nanobodies) derived from hcAbs (Camelid and Shark) can be used as crystallography chaperones for stabilization of the protein conformations during crystallization trials [108]. Immunoaffinity chromatography is another potential area for sdAb fragment application due to the fact, that sdAb fragments are highly resistant to effects of the organic solvents than that present in the elution buffer [109]. In addition to this, the development of humanized sdAb fragments have been described for decreasing their immunogenicity risk [58,110]. In a study, Van de Broek et al. designed branched gold nanoparticles and biofunctionalized them with anti-HER2 Nb [98]. Nanobodies can be used as

anti-toxin agents, anti-venom agents, anti-virus agents, anti-bacterial agents, anti-parasitic agents, anti-inflammatory agents, immune modulating agents, anti-cancer agents, anti-fungal agents, anti-haptens and diagnostic and biosensor tools. Here we will discuss them in greater detail.

2.3.1. Nanobodies as anti-toxin

The first Nobel Prize in Medicine was awarded in 1901 to Emil von Behring for his discovery of passive immune therapy as a cure for diphtheria, a devastating epidemic disease in those days. The molecular structure of antibodies, the effective agent in the diphtheria antiserum that von Behring obtained from immunized horses, was solved only half a century later. Today another half century later, we have the tools for cloning and reformatting antibodies from different animals and even for assembling antibodies semi-synthetically. sdAbs, in particular, hold great promise to fulfill Paul Ehrlich's vision of "magic bullets" for combating toxins and pathogens [111,112]. Some of the most potent toxins found in nature are enzymes including numerous proteins secreted by bacteria and the venomous glands of snakes and scorpions [113].

Botulinum neurotoxin, a protein produced by *Clostridium botulinum*, is known to be the most toxic substance in existence and is 15,000 times and 100,000 times more toxic by weight than the nerve gases VX and sarin respectively both of which are considered to be weapons of mass destruction [114]. Due to botulinum neurotoxin's extreme toxicity and ease of production, its potential use as a biothreat agent is a legitimate threat and has led to the toxin's designation as a Category A risk agent by the CDC. Single-domain antibodies (sdAb) specific for botulinum neurotoxin serotype A (BoNT A) were selected from an immune llama phage display library derived from a llama that was immunized with BoNT A toxoid. Sandwich assays that incorporated selected sdAb as capture and tracer elements demonstrated that all of the sdAb were able to recognize soluble ("live") BoNT A Tx and BoNT Ac Tx with virtually no cross-reactivity with other BoNT serotypes. These high-affinity binders may prove beneficial to the development of recombinant reagents for the rapid detection of BoNT A, particularly in field screening and monitoring applications [114].

Other toxin-specific sdAbs have been isolated from immune llama phage display libraries. Like murine ART2.2, the SpvB Salmonella toxin is an ADP-ribosyltransferase that transfers ADP-ribose from NAD onto specific amino acid side chains of proteins [82,86]. Upon translocation into the cytosol of animal cells, SpvB blocks cytoskeletal functions by ADP-ribosylating actin and thereby blocking its polymerization. When expressed as intrabodies in transfected cells, these nanobodies effectively block SpvB-mediated disruption of the cell cytoskeleton. Several other important human pathogens, including Vibrio cholera, Bordetella pertussis and Salmonella enteric, which induce disease by secreting toxin ARTs might similarly be suitable for targeting by enzyme-blocking sdAbs [115]. Bacterial toxin-specific sdAbs have been successfully isolated from naive llama and naive shark libraries, including sdAbs specific for cholera toxin [104], staphylococcal enterotoxin B and botulinum neurotoxin A [116,117]. It is likely that many of these sdAbs possess only low affinities and their neutralization capacities have not been reported. Notwithstanding, some have been shown useful for diagnostic purposes e.g., upon conversion to a multivalent format by the attachment to beads. These sdAbs thus hold promise for biosensor applications. Endotoxin, i.e., lipopolysaccharide (LPS), released from the outer membrane of gram-negative bacteria into the blood circulation after infection can mediate severe septic shock. LPS-specific sdAbs, selected from a non-immune llama VHH phage display library, effectively removed LPS from serum and other aqueous solutions [118].

2.3.2. Nanobodies as anti-venom agents

Venom of snakes, scorpions, spiders and frogs represent significant threats to human health in many countries. Intravenous administration of conventional IgG or Fab fragments prepared from immunized horses or sheep can provide alleviation for some cases of systemic envenoming and recent studies indicate that corresponding hcAbs prepared from immunized camels and llamas are similarly or even more effective [119–121]. An sdAb against alpha cobra toxin was derived from a naive llama phage display library and engineered into a pentameric format by genetic fusion to the non-toxic B-subunit of verotoxin, yielding a reagent with higher avidity and neutralization capacity [122]. An sdAb directed against scorpion toxin (NbAahI′22) derived from an immunized dromedary showed potent neutralizing capacity already in a monovalent format which was enhanced further upon conversion into a bivalent heavy-chain format by genetic fusion to the Fc region of human IgG1 [123].

2.3.3. Nanobodies as anti-virus

The sdAbs have demonstrated their application also

in the treatment of viral diseases. Numerous viruses are being targeted by sdAbs, among these is HIV, for which cross-type specific HIV-neutralizing sdAbs were recently derived from an immunized llama [124]. Several groups have succeeded in raising sdAbs directed against rotavirus, the causative agent of severe childhood diarrhoea. Immunization of a llama with a more internally located capsid protein of rotavirus yielded sdAbs that recognize and neutralize several different strains of rotavirus [125]. In a mouse model, oral application of these sdAbs provided significant amelioration of diarrhoea. In an elegant approach to increase the local dose of rotavirus-absorbing sdAbs, a rotavirus-specific sdAb derived from an immunized camel was cloned in-frame with a lactobacillus cell surface protease into a lactobacillus expression vector [90]. Transformation yielded recombinant versions of this commensal gut bacterium that express a high density of rotavirus-specific sdAbs on the cell surface (dubbed lactobodies). Feeding of the recombinant lactobacillus also provided significant protection from rotavirus-mediated disease.

Foot and mouth disease virus (FMDV) neutralizing sdAbs were isolated from a llama immunized with a crude extract of FMDV-infected BHK cells [126]. The *in vivo* half-life of these sdAbs was enhanced by genetic fusion to a second VHH specific for pig IgG. Intramuscular administration of these bispecific sdAbs significantly reduced viremia in subsequently infected piglets, but did not prevent transmission of FMDV [127]. Virus-neutralizing sdAbs have also been developed for industrial applications, for example, the neutralization of phages that infect lactobacilli and thereby present a significant threat in the milk and cheese industry [128–130]. sdAbs have been generated and applied successfully also against intracellular viral proteins. Porcine endogenous retroviruses (PERV) reside within the pig genome and are capable of transfecting human cells *in vitro*, thereby presenting a potential hazard for xenotransplantation. An sdAb, isolated from a llama immunized with the 60 kDa Gag protein of porcine endogenous retrovirus [131] and expressed as an intrabody, effectively inhibited production of virus particles. Transgenic expression of such sdAbs could contribute to solving safety problems associated with PERVs. Similarly, an sdAb derived from a llama immunized with hepatitis B virus particles was converted into a format targeted to the lumen of the endoplasmic reticulum (ER) by fusion to the KDEL ER-retention signal. Cells co-transfected with expression constructs for this sdAb reagent and hepatitis B showed enhanced intracellular retention and correspondingly diminished secretion of hepatitis B particles. In mouse models, the rapid intravenous bolus injection of an expression vector for the hepatitis-specific sdAb in a large volume of buffer resulted in efficient hydrodynamic transfection of liver cells and a therapeutic benefit [93]. Four sdAbs were selected from a non-immune llama library by panning against immobilized Marburg virus [132]. Even though these sdAbs from a naive library displayed only low affinities, a sensitive diagnostic assay system could be developed by converting the nucleoprotein-specific sdAbs into multivalent formats. Capture of the virus was achieved by simple immobilization of the sdAbs on the wells of an ELISA plate. Detection was achieved upon genetic fusion to bacterial alkaline phosphatase which forms homo-dimers, thus yielding a bivalent enzyme-linked detection reagent. The assay allows detection of three Marburg virus variants without cross reactivity to the four Ebola virus species.

2.3.4. Nanobodies as anti-bacteria

Nanobodies targeting pathogenic bacteria have been developed and with the goals of blocking the attachment of bacteria to host cells and/or for more effective delivery of prodrugs. For example, a VHH (K609) against *E. coli* F4 fimbriae, applied at high doses, reduced *E. coli* induced diarrhoea in piglets [133]. Using a DNA-shuffling strategy involving the random recombination of gene segments obtained from VHH K609 and two other F4-specific VHHs, following DNA fragmentation and PCR amplification, a variant VHH (K922) was selected that exhibited $>$ 100-fold increase in resistance to *in vitro* degradation by gastric and jejunal fluid.

Streptococcus mutans, the main cause of dental caries, achieves virulence in part via its ability to adhere to and to accumulate on tooth surfaces. An sdAb (S36-VHH) was developed from a llama immunized with a mixture of disrupted and intact *Streptococcus mutans* and genetically fused to fungal glucose oxidase [134]. In a rat model of strong cariogenic challenge, daily application of the sdAb monomer as well as the VHH-glucose oxidase fusion protein resulted in a reduced level of bacterial recovery 21 days after infection [135].

The sdAbs have also been used successfully to improve the probability of obtaining crystals for proteins that are difficult to crystallize. A sdAb can assist in the crystallization process by stabilizing the protein of interest and/or by creating additional crystal contact

surface. VHHs isolated from immunized llamas were used to assist the crystallization of major components of the type 2 secretion system of enteropathogenic *E. coli* and *Vibrio*. This 11 component extracellular protein secretion system is used for secretion of cholera toxin and heat labile enterotoxin [136,137]. Similarly, the phage P2 receptor-binding protein was successfully co-crystallized with an sdAb [138].

Many bacteria secrete enzymes that inactivate antibiotics as an efficient escape mechanism from antibiotic therapy. Enzyme-blocking sdAbs were generated from immunized dromedaries that effectively block β-lactamases which catalyze the hydrolysis of β-lactam antibiotics with the potential utility of improving antibiotic therapies [56]. Recent research has led to the efficient production of nanobodies against urease activity of *Helicobacter pylori* in Pichia pastoris [139,140]. *H. pylori* colonizes human gastric mucosa and causes gastritis, duodenal ulcers and even gastric cancer [141]. Treatment of *H. pylori* infection with antibiotics leads to increased risk of antibiotic resistance. The high cost of available treatment measures and the increased number of reported relapses generate the need for new alternative therapeutic approaches [142]. Urease enzyme is an important virulence factor since it allows for survival under acidic conditions and the possibility of *H. pylori* colonization [143,144]. The UreC subunit of urease enzyme exists in catalytic sites, showing great vaccine potential and antibody development for treatment of *H. pylori* infection [142,145]. Since mAbs are not very stable and poorly immunogenic, the development of a new class of antibodies seems necessary [146]. Previously, VHH nanobody against UreC recombinant protein was produced using an *E. coli* host. For better expression and posttranslational modifications, VHH with high affinity and specificity was produced in *P. pastoris* against UreC. The results suggest attribution of the enhanced quality and quantity of the nanobody produced in *P. pastoris* to better posttranslational modification and folding in the yeast cell. The findings suggest that nanobody produced in yeast against the UreC subunit of *H. pylori* is specifically successful in inhibition of *H. pylori* infection. Thus, the nanobody produced could lead to a therapeutic and prophylactic approach in the management of *H. pylori-associated disease* and should be further investigated [147].

2.3.5. Nanobodies as anti-parasite

Targeting parasites with Single domain antibodies (sdAbs) are also being developed as anti-parasite reagents. Trypanosomes are coated with variable surface glycoproteins (VSGs) and evade host immune responses by changing the VSGs that cover the entire membrane. Owing to their smaller size, sdAbs might better penetrate the VSG coat to gain access to conserved epitopes that are inaccessible for conventional antibodies. An sdAb (NbAn33) recognizing an oligomannose cryptic epitope on trypanosomes was isolated from a dromedary immunized with purified VSG [148]. In contrast to conventional antibodies and lectins, this sdAb was able to penetrate the dense VSG coat. A fluorochrome-conjugate of this sdAb allowed the direct sensitive detection of parasites in blood smears. Immunotoxins were generated by genetically fusing this sdAb (An33) to either β-lactamase or a truncated version of the pore forming human serum protein apolipoprotein L-I. Fusion to β-lactamase, which can convert a cephalosporin-based prodrug into the potent toxic phenylenediamine mustard (PDM), yielded a conjugate with both, retained capacity to bind to trypanosomes and retained enzyme activity. Sub-lethal doses of the pro-drug proved highly toxic to conjugate treated parasites. Genetic fusion to a truncated form of human apolipoprotein L-I yielded an immunotoxin with lytic activity against a range of different trypanosomes [149]. In a mouse model the VHH-apoL-I fusion protein had curative and alleviating effects on acute and chronic infections with trypanosomes. Further sdAbs directed against VSG and other trypanosome proteins were selected from an immune library obtained from a dromedary infected with live trypanosomes [150]. sdAbs directed against trans-sialidase from *Trypanosoma cruzi* have also been obtained and characterized [151]. Even though these sdAbs are unable to interfere with the crucial role of trans-sialidase in cell entry of the parasite, they served as a tool to unmask a novel strategy of *Trypanosoma cruzi* to evade the host immune system.

The larval form of the pork tapeworm *Taenia solium* is the cause of cysticercosis, the most common parasitic infection of the central nervous system and a major cause of epilepsy in developing countries. Immunization of two dromedaries with *Taenia solium* extracts yielded an sdAb (Nbsol52) recognizing the 14 kD diagnostic glycoprotein Ts14 [152]. This sdAb showed sub-nanomolar affinity for Ts14 and demonstrated capability of binding antigens present in the serum of infected pigs. Further studies are required to assess the potential therapeutic use of such sdAbs in cysticercosis.

The sdAbs have also been found to inhibit fungal infections. For example, *Malassezia furfur* is a fungus

implicated in dandruff and sdAbs specific for a cell wall protein of *M. furfur* were derived from an immunized llama [153]. Some of these sdAbs were found to be resistant to detergents in shampoo and may provide a possible antifungal additive to shampoos.

2.3.6. Nanobodies as anti-inflammatory and immune modulating agents

Nanobodies (sdAbs) also demonstrate potential in neutralizing soluble extracellular proteins including toxins, cytokines and blood clotting components. The capacity of sdAbs to block leukocyte ecto-enzymes indicates their potential utility as anti-inflammatory and immune modulating agents. Notwithstanding, a number of obstacles still have to be surmounted before widespread clinical application of sdAbs becomes possible. A major concern for repeated and/or long-term clinical use is the potential immunogenicity of sdAb reagents. While the systemic application of small monovalent sdAbs seems to induce little, if any, neutralizing antibody responses, protein immunogenicity generally increases with size and complexity. This will likely pertain also for the fusion constructs. Enhanced immunogenicity, of course, is desired in case of vaccines, but not for therapeutic applications [154].

Recently, a strategy to humanize camelid sdAb was described in which the surface of a camel sdAb was reshaped by mutating 12 out of the 14 surface-exposed residues in which camel sdAbs differ from human VH domains to the corresponding human residue [58]. It remains to be determined whether these humanized VHH display lower immunogenicity than the camelid sdAb. Immunogenicity is a particular concern for intravenous and other systemic injections, but may be less problematic for other routes of application, in particular gastrointestinal and intraocular injections. Moreover, single injections, such as for the treatment of acute toxin exposure, are less immunogenic than the repeated applications required for the treatment of chronic inflammatory diseases. For intracellular targets, more efficient delivery tools will need to be developed for sdAbs to reach the cytosol and other intracellular compartments.

Transfection-mediated expression of intracellular sdAbs appears to be a feasible strategy for cell/gene therapy regiments and for transgenic plants and animals. For humans, however, *in vivo* transfection so far seems feasible only for accessible tissues, such as skin and possibly the liver. Considering that antigen-binding of many camelid and shark sdAbs is mediated in large part by their long CDR3, it may be possible in some cases to develop peptide-mimetics of the antigen-binding principle [155]. Presently, the immunization of llamas and dromedaries is cumbersome and costly in comparison to immunization of smaller animals. Transgenic mice expressing camelid antibodies, analogous to the transgenic mice expressing human antibodies could provide more economic sources of sdAbs [94]. A recent study provided a first proof of principle that single-chain antibodies, indeed, can be correctly assembled and expressed by B-cells of transgenic mice carrying a mini-Ig construct encoding a dromedary VHH and the Fc-domain of human IgG1 [156,157]. Assuming that progress will continue at the present pace, it is likely that the future repertoire of researchers and clinicians will include a battery of sdAb reagents directed against cytokines, ecto-enzymes, tumor antigens, toxins, and microbes.

2.3.7. Nanobodies as anti-cancer agents

The nanobodies are not restricted to immune-modulation, but are also demonstrating potential in the treatment of cancer. For example, nanobody with sub-nanomolar affinity for the human tumor-associated carcinoembryonic antigen has been identified by researchers. This nanobody was conjugated to *Enterobacter cloacae* ß-lactamase, and its site-selective anti-cancer prodrug activation capacity was evaluated both *in vitro* and *in vivo* [158]. The *in vivo* studies demonstrated that the conjugate had an excellent biodistribution profile and induced regressions and cures of established tumor xenografts. The easy generation and manufacturing yield of nanobody-based conjugates together with their potent antitumor activity make nanobodies promising vehicles for new generation cancer therapeutics. Carcinoembryonic antigen (CEA) expression profile on cancer cells of epithelial origin, such as colorectal, lung, breast, and ovarian carcinoma makes it an attractive target for tumor therapy [158].

Critical factors of nanobodies in the development of effective conjugates for cancer therapy are their homogeneity, absence of regions prone to aggregation or susceptible to proteolysis, affinity and specificity, high solubility and stability. The hunt for the smallest antibody fragment still capable of binding to antigens has progressed from full antibody molecules to Fab and recombinant single-chain Fv fragments. These smaller molecules have improved tumor penetration, faster blood clearance, and reduced immunogenicity compared with the complete antibody. Despite their beneficial properties, single-chain fragment variables

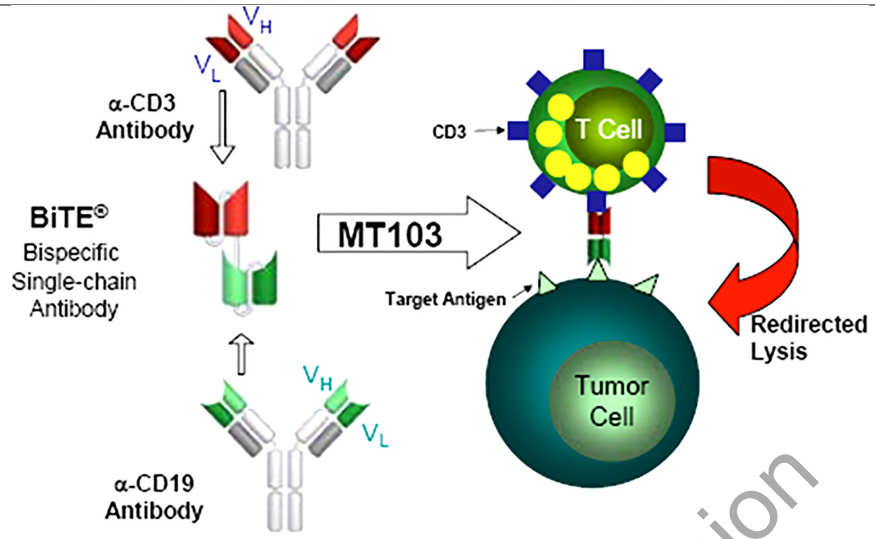

Fig. 7. The molecular mechanism of nanobody induced tumor cell lysis.

(scFv) and their conjugates are still amenable for improvement in terms of stability [159], expression yield, protease resistance and aggregation caused by synthetic linkers [160].

Moreover, nanobodies derived from camelids show high homology with the human VH3 gene family [161]. It may be demonstrated that cAb-Lys3, a nanobody that inhibits lysozyme activity *in vitro* [48], effectively targets tumours and metastatic lesions transgenic for hen egg lysozyme in a scid mouse model [85]. The isolation of nanobodies specific to CEA and their use as modular building units for the construction of immunoconjugates, using the hinge of llama heavy-chain antibodies as natural linker has also been reported. Conjugates are readily purified in high yields without aggregation or loss of functionality. Conjugate treatment in a targeting strategy known as antibody-dependent enzyme prodrug therapy effected immunologically specific cell kill and led to regressions and cure of established tumor xenografts (Fig. 7) [162]. Tumour vasculature and diffuse infiltrative growth in high grade gliomas limit the effectiveness of anti-angiogenic compounds. Therefore, additional destruction of existing tumour vasculature may be a promising alternative avenue to effectively deprive tumours from blood. This approach requires the identification of novel tumour vascular targeting agents, which have broad tumour vessel specificities, i.e. are not restricted to newly formed vessels. Here, the generation of a phage library displaying nanobodies that were cloned from lymphocytes of a llama which had been immunized with clinical glioma tissue is described. Recent research suggests that the generation of nanobody-displaying immune phage libraries and subsequent *in vivo* biopanning in appropriate animal models is a promising approach for the identification of novel vascular targeting agents [163].

2.3.8. *Nanobodies as anti-fungal agents*

Fungi secrete small chemical compounds that can be toxic to animal cells. Recently, an sdAb (NAT-267) was developed from an immunized llama against BSA-conjugated deoxynivalenol (300 Da), a mycotoxin produced by common grain mould [16]. The high affinity of this sdAb paves way for developing low-cost toxin detection systems and potentially also for immunomodulation of deoxynivalenol's cytotoxic effects.

2.3.9. *Nanobodies as anti-haptens*

Haptens have been targeted by sdAbs, including red azo dyes (728 Da) found in red wine (with the potential use of targeting enzymes to stains during washing), caffeine (with the potential use of detecting caffeine in beverages), the explosive TNT (biosensor) and the chemotherapeutic agent methotrexate (MTX, 454 Da) [165–168]. Anti-hapten sdAbs also demonstrate potential in cancer therapy for example, in a bi-specific format to help target radiolabelled haptens to tumours for imaging and radioimmunotherapy [169].

2.3.10. *Nanobodies in diagnostics and biosensor tools*

Approximately 15 years have passed since the

Table 5
Nanobodies based probes in pre-clinical molecular imaging

Target	Nanobody		Disease model
EGFR	8B6	SPECT (^{99m}Tc)	Human epidermoid carcinoma (A431), Human prostate carcinoma (DU145)
	7C12	SPECT (^{99m}Tc)	Human epidermoid carcinoma (A431)
	7D12	SPECT (^{99m}Tc) PET (^{68}Ga) Optical (IRDye800CW)	Human epidermoid carcinoma (A431)
HER-2	2Rs15d	SPECT (^{99m}Tc)	Human colon carcinoma (LS174T), human breast cancer (SKBR3), human ovarian cancer (SKOV3)
	2Rs15d	PET (^{68}Ga)	Human ovarian cancer (SKOV3)
	11A4	Optical (IRDye800CW)	Human breast cancer (SKBR3)
HGF	1E2 and 6E10	PET (^{89}Zr)	Human glioblastoma (U87 MG)
MMR	α-MMR	SPECT (^{99m}Tc)	Mammary adenocarcinoma (TS/A), Lewis lung carcinoma (3LL-R)
		SPECT (^{99m}Tc)	Rheumatoid arthritis
VCAM-1	cAbVCAM1-5	SPECT (^{99m}Tc) Ultrasound (microbubble)	Atherosclerosis (ApoE-deficient mice) Murine adenocarcinoma (MC38)
CEA	CEA1	SPECT (^{99m}Tc)	Human colon adenocarcinoma (LS174)

serendipitous discovery of the peculiar hcAbs in camels [40], followed by the elucidation of the similar, heavy-chain-only structure of shark new antigen receptors [154]. The remarkable proof of principle studies published since then point to a tremendous diagnostic and therapeutic potential of single domain antibody reagents derived from these hcAbs. The discovery of naturally occurring, heavy-chain only antibodies in camelidae and their further development into small recombinant nanobodies, presents attractive alternatives in drug delivery and imaging [170].

Nanobodies demonstrate most of the requisites of an ideal probe for successful molecular imaging. Owing to their small molecular size, they can easily reach their targets within a few hours post injection and exhibit great potential in tumor detection, confirmation of target expression, and selection of patients who have the highest chance to benefit from targeted therapies. Several imaging techniques such as SPECT, PET, optical imaging and ultrasound have been successfully employed for molecular imaging using nanobodies, with the majority using radionuclide-based techniques (i.e. SPECT and PET) since these techniques are sensitive, quantitative, and clinically relevant [171,172]. SPECT and PET have the sensitivity needed to visualize most interactions between physiological targets and ligands, which can enable non-invasive detection down to the picomolar level [172]. Table 5 lists the nanobodies employed and the detection techniques used in the study of diseases.

For SPECT imaging, the nanobody needs to be labelled with a suitable γ-emitting radionuclide (e.g. 99mTc or 111In), having an ideal γ-energy of 100–250 keV [173]. These γ-rays are recorded by the detectors of a dedicated γ-camera or SPECT instrument, which can be converted into an image upon signal processing to pinpoint the localization of the radiolabelled nanobody. On the other hand, PET requires radiolabelling of the nanobody with a suitable positron-emitting radionuclide (e.g. 18F, 64Cu, 68Ga or 89Zr) [173]. Compared with SPECT, PET has greater advantages with respect to sensitivity and resolution hence has been gaining significantly more clinical popularity over the last decade [174,175]. However, most of the literature reports on radionuclide-based imaging with nanobodies have used 99mTc for SPECT imaging. The widespread interest in the use of 99mTc is primarily due to its excellent nuclear decay characteristics, viable coordination chemistry for radiolabelling a wide variety of biomolecules and convenient availability from cost-effective 99Mo/99mTc generators [176]. Also, due to the presence of the hexahistidine tag on the nanobody, it can easily be radiolabelled with 99mTc (CO)3 without any chemical modification of the protein [177]. However, the hexahistidine tag on the nanobody poses some concerns as it might reduce immune responses and thus, the necessity for its removal has also been suggested [178]. The high operational cost of on-site cyclotrons needed for production of PET isotopes might be a deterrent for their use, which could be overcome with the use of 68Ga derived from 68Ge/68Ga generators [173,178].

Nanobodies raised against haptens using strong selection systems exhibit extremely high affinities that could be utilized in low-cost detection systems and in immunomodulation. Nanobodies have been shown to

bind to copper ions raising new possibilities for the development of redox protein-based detection systems, such as solid-state electrochemical sensing and without the need for mediators [165]. The ease of manipulation and the harsh engineering that nanobodies can sustain make them perfect candidates as biorecognition elements in biosensor platforms.

Biosensors, using natural recognition processes for detecting targets with extremely low detection limits, have enjoyed low marketability since they have an operational versatility and stability that is largely dictated by the biological moiety that is immobilized on top of the physicochemical transducer [179]. Nanobodies can confer their extended shelf-life and ruggedness to biosensors, broadening their applicability to production control and environmental monitoring-two areas that (to date) only microorganisms can adequately serve, at the expense of selectivity, sensitivity and specificity. The ability of Nanobodies to recognize cryptic epitopes may open new avenues for environmental detection, especially in assessing pollution impact at the cellular or subcellular level, or even when elucidating the mechanisms of metabolic degradation and assimilation of endocrine disruptors or of accumulating pesticides in sensitive ecosystems [180]. The development of immunosensors using conventional Ab fragments has been proven to be challenging due to the low affinities expressed in the presence of binders, but the nanobodies that can be accommodated in the nanometre space may certainly enhance the simultaneous detection of many compounds with a single instrument [180]. The high solubility and low aggregation potential of nanobodies could reduce the use of reagents during measurements while in situ regeneration of the sensor might be further facilitated for uses in long-term monitoring [181].

In the post-genomic era and with the high-throughput techniques available, the goal is to discriminate between all individual proteins from the proteome, including their splice variants and post-translationally modified derivatives [150]. Nanobodies may offer the means to attain this goal. More nature-mimicking surfaces, such as lipid bilayers may provide a more responsive host to Nbs and extend the range of applications in clinical diagnosis and environmental monitoring, where Nbs could be proven advantageous for maximizing protein loading to lower detection limits, reducing steric hindrance to lower noise levels and optimizing dipolar and transmembrane potentials to increase sensitivity [71].

3. Conclusion

The discovery of naturally occurring, heavy-chain only antibodies in Camelidae and their further development into small recombinant nanobodies, presents attractive alternatives in therapeutics, drug delivery and imaging techniques. They are easily expressed in microorganisms and amenable to engineering. Nanobody derivatives are stable, soluble and versatile having reduced aggregation tendencies, unique refolding capacities and high-target binding capabilities. These unique physicochemical and pharmacokinetic properties of nanobodies match the requirements of many biomedical applications and offer several advantages over conventional antibody (Ab) technology for immunotherapy, drug delivery and diagnostics. Nanobodies provide a rich pool for intracellular signalling molecules, protein-protein interactions and cancer biomarkers. Nanobodies can be fused with fluorescent proteins to produce chromobodies that may be utilized in single-molecule localization with super-resolution imaging techniques. Since they can be engineered to induce conformation changes or to discriminate between conformational variants, nanobodies may prove to be a beneficial research tool for monitoring protein expression, translocation and subcellular localization. Nature-derived nanobodies represent a beneficial tool in Ab-based markets with ready-to-use potential. Numerous Nanobodies have already proven useful for basic and advanced research in therapy and diagnostics. *In vitro* and *in vivo* studies have shown, at least in principle, deep penetration into dense tissues, favourable kinetics for drug delivery, recognition of hidden epitopes, enhanced thermodynamic stability and rapid elimination.

Future studies in humans will be required to confirm their performance, benefits and their efficacy in cancer immunodiagnostics and/or therapy. Bringing to the field, nanobodies with specificities to other targets of biological relevance will be another future challenge, not only to address fundamental understandings about their molecular characteristics and structural stabilities, but also to position them as clinically useful pharmaceuticals. Notwithstanding, a number of obstacles including immunogenicity and functionalization still have to be clinically proven and/or surmounted before clinical implementation becomes feasible. But the number of possibilities and prospects for therapeutic and diagnosis use of nanobodies are numerous with the technology at hand. Therefore. the ability to tailor properties and pharmacokinetics with multivalent and

fused constructs to suit the needs of the intended use places nanobodies are at the forefront of biomedical applications.

Acknowledgments

The authors are thankful to University Grants Commission (UGC) Govt. of India New Delhi and University of Kashmir Srinagar. Mr. Umar Mehraj is the recipient of Junior Research Fellowship (JRF) from UGC-CSIR Govt. of India, New Delhi.

Conflict of interest

The author(s) declare no conflict of interest.

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