Review

Nanobodies - the new concept in antibody engineering

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Nanobodies are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally occurring heavy-chain antibodies. The Nanobody technology was originally developed following the discovery that *camelidae* (camels and llamas) possess fully functional antibodies that lack light chains. These heavy-chain antibodies contain a single variable domain (VHH) and two constant domains (CH_2 and CH_3). Importantly, the cloned and isolated VHH domain is a perfectly stable polypeptide harboring the full antigen-binding capacity of the original heavy-chain antibody. These newly discovered VHH domains with their unique structural and functional properties form the basis of a new generation of therapeutic antibodies which were named Nanobodies. The aim of this paper is to show the properties of Nanobodies, their production and expression, applications and their clinical status.

Key words: Nanobodies, *camelidae*, antibody engineering, Nanoclone.

INTRODUCTION

The mouse hybridoma technology described by Köhler and Milstein in 1975 was an important step in the development of antibody technology and paved the way for the emergence of therapeutic monoclonal antibodies (mAbs) (Köhler and Milstein, 1975). In 2005, 18 monoclonal antibody products were on the market and more than 100 in clinical trials; it was clear that engineered antibodies have become of age as biopharmaceuticals. In fact, by 2008, engineering antibodies were predicted to account for > 30% of all revenues in the biotechnology market showed in Table 1, which contain safety information on monoclonal antibody drugs already approved by the Food and Drug Administration (FDA) for clinic use, most of them posted on the FDA website http://www.fda.gov) (Holliger and Hudson, 2005; Pavlou and Beslsey, 2005; Reichert and Pavlou, 2004). Recently, smaller recombinant fragments, for example, classic monovalent antibody fragments (Fab, scFv and engineered variants; diabodies, triabodies, minibodies and single-domain antibodies) are now engineering as credible alternatives. These fragments retain the targeting specificity of whole mAbs but can be produced more economically and possess other unique and superior properties for a range of diagnostic and therapeutic applications (Holliger and Hudson, 2005).

Single-chain Fvs are a popular format in which the V_H and V_L domains are joined with a flexible polypeptide linker preventing dissociation. Antibody Fab and scFv fragments, comprising both V_H and V_L domains, usually retain the specific, monovalent, antigen binding affinity of the parent IgG, while showing improved pharmacokinetics for tissue penetration (Harmsen and De Haard, 2007; Holliger and Hudson, 2005). Interest was revived when it was discovered that at least two types of organisms the camelids (camels and llamas) and cartilaginous

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Abbreviations: HcAb's, Heavy-chain antibodies; **VHH,** variable domain of heavy-chain antibody; **mAbs,** monoclonal antibodies; **FDA,** food and drug administration; **Fab,** fragment-antigen binding; **Fc,** fragment crystalline; **scFv,** single-chain variable fragment; **V_H,** variable domain of the heavy chain; **V_L,** variable domain of the light chain; **IgG,** immunoglobulin class G; **V-NAR,** variable region of new or nurse shark antigen receptor; **Ag,** antigen; **CDR,** complementarity-determining regions; **CH,** constant heavy domain; **CEA,** carcinoembryonic antigen; **PSA,** prostate-specific antigen; **EGFR,** epidermal growth factor receptor; **Aah,** *Androctonus autralis hector* scorpion; **SPECT,** single photon emission computed tomography; **TNFα,** tumour necrosis factor; **NCC,** neurocysticercosis and **DARPins,** designed ankyrin repeat proteins.

Table 1. Therapeutic mAbs approved by FDA.

Brand/ Generic name	Ag specificity	mAb Type	Therapeutic category	Year
Orthoclone OKT3 (MuromonabCD3)	CD3	Murine	Transplant antirejection	1986
ReoProT (Abciximab)	Platelet gpIIb/IIIa	Chimeric	Prevents blood clotting	1994
Rituxan (Rituximab)	CD20	Chimeric	Non-Hoodgkins lymphoma	1997
Zenapax (Daclizumab)	IL-2 receptor	Humanized	Transplant immune suppression	1998
Simulect (Basiliximab)	IL-2 receptor	Chimeric	Novartis transplant	1998
Synagis (Palivizumab)	RSV	Humanized	Anti-respiratory syncytial virus	1998
Remicade (Infliximab)	TNF-α	Chimeric	Contocor anti arthritis	1998
Herceptin (Transtuzumab)	Her-2	Humanized	Breast cancer	1998
MyLotarg (Gemtuzumab)	CD33	Humanized	Acute myloid leukemia	2000
Campath (Alemtuzumab)	CD52	Humanized	leukemia	2001
Zevalin (Ibritumomab tiuxetan)	CD20	Murine	B cell non-Hodgkin's lymphoma	2002
Xolair (Omalizumab)	IgE-Fc	Humanized	Allergic asthma	2002
Humira (Adalimumab)	TNF-α	Human	Arthritis treatment	2003
Bexxar (Tositomomab-l131)	CD20	Murine	B cell non-Hodgkin's lymphoma	2003
Raptiva (Efalizumab)	CD11a	Humanized	Psoriasis	2003
Erbitux (Cetuximab)	EGFR	Chimeric	Cancer therapy	2004
Avastin (Bevacizumab)	VEGF	Humanized	Colorectal cancer	2004
Tysabri (Natalizumab)	TNF-α	Humanized	Multiple sclerosis (MS)	2004
Lucentis (Ranibizumab)	VEGF	Humanized	Mascular degradation	2005
Lymphacide (Epratuzumab)	CD22	Humanized	Non-Hodgkin's lymphoma (NHL)	2005
Antegren (Natalizumab)	SAM	Humanized	Multiple Sclerosis MS	2005
HuMax-IL-15	IL-15	Human	Inflammation and Arthritis treatment	2007
ABT-874	IL-12	Human	Psoriasis	2007
CAT-213	Protein eatoxin 1	Human	Allergy	2008
CAT-192	TGF beta1	Human	Dye-sensitized solar cell (dssc)	2008
HuMaxCD4	CD4	Human	Cutaneous T-cell lymphoma (CTLC), Non-cutaneous T-cell lymphoma (NCTLC)	2008

CD, Cluster of Differentiation; IL, Interleukin; RSV, Respiratory Syncytial Virus; TNFα, Tumour Necrosis Factor; Her, Herceptin; EGFR, Epidermal Growth Factor Receptor; VEGF, Vascular Endothelial Growth Factor; SAM, Selective Adhesion Molecule and TGF, Transforming Growth Factor.

fish (Wobbegong and nurse sharks), have evolved high affinity single V-like domains called VHH in camelids or Nanobody (Figure 1b) and V-NAR (Figure 1c) in sharks, mounted on an Fc equivalent constant domain framework as an integral and crucial component of their im-mune system (Hamers-Casterman et al., 1993; Harmsen and De Haard, 2007; Holliger and Hudson, 2005; Roux et al., 1998). Nanobodies are the smallest available intact antigen binding fragment, only 15 kDa, with 2.5 nm in diameter and ~ 4 nm in high (Cortez–Retamozo et al., 2004; Revets et al., 2005). Nanobodies possess significant advantages for biotechnological and medical applications. They are well expressed in micro-organisms and have a high stability and solubility. Furthermore, they are well suited for construction of larger molecules and selection systems such as a phage, yeast, or ribosome display (Harmsen and De Haard, 2007). This mini-review offers an overview of the properties of Nanobodies, their production, expression, applications and their clinical status.

STRUCTURE OF NANOBODIES

The conventional antibodies are made up of two identical heavy and two identical light chains held together by interchain disulfide bonds. In the most abundant type of antibody in circulation, immunoglobulin G, the exposed hinge region is extended in structure due to the high proline content and is therefore vulnerable to proteolytic attack; thus the molecule can be easily split in the labo-ratory using papain to yield two identical Fab fragments, each with a single combining site for antigen, and a third fragment, Fc, which lacks the ability to bind antigen (Roitt and Delves, 2001) (Figure 1a). The IgG antibodies from species of *camelidae* (that is *Camelus dromedarius, Camelus bactrianus, Lama glama, Lama guanaco, Lama alpaca* and *Lama vicugna*) form a surprising exception to this paradigm as their serum contains also a considerable fraction of heavy-chain antibodies (HcAbs). The HcAbs of *Camelidae* have a unique structure consisting of a single variable domain (VHH or Nanobody), a hinge region and
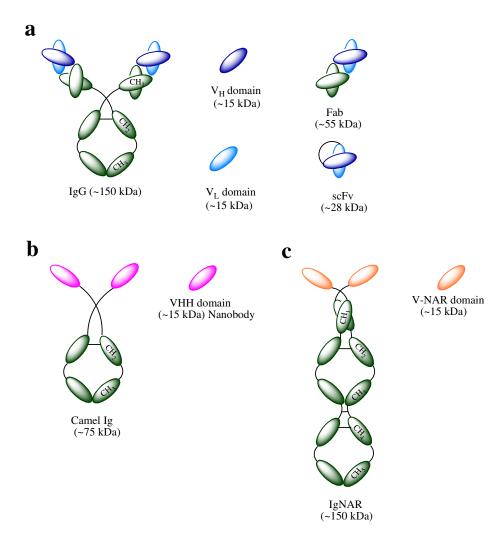
Figure 1. Schematic representation of a variety of antibodies and antibody fragments. a. The conventional antibody, containing two variable regions (each composed of a V_H and V_L domains) that confer antigen-binding specificity of antibody, and an Fc fragment in the constant region that recruits effecter functions of the immune system. b. The camelid HcAb is unusual immunoglobulin-like structure comprising a homodimiric pair of two chains of V-like domain (neither has light chain), in which the displayed V domain bind target independently. c. The shark Ig-NAR comprises a homodimer of one variable domain (V-NAR) and five C-like constant domains (C-NAR). (Sizes given in kilodaltons are approximate).

two constant domains (CH_2 and CH_3) (Figure 1b), the two constant domains are highly homologous to the Fc domains (CH_2-CH_3) of classical antibodies (Muyldermans et al., 2008). These HcAbs lack the first domain of the constant region (CH_1) which is present in the genome, but is spliced out during mRNA processing. The absence of the CH_1 domain explains the absence of the light chain in the HcAbs, at this domain is the anchoring place for the constant domain of the light chain. Consequently, HcAbs naturally evolved to confer antigen-binding specificity and high affinity by three CDRs from conventional antibodies or fragments thereof (Muyldermans, 2001; Revets et al., 2005).

SO WHAT ARE NANOBODIES?

Nanobodies are the smallest available intact antigen binding fragments, only 15 kDa, harboring the full antigen-binding capacity of the original heavy chain of naturally occurring heavy-chain antibodies that have evolved to be fully functional in the absence of light chains (Cortez-Retamozo et al., 2004; Revets et al., 2005). They were discovered in the blood of camels at the end of the 1980s by professor Raymond Hamers from the Vrije University of Brussel (Belgium) (De Haard, 2008). The crystal and solution structures of several Nanobodies have been solved and show that their
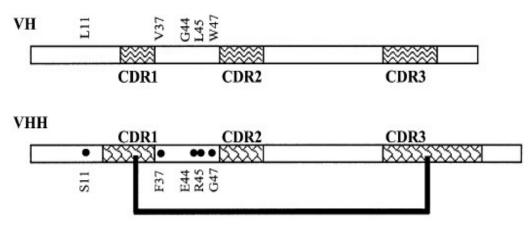
Figure 2. Schematic representation of the differences between VH and VHH based on the sequence comparison of cDNA clones. The position of the CDR in between the framework regions is indicated. The CDR1 and CDR3 of a VHH are larger than in VH genes, and they are often connected by a disulfide bond (thick line). The hallmark amino acid substitutions in framework-one and -two are given. The numbering refers to the position of the amino acid along the sequence according to the Kabat numbering (Kabat et al., 1991) "Reprinted from Molecular Biotechnology, 74, Muyldermans Serge, Single domain camel antibodies: current status/ Results and discussion, Differences between VH and VHH, 277-302., 2001, with permission from Elsevier).

scaffolds consist of two α-sheeted structures similar to a VH immunoglobulin fold in a conventional antibody (Ahmadvand et al., 2008; Revets et al., 2005). By constant the structures of the antigen-binding loops of Nanobodies deviate importantly from the sets found in mouse and human antibodies. This finding provides evidence that the antigen-binding loops of Nanobodies exhibit a much larger structural repertoire than observed for conventional VH. In addition, the CDR3 regions of Nanobodies are on average longer than those of VH in 12 and 9 amino acids, respectively, whereas in dromedary-derived Nanobodies, a length of 16-18 amino acids is frequently observed, although in the llama, a considerable fraction of the Nanobodies seem to have a much shorter CDR of < 6 amino acids. Nanobody sequences also contain amino acid substitutions in the framework regions that are not observed in VH domains that pair with VL domains. Hence, these Nanobody hallmark substitutions might have evolved to compensate for the absence of the VL domain in the antigen binding site, and they might accommodate more flexible loops than a VH.

The hydrophobic to hydrophilic amino acid substitutions (V37F or V37Y, G44E, L45R and W47G) within the framework-2 region (the residues in this region of the VH normally interact with the VL domain and are well conserved throughout evolution) are solvent accessible in the Nanobodies (Figure 2). Consequently, they have been allocated to improve the solubility of Nanobodies (Harmsen, 2007; Muyldermans, 2001; Revets et al., 2005; Riechman and Muyldermans, 1999; Roovers, 2007). It has demonstrated that Nanobodies naturally have a high homology (approximately 90%) with human VH frameworks and, for use as therapeutics, can be further humanized with 95-99% homology by making a small

number of amino-acid substitutions in the framework region (Harmsen and De Haard, 2007; Vaeck, 2004). This homology between the Nanobodies and human VH frameworks paved the way to produce human single domain antibodies that contain a minimum of non-human residues, thereby making them particularly valuable as therapeutic reagents (Davies and Riechmann, 1994; Tanha., 2001).

PROPERTIES OF NANOBODIES

Conventional antibodies have many important features, such as their high affinity and selectivity for a target, their ease of discovery and their low inherent toxicity (Ablynx, 2005). Nanobodies are unique because, in addition to these benefits, they have other technological and biophysical advantages that enable them to outperform conventional antibodies in several areas (Ablynx, 2005; Revets et al., 2005). Firstly, Nanobodies are small proteins only tenth the size of a conventional antibody (De Genst et al., 2006; Harmsen and Haard, 2007), so they penetrate tissues more effectively than conventional antibodies and they can recognize uncommon or hidden epiopes. Secondly, Nanobodies samples are more homogenous showing no sign of spontaneous dimerisation in contrast to scFv that often dimerises to $scFv_2$. Furthermore, the compulsory single domain mature of the small VHH makes it the best candidate to develop bi-specific antibodies or immuno-conjugates by joining the genes of a VHH with another VHH, an enzyme or a toxin in the expression unit.

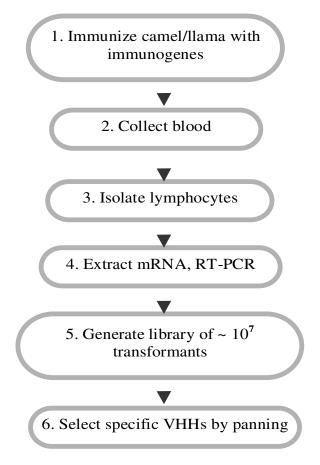Nanobodies are naturally soluble in aqueous solution and do not have a tendency to aggregate, probably due

1. Immunize camel/llama with immunogenes

▼

2. Collect blood

▼

3. Isolate lymphocytes

▼

4. Extract mRNA, RT-PCR

▼

5. Generate library of ~ 10^7 transformants

▼

6. Select specific VHHs by panning

Figure 3. Illustration of the cloning and selection of Nanobodies from an immunized dromedary (or llama).

PRODUCTION OF RECOMBINANT NANOBODIES

Cloning the repertoire of antigen-binding fragments from an immunized animal into a phage display vector and selection of antigen-specific clones by panning has become in the past decade a routine method for selecting antigen-specific molecules (Muyldermans, 2001; Revets et al., 2005). Now, by applying the Nanoclone technology it is possible to directly clone Nanobodies from individual, target-specific B cells. Generally, cDNA is prepared from peripheral blood lymphocytes, isolated from an immunized dromedary or llama (Ablynx, 2005). Consequently, the complete *in vivo* matured Nanobody repertoire of a single immunized animal can be amplified by a single set of primers. A secondary polymerase chain reaction with nested primers is then performed to produce more material and to include restriction enzyme sites for cloning purposes (Muyldermans, 2001). Following cloning of the amplified Nanobody gene fragments in the appropriate expression vector, a Nanobody library containing the repertoire of the intact *in vivo* matured antigen-binding sites is obtained (Arbabi Ghahroudi et al., 1997). Because of the *in vivo* maturation of the HcAbs, relatively small libraries of only 10^6–10^7 individual Nanobody genes have routinely resulted in the isolation of Nanobodies with nanomolar affinity for their antigen (Arbabi Ghahroudi et al., 1997; Cortez-Retamozo, 2004; Muyldermans, 2001). Nanobody libraries can be screened for the presence of antigen-specific binders either by direct colony screening or by panning (Muyldermans, 2001) (Figure 3). Retrieval of binders by panning is the preferred method, as panning allows selection for binders with the highest affinities and those that express better in bacteria. Soluble Nanobodies can then efficiently be produced in bacteria or lower eukaryotes (Revets et al., 2005). Despite the success of retrieving high-affinity lead compounds in a short period of time, the method sometimes suffers from the requirement of sufficient amounts of target antigen for immunization, although good immunizations have been obtained with as little as 100 µg of antigen for entire immunization. The availability of synthetic Nanobody libraries should offer a solution to identify antigen binders in cases where difficulties to immunize are encountered, lack of antigen, low immunogenic or toxic antigen (Revets et al., 2005).

Nanobodies are expressed from a single gene requiring no post-translational modifications. The recombinant Nanobodies are routinely obtained at levels up to 10 mg/l when expressed in *Escherichia coli* grown in shake-flask cultures (Arbabi Ghahroudi et al., 1997). The production process is scalable and expression systems other than bacteria can be used. The high-level secretion of Nanobodies from a range of fungal (Joosten et al., 2005) and yeast species has been shown (Frenken et al., 2000; Rahbarizadeh et al., 2006; Thomassen et al., 2002), with secretion by *Saccharomyces cerevisiae* at levels > 100 mg/l from shake-flask cultures and > 1 g/l from a 10L fed-batch fermentation. Scaled-up yields of > 1 kg of Nano-

to the substitution of hydrophobic by hydrophilic residues in the framework-2 region compared with VH from conventional antibodies interact through hydrophobic areas with the CH_1 and VL domains. As a consequence, the separate expression of the VH domain only leads to the formation of inclusion bodies or to folded domains exposing hydrophobic patches which render them sticky (Muyldermans, 2001).

Nanobodies are highly stable to heat which retain > 80% of their binding activity after 1 week of incubation at 37°C, indicating that Nanobodies have a very good shelf-life. Melting points of Nanobodies are in the range of 67-78°C (De Genst et al., 2006). Besides their thermal resistance, Nanobodies were shown to be stable against the denaturing effect of chaotropic agents, in the presence of proteases and to extremes of pH. Therefore, Nanobodies are expected to be able to survive in harsh conditions, such as those found in the stomach, and remain biologically active in the gut, creating opportunities for the oral delivery of Nanobodies to treat gastrointestinal diseases (Ablynx, 2005; Harmsen and De Haard, 2007; Revets et al., 2005).

This combination of properties is unique to Nanobodies and is not shared by other domain antibodies.

body were obtained from a 15 m^3 fermentation (Harmsen and De Haard, 2007; Muyldermans, 2001).

BIOTECHNOLOGICAL AND MEDICAL APPLICATIONS OF NANOBODIES

Nanobodies are distinguished from other conventional antibodies by their unique properties of size, solubility, intrinsic stability, easy tailoring into pluripotent constructs, recognition of uncommon or hidden epitopes, binding into cavities or active sites of enzyme targets, ease and speed of drug discovery and ease of manufacture. These features should lead to a number of biotechnological and medical applications in which Nanobodies should excel other antibody formats (Revets et al., 2005). Today, in several laboratories, the nanobodies have been used as a research tool and in a variety of diagnostic or thera-peutic applications (Muyldermans et al., 2008). Many diseases were successfully treated with Nanobodies; these Nanobodies either are used as targeting devices for toxic enzymes or block a specific molecular interaction. For example, sleeping sickness was successfully treated with Nanobodies that bind to a trypanosome coat protein and were fused to the apolipoprotein L-1(ApoLI) enzyme, resulting in trypanosome lysis (Baral et al., 2006, Stijlemans et al., 2004). In oncology, a VHH directed against carcinoembryonic antigen (CEA) was used for targeting the genetically fused β-lactamase to tumor cells. This enzyme then converts an injected nontoxic prodrug into a toxic drug in the vicinity of the targeted tumor cells, leading to their killing (Cortez-Retamozo et al., 2004). Several Nanobody therapies are also being developed for treatment of oncology or inflammatory diseases based on blocking molecular interactions. NANOBODIES binding to epidermal growth factor receptor (EGFR) can block epidermal growth factor (EGF) binding to its receptor, which can be used to treat solid tumors (Roovers et al., 2007). Furthermore, by blocking receptor interaction, Nanobodies binding to ovine tumor necrosis factor-α can be used for treatment of rheumatoid arthritis and Crohn's disease (Maass et al., 2007). In 2008, a new Nanobody has been produced and this Nanobody can recognize and neutralize the *Androctonus australis hector* Aahl' toxin, which can cause serious public health problem in many countries (Hmila et al., 2008).

In addition to all these applications in tumour treatment, Nanobodies may add value to cancer diagnostic tests used at present. For example, early detection and staging of prostate cancer is based on the detection of prostate-specific antigen (PSA) in the blood circulation. However, different isoforms of PSA are present in the blood, of which some correlate better with prostatic disorders than others. New Nanobodies have been generated that can discriminate between different isoforms of PSA. Remark-ably, these Nanobodies seem to sense or induce confor-mational changes on different PSA isoforms, a feature that may be exploited to discriminate different stages of

prostate cancer (Saerens et al., 2004). In 2008, Lieven Huang and his team described the use of a llama single domain antibody fragment for the *in vivo* radio-immuno-detection of EGFR over expressing tumors using single photon emission computed tomography (SPECT) in mice. The EGFR-binding Nanobody investigated in that study showed high specificity and selectivity towards EGFR over expressing cells. Pinhole SPECT analysis with ^{99m}Tc-8B6 Nanobody enabled *in vivo* discrimination bet-ween tumors with high and moderate EGFR over expres-sion. The favorable biodistribution further corroborates the suitability of Nanobodies for *in vivo* tumor imaging (Huang et al., 2008). The cysticercosis is caused by larval stage of pork tapeworm *(Taenia solium).* Humans are the definitive host, harboring the adult tapeworm in the intestine. The infections cause by *T. solium,* neuro-cysticercosis (NCC) which cause a big health problem and economic burden in developing countries. Today, serodiagnosis of cysticercosis can be done using Nanobodies generated against the *T. solium* (Deckers et al., 2008).

The Nanobodies may also be used for several biotech-nological applications. For example, the targeting and tracing antigens in live cells can be done using fluore-scent Nanobodies especially for the endogenous proteins, their post-translational modifications and non protein cell components remain invisible and cannot be studied. Recently, fusion proteins (termed 'chromobodies') com-prised an antigen binding Nanobody and fluorescent proteins have been generated to overcome these limita-tions. These chromobodies can recognize and trace anti-gens in different sub-cellular compartments throughout S phase and mitosis (Rothbauer et al., 2006). Of all the bottlenecks of structure determinations through X-ray cry-stallography, arguably, the most critical is the actual production of crystals. Thus far, numerous techniques have been developed to circumvent this major obstacle. Natural partner proteins can greatly improve the pro-bability of obtaining crystals by stabilizing the protein of interest, and by creating additional crystal contact sur-faces. However, not all proteins have natural partners with whom they interact strongly, or these partners are not yet known, therefore alternative binders has been ex-plored, including "Designed Ankyrin Repeat Proteins" (DARPins), and a diversity of antibody domains, in particular Fab's and single-chain Fv's. Recently, The cry-stallization of the EpsI: EpsI pseudopilin, which form the central pseudopilus of the sophisticated bacterial type 2 secretion systems, heterodimer from Vibrio vulnificus, was greatly accelerated by the use of Nanobodies (Lam et al., 2009). The VHH-pigFc is a chimeric HcAb com-posed of a pig Fc isotypes and a Nanobody, this chimeric antibody is used to generate monoclonal antibodies against pig IgG isotypes (Muyldermans et al., 2008).

Therefore, Nanobodies with their small size which makes them particularly suitable for targeting antigens in obstructed locations, such as tumour. Their low immuno-

genic potential and their high affinity and specificity Nanobodies should be ideally placed to become a new class of antibodies for biotechnological and medical applications.

PERSPECTIVES

Nanobodies are a unique class of antibody-derived therapeutic proteins that combine the beneficial features of conventional antibodies with desirable properties of small-molecule drugs. Nanobodies are uniquely versatile single domain antibody fragments; they have the affinity and selectivity of conventional antibodies yet are only a fraction of their size. All Nanobodies contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. They have a naturally low immunogenicity and high homology to human V_H frameworks which makes them ideally suited for therapeutic applications. Because of their unique structure and high stability, Nanobodies can access targets and opportunities that are beyond the reach of conventional antibodies and small-molecule drugs. Ablynx, in Ghent, Belgium which is a bio-pharmaceutical company is engaged in the discovery and development of Nanobodies and it is developing a port-folio of Nanobody-based therapeutic programs in a number of major disease areas, including inflammation, thrombosis, oncology and Alzheimer's disease. Today, the Nanoclone which is Ablynx's proprietary technology platform enables the rapid discovery of large numbers of high-potency Nanobodies against therapeutic targets. Ablynx uses Nanoclone to identify antigen-specific Nanobodies directly from immunized llamas. The elegance of Nanoclone obviates the need for large expression libraries or phage display. Individual Nanobodies are easily expressed in microbial cells and can immediately be screened for functional activity in a bioassay. Future studies will be done in humans and it will be required to confirm their performance, benefits and their efficacy in cancer immunodiagnosis 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.

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