

## **Novel therapy based on camel nanobodies**

### **Abstract (The journal requires max 120 words).**

Nanobodies (Nbs) are small antibody fragments derived from camelid heavy chain antibodies through recombinant gene technology. Their exceptional physicochemical properties, low immunogenicity, possibility of humanization and unique antigen recognition properties make them excellent candidates to target biologically active components. In this context, different therapeutic approaches based on the novel camelid Nanobodies have been developed to combat a wide range of diseases. Based on the reported information, the aims of this review is to provide a comprehensive overview of the current state of the use of camelid-derived Nbs as novel therapeutic agents against multiple diseases.

## **Defined key words (5-10 key words).**

**Nanobody** (The term Nanobody® is a registered trademark [201]).

A nanobody (Nb) is small antibody fragment that can be used to target biologically active components due to its unique antigen recognition properties. Nanobodies are the recombinantly produced variable single-domain fragment of heavy chain camelid antibodies.

## **Immunotherapy**

Immunotherapy refers to the set of treatments which seeks to stimulate, enhance or suppress the immune system to combat different kinds of diseases or conditions. The use of antibodies (including nanobodies) as therapeutic tools is also considered.

## **Formatted nanobodies**

Formatted Nbs are entities (linked Nbs) obtained by recombinant gene technology: multivalent (two or more linked Nbs), multispecific (linked Nbs with binding sites in different antigens) and multiparatopic (linked Nbs with different antigen-binding sites in the same antigen).

## **Engineering nanobodies**

Nbs can be fused with different proteins by recombinant technology to obtain immunotherapeutic tools.

## **Targeted drug delivery system**

Targeted drug delivery systems are special pharmaceutical carriers aimed to increase the concentration of the medication in some parts of the body relative to others.

## **Nanobody-based delivery system**

Nanobody-based delivery systems are any formulation targeted by a specific Nb to its site of action.

54 **Executive summary**

55

56 **1. Nanobodies.**

- 57 • Nanobodies are camelid derived small antibody fragments obtained by recombinant  
58 gene technology.
- 59 • Nbs can be used to target biologically active components.
- 60 • Nbs are economically produced.
- 61 • Nbs have unique structural and functional properties suitable for biotechnological  
62 and biomedical applications.
- 63 • The high capacity to target biological active occluded epitopes, easy tailoring by  
64 genetic engineering and coupling to drug delivery systems; make them ideal for a  
65 new generation of targeted-based therapies.
- 66 • Nanobodies have low immunogenicity by themselves and as such do not evoke an  
67 anti-idiotypic host response in the recipient.

68

69 **2. Engineering and coupling of Nbs to small molecules.**

- 70 • Nbs can be used to generate immunotoxins by genetic engineering.
- 71 • Anti-*T. brucei* Nb fused to truncated human trypanolytic factor presents lytic activity  
72 against African trypanosomes.
- 73 • Anti HIV-Nef virulence factor Nb fused to SH3 domains blocks HIV-Nef functions.
- 74 • Fusion protein between anti-VEGFR2 Nb and truncated PE inhibits the spread of  
75 VEGFR2 expressing tumoral cell line.
- 76 • Anti-CEA Nb fused to  $\beta$ -lactamase is effective as Ab-directed enzyme prodrug  
77 therapy.

78

79 **3. Coupling Nbs to drug carriers.**

- 80 • Nbs can be coupled to drug delivery systems for specific targeting.
- 81 • Anti-EGFR Nb-liposomes conjugates produce a strong inhibitory effect on tumour  
82 cell proliferation after EGFR downregulation. The system loaded with IGF-1R  
83 kinase inhibitor shows higher effectiveness.
- 84 • Anti-EGFR Nb-micelles loaded with doxorubicin almost suppress tumour growth.
- 85 • Anti-EGFR Nb-PEG-Albumin nanoparticles loaded with the multikinase inhibitor  
86 17864 produce a complete inhibition of tumour cell growth.
- 87 • Anti-VCAM1 Nb-microbubbles conjugates are efficient in tumours detection,  
88 opening a new type of nanobody-based drug delivery system.

- 89 • Gold nanoparticles conjugated to an anti-HER2 Nb destroy carcinoma cells after  
90 laser irradiation.
- 91 • Anti-MUC1 Nb tagged polyplex nanoparticles carrying *tBid* transgene  
92 transcriptionally driven by a cancer promoter induce selectively cell death in MUC1  
93 expressing cell line.
- 94 •  $\beta$ -cyclodextrins coupled to an anti-*T. brucei* Nb, loaded with antitrypanosomal drug,  
95 enhance drug effectiveness.
- 96 • PLGA and chitosan nanoparticles coated with an anti-*T. brucei* Nb, loaded with  
97 antitrypanosomal drug, remarkably decrease the curative dose.

98

#### 99 **4. Other applications of Nbs in medicine.**

##### 100 **4.1. Viruses.**

- 101 • An anti-Influenzavirus M2 Nb and formatted anti-influenzavirus HA Nbs protect  
102 mice from viral infection.
- 103 • Anti-HIV envelope glycoproteins Nbs neutralize various HIV subtypes.
- 104 • Anti-RSV Nbs protect mice against RSV infection.

##### 105 **4.2. Rheumatoid arthritis (RA).**

- 106 • A trivalent anti-TNF- $\alpha$  Nb is more efficient than currently TNF- $\alpha$  antagonists.
- 107 • An anti-human IL-6R Nb shows anti-inflammatory effects in clinical trials.

##### 108 **4.3. Thrombotic thrombocytopenic purpura (TTP).**

- 109 • A formatted anti-vWF Nb shows higher efficacy than currently antiplatelets  
110 drugs.

##### 111 **4.4. Alzheimer's disease (AD).** Anti-A $\beta$ Nbs avoid amyloid fibrils formation.

##### 112 **4.5. Scorpion venom.** Anti-Aah toxins Nbs prevent scorpion envenoming.

##### 113 **4.6. Malaria.** An anti-DARC Nb interferes in *P. vivax* infection.

##### 114 **4.7 Botulinum toxin.** Anti-BoNTs Nbs block BoNT protease activity.

##### 115 **4.8. Bone disorders.** An anti-RANKL Nb blocks osteoclast differentiation and bone 116 resorption.

##### 117 **4.9. Cancer.**

- 118 • An Anti-endoglin Nb inhibits proliferation of human endothelial cells and  
119 angiogenesis.
- 120 • Anti-EGFR Nbs inhibit tumour growth.
- 121 • An Anti-VLA-3 inhibits cell adhesion in a tumour cell line.
- 122 • An anti-CD16 Nb activates NK cells opening the possibility to destroy tumour  
123 cells. An anti-MUC1 Nb expressed in T cells as chimeric antigen receptor  
124 produces cell death of tumour cells.

- 125           • An anti-CXCR4 Nb in clinical trials for cancer therapy.
- 126       **4.10. Bacteria.** Anti- $\beta$ -lactamases Nbs increase ampicillin sensitivity.
- 127       **4.11. *Trypanosoma brucei*.** Anti-VSG Nbs are potent trypanolytic agents.
- 128

## 1. Nanobodies.

Antibodies (Abs) of all vertebrates have the same basic molecular structure, consisting in tetrameric molecules composed of two identical large heavy (H) and two identical small light (L) chains [1-3]. Each chain is constituted by globular domains: one variable ( $V_H$ ) and three constant domains ( $C_H1-3$ ) in the case of H-chains; and one variable ( $V_L$ ) and one constant domain ( $C_L$ ) in the case of L-chains (Figure 1A). Once folded, antibodies are organized in three large structural regions. Two of them are identical and include the L-chains paired with the  $V_H$  and  $C_H1$  domains of the H-chains. These two regions, termed Fab fragments (Fab, antigen-binding fragment), contain the antigen binding activity. The other region is called Fc fragment because was originally observed to crystallize easily (Fc, fragment crystallizable region). Fc fragment is formed by the  $C_H2$  and  $C_H3$  domains of the two H-chains and is identical in all Abs of the same isotype (Figure 1A) [3].

Although some anomalies in the vertebrates immunoglobulins structure have been described, such as Abs without L-chains in the called H-chain disease in humans [4], it was not until 1993, when the group of Raymond Hamers, at the Free University of Brussels (Belgium) reported that *Camelidae* species (*Lama glama*, *Lama guanicoe*; *Vicugna pacos*, *Vicugna vicugna*; *Camelus bactrianus* and *Camelus dromedarius*) produced naturally a fraction of unique isotype of G-like immunoglobulins that devoid of the L-chains (Figure 1A) [5]. These homodimeric immunoglobulins, referred as heavy-chain Abs (HcAbs), are also present in cartilaginous fishes [6-7].

At structural level, camelid HcAbs differ from conventional immunoglobulins in several aspects. Each monomer unit (H-chain) contains a Fab fragment reduced to one single variable domain, termed  $V_{HH}$  [5, 8-9]. The  $V_{HH}$  domain represents the smallest intact antigen-binding site in nature [10]. The second structural difference is the absence of the  $C_H1$  domain [5, 11]. The complete sequence analysis of the cDNA, obtained from peripheral blood mononuclear cells RNA isolated from llama, confirmed the absence of the entire  $C_H1$  domain. This imply the direct join of the maturated  $V_{HH}$  with the Fc domains ( $C_H2-C_H3$ ) via the hinge region [12]. The sequence of the  $C_H1$  domain is present in the camelid genome, however is spliced out during the messenger RNA processing of IgG2 and IgG3 due to a single mutation of the canonical splicing site after the  $C_H1$ /intron border [11, 13]. In IgG1 the  $C_H1$  domain is not spliced, and hence, this is a conventional camelid Ab.

The organization of the  $V_H$  domain of conventional Abs and the  $V_{HH}$  domain of HcAbs shares many features (Figure 1B). Both are composed of four conserved sequences, the framework regions (FR1 to FR4), and three hypervariable regions, the complementarity determining regions (CDR1 to CDR3) (Figure 1B). FRs compromise the domain core structure and CDRs form loops involved in antigen binding. The diversity of recognized antigens is determined by CDRs variable amino acid sequence (Figure 1B). However, some differences are clear. On average, the lengths of  $V_{HH}$  CDRs, in particular the CDR3, than provides the bulk of antigen specificity, are longer than CDRs of  $V_H$  domains [8, 12-15]. Moreover,  $V_H$  conformation is flat or concave, unlike the  $V_{HH}$  domain which has a convex conformation that allows interactions with concave antigens [16]. Finally, the  $V_{HH}$  sequence carries important substitutions of highly conserved amino acid residues located in the FR2 region [17]. The hydrophobic residues Val42, Gly49, Leu50 and Trp52 in the  $V_H$  domain of conventional Abs are substituted in the  $V_{HH}$  of HcAbs by the hydrophilic residues Phe/Tyr42, Glu/Gln49, Arg/Cys50 and Leu/Gly52 [8, 18] (Numbering of the international ImMunoGeneTics-IMGT information system [19]) (Figure 1B). The presence of these hydrophilic amino acids residues explains the absence of interaction with the  $V_L$  domain as well as the high solubility of the  $V_{HH}$  as a single-domain fragment [8, 18, 20]. In summary, the CDR loops of the  $V_{HH}$  domain are substantially different in conformation, length and repertoire to those loops present in conventional  $V_H$  domain. These differences could act as a mechanism to compensate the absence of the combinatorial diversification provided by  $V_H$ - $V_L$  interaction [21-23].

Recombinant gene technology allows a high-yield isolation of the genes encoding the antigen-specific  $V_{HH}$  repertoire from camelid animals. The method consists of cloning, in phage display vectors, of the  $V_{HH}$  repertoire produced by peripheral blood lymphocytes of an immunized camelid to finally select antigen-specific  $V_{HH}$  fragments (also called nanobodies –Nbs–) (Figure 1 and 2) [24, 201]. Nbs are the smallest (2.5 nm of diameter and 4 nm of length) antigen-binding fragments that can be obtained [25-27]. Their small dimension allows them a fast tissue penetration. However, it can be a disadvantage due to their short half life [25, 28-30]. As result of the small size and the special conformation, Nbs can recognize uncommon hidden non-accessible antigenic epitopes with high affinity and specificity, such as enzyme active sites [14, 24]. Moreover, the presence of an additional disulfide bond between the CDR1 and CDR3 provides stability by increasing loops resistance to unfolding [15, 31]. Therefore, Nbs are more stable than conventional Abs, remaining full functional at high temperature [32-34] and extreme pHs [35]. Furthermore, through genetic engineering,

Nbs can be modified to improve their features in a wide range of formats according to three characteristics: multivalent (two or more linked Nbs), multispecific (linked Nbs with binding sites in different antigens) and multiparatopic (linked Nbs with different antigen-binding sites in the same antigen) (Figure 3) [29, 36-37]. In short, the isolation of Nbs from camelid HcAbs offers the possibility to generate fully active, stable, soluble, format flexible, specific and high-affinity Ab entities without the common drawbacks of single domains fragments isolated from conventional Abs.

The camelid origin of Nbs could in theory lead to the induction of an immune response when injected into non-camelid hosts. However, so far such anti-Nbs is not reported yet. Nbs are expected to produce low immunogenicity for two reasons: first, due to high sequence homology (more than 80%) between the human V<sub>H</sub> framework and Nb framework [38-39] and second, because their short half-life due to their rapid clearance from the blood [38]. Moreover, Nbs can be customized by altering specific amino acid sequences [40]. In the case of humans, Nbs can be humanized changing the specific positions 49 (Glu to Gly) and 50 (Arg/Cys to Leu) in the framework-2, without significant loss of their properties and specificity [40].

In summary, Nbs are economically produced in bacterial, fungal, yeast or plant expression systems which together with its ease manufacture has contributed to increase of its use [38]. Currently, several diseases such as infections, cardiovascular disorders, autoimmune diseases and cancers have Ab-based therapies. However, the high cost of conventional Ab technology and their limitations associated with its use, becomes Nbs in a good alternative to use in immunotherapy. In addition, their ability to target biological occluded active epitopes, their easy manipulation (by genetic modification into multifunctional therapeutic proteins) and their coupling to drug delivery systems, make them ideal for a new generation of targeted-based therapies (Figure 3).



## 2. Engineering nanobodies to generate fusion therapeutic proteins.

To date, the use of immunotoxins in infection diseases and some types of cancer is becoming more widely applied. The mechanism of action of these therapies is based on the attachment of toxins to specific Abs to generate targeted immunotoxins. Therefore, Nbs can also be used for the same purpose. One of the applications of engineered Nbs as immunotoxin has been their use in an experimental model for Human African trypanosomiasis therapy. The disease is caused by the protozoan parasite *Trypanosoma brucei* whose cellular surface is composed mostly by a monolayer coat of a single, tightly packed and highly immunogenic protein, the VSG (Variant Surface Glycoprotein). These parasites have developed an antigenic variation strategy, whereby spontaneously change the VSG, evading the B-cell mediated Ab host immune response [41]. This mechanism hampers the generation of conventional vaccines. However, the identified nanobody NbAn33 binds specifically to an invariant occluded epitope of the VSG which is non accessible to conventional Abs. This ability makes NbAn33 an useful tool to target trypanosomes [42-43]. A recombinant fusion protein of NbAn33 and a truncated form of human apoL1 (human trypanolytic factor [44]) generated an immunotoxin with lytic activity against a wide range of African trypanosomes [43]. This anti-*T. brucei* immunotoxin Nb-based cured mice from parasite infection. This study reports by first time an effective alternative to the outdated drugs used in the therapy of Human African trypanosomiasis.

A similar strategy using genetically modified Nbs has been developed for experimental HIV therapy. The negative regulatory factor (Nef) is an essential HIV virulence protein for AIDS pathogenesis and an attractive target for drug discovery. HIV-Nef virulence factor is inhibited by compounds that block the interaction of Nef with the SH3 domain of some members of Src family protein-tyrosine kinases, such as Hck kinase [45]. A Nb against HIV-Nef [46] was fused to modified human Hck-derived SH3 domains with high affinity to Nef [45]. These engineered Nbs, termed as neffins, were able to improve the inhibitory activity of the native anti-Nef Nb, opening a new path in the development of antiretroviral agents [47-48].

In cancer therapy, the use of targeted immunotoxins is intended to kill specifically tumour cells. Behdani *et al.* were the first to employ an immunotoxin Nb-based in anti tumours therapy [49]. They selected the vascular endothelial growth factor receptor 2 (VEGFR2) as target and the *Pseudomonas* exotoxin A (PE) as cytotoxic agent. VEGFR2 is over-expressed in the angiogenesis process by endothelial cells in tumour

267 vasculature [50-51]. *Pseudomonas* exotoxin A (PE) is a potent toxin which kills  
268 eukaryotic cells [52-54]. An anti VEGFR2-specific Nb [55] was genetically fused to a  
269 truncated form of PE. The generated immunotoxin, referred as VGRNb-PE, showed a  
270 high efficiency to bind the VEGFR2 receptor *in vitro* and, interestingly, was able to  
271 inhibit the spread of VEGFR2-expressing cells. However, the therapeutic efficacy of  
272 this chimera *in vivo* has been not tested.

273  
274       Engineered Nbs have been also used in Ab-directed enzyme prodrug therapy.  
275 For instance, a nanobody that recognises the carcinoembryonic antigen (CEA) was  
276 conjugated to *Enterobacter cloacae*  $\beta$ -lactamase ( $\beta$ L) [25]. This enzyme was chosen  
277 for its ability to convert many substrates into potent cytotoxic compounds *in situ* [56].  
278 The therapeutic efficiency of the Nb- $\beta$ L conjugate was demonstrated *in vitro* and *in*  
279 *vivo*. On a cancer colon cell line, the prodrug presented a 40-fold lower toxicity than the  
280 drug. However, in the presence of the conjugate, the prodrug was converted into the  
281 active compound, killing the cells as efficiently as the active drug. In an *in vivo* assay  
282 on xenograft colon tumour murine model, the conjugate showed excellent  
283 biodistribution properties and high specificity for the target cells. Moreover, the anti-  
284 CEA Nb- $\beta$ L conjugate cured established xenograft tumour in all animals.

### 3. Coupling Nbs to carriers.

Nanobodies have been conjugated to different types of drug carriers for active targeting of specific cells or tissues. Most of these formulations were designed for cancer treatment and directed against over-expressed molecules in tumour cells. Also, we have devised nanobody based drug delivery systems for active targeting of infectious protozoan agents.

Among drug delivery systems, liposomes are the most broadly used. Liposomes are lipid bilayers which enclose an internal volume suitable for entrapment of biological active molecules. Liposomes are the drug delivery system of choice in systemic administration due to their excellent pharmacokinetics properties. Nanobodies targeted liposomes have been used to downregulate the epidermal growth factor receptor (EGFR), a tumoral marker over-expressed in epithelial tumours, non-small-cell lung cancer, colorectal cancer and pancreatic cancer [57]. EGFR signalling pathway is a target for therapy of many human epithelial tumours [58-59]. There are two different approaches to act against EGFR: i) extracellularly, using monoclonal antibodies to avoid ligand binding to the receptor and ii) intracellularly, with small tyrosine kinase inhibitors that compete with ATP for active binding sites on the receptor [59]. Oliveira *et al.* utilized nanobodies as EGFR antagonist to prevent binding of the epidermal growth factor ligand (EGF) to the receptor [57]. A Nb named EGa1, which recognizes the EGFR ectodomain [60-61], was coupled to polyethylene glycol (PEG) coated liposomes (PEGylation). This nanobody-liposome formulation, referred as EGa1-L, induced the almost complete disappearance of EGFR on the surface of tumour cells *in vitro*. This effect was due to receptor internalization and degradation, which resulted in a strong inhibitory effect on tumour cell proliferation. However, no therapeutic effect was observed in a xenograft carcinoma murine model. The authors suggested that the encapsulation of chemotherapeutic agents might improve the anti tumour effect of the nanobody-liposome formulation [57].

Tumours can develop resistance to EGFR inhibitors [62]. The resistance mechanism is associated to the overexpression of the insulin-like growth factor 1 receptor (IGF-1R) [63]. Thus, an anti-EGFR nanobody-liposome loaded with an IGF-1R kinase inhibitor was used to target both factors in cancer cells [64]. This nanobody-based dual tool blocked both EGFR and IGF-1R activation and induced EGFR downregulation. Moreover, this formulation inhibited tumour cell proliferation even upon short-term exposure.

Another drug delivery system frequently used for targeting therapy are polymeric micelles. These are flexible carriers whose physicochemical and biological properties can be modulated by modifying their synthesis procedure. Furthermore, these micelles are thermosensitive, biodegradable and can be easily loaded with hydrophobic drugs [65-67]. Their small size (60-80 nm) and the possibility of PEG coating provide them a prolonged circulation time required for passive targeting of tumours. Passive targeting is mediated by the enhanced permeation and retention (EPR) effect [68]. PEG coating prevents micelles opsonization by cells of the reticuloendothelial system, but also inhibits their uptake by the target cells. Antibody conjugation leads to receptor mediated endocytosis of the micelles by tumour cells [69-70]. Core-crosslinking of micelles increases their stability and circulation time [71]. Micelles have been used in nanobody targeting technology. Polymeric micelles based on PEG-methacrilamide-lactate were assembled in aqueous solutions by rapid heating above the critical micelle temperature and with strong stirring. Core-crosslinking was done by derivatizing part of the lactate groups with methacrylic anhydride. Micelles were decorated with the EGa1 (anti-EGFR) nanobody by modifying part of the Nb lysines with N-succinimidyl-S-acetylthioacetate, deprotected with hydroxylamine hydrochloride, and subsequently coupled to the micellar surface [72].

The effectiveness of EGa1 (anti-EFGR) nanobody-conjugated core-crosslinked polymeric micelles loaded with doxorubicin was evaluated *in vivo* in murine model [73]. Doxorubicin was covalently bound via hydrazone-based linkers, which are pH-responsive, releasing the drug in the acidic environment of the lysosome. Empty nanobody-coated polymeric micelles (without doxorubicin loading), inhibited tumour growth, possibly by blocking uptake and inducing degradation of EFGR as it was described for EGa1-Liposomes [57]. However, doxorubicin loading increased the formulation effectiveness almost suppressing tumour growth. Animals treated with nanobody-coated doxorubicin loaded micelles showed lower average tumour volume and higher survival than animals treated with doxorubicin loaded micelles (without nanobody coating). The combination of these two therapeutic strategies, blocking the receptor and the incorporation of a chemotherapeutic agent, provides a substantial improvement of treatment effectiveness.

Novel nanobody conjugated albumin nanoparticles have been recently described for active targeting of tumour cells [74]. Albumin nanoparticles (approx. 100 nm in size) were obtained by desolvation with ethanol and crosslinking with glutaraldehyde. Nanoparticles were PEGylated and conjugated to EGa1 (anti-EGFR) nanobody.

Albumin nanoparticles were loaded with the multikinase inhibitor 17864 (a sunitinib analogue) via formation of platinum adducts, with the pyridil moiety of the inhibitor (17864-Lx) that coordinate with the albumin methionine or cysteine residues. This tyrosine kinases inhibitor blocks important signalling pathways and stops tumour growth and angiogenesis [74-75]. The broad spectrum of 17864 inhibitor increased the efficacy but also side effects of the formulation. However, active targeting with the nanobody EGa1 enhanced the accumulation of nanoparticles in tumour cells, decreasing side effects. EGa1-PEG-albumin nanoparticles were endocytosed via clathrin-mediated mechanism and accumulated in the lysosome, where released the inhibitor. EGa1-PEG-albumin nanoparticles loaded with 17864 showed a complete inhibition of tumour cells proliferation, whereas PEG-albumin nanoparticles (without nanobody coating) had no antiproliferative effects. However, as reported in EGa1-liposomes and EGa1-polymeric micelles, empty EGa1-PEG-albumin nanoparticles showed strong inhibition effect on EGFR but insufficient to stop tumour cell proliferation. Only the combined effect of EGa1-nanoparticles loaded with an antiproliferative drug resulted in complete inhibition of tumour cell growth [57, 64, 73-74]

Nowadays, microbubbles are used in medical diagnostics as a contrast agent for ultrasound imaging and have been proposed as new class of drug delivery system [76]. Anti-inflammation marker Vascular Cell Adhesion Molecule-1 (VCAM-1) nanobodies have been used in a microbubbles-based system for diagnosis of vascular endothelial tumours. These anti-VCAM1 Nbs were biotinilated and then coupled to the surface of biotinylated lipid microbubbles via streptavidin-biotin bridging system [77]. This formulation showed excellent results in tumours detection, and opens a new type of nanobody-based drug delivery system.

Nanobody conjugated branched gold nanoparticles have been developed for targeted photothermal therapy of cancer [78]. This therapy is mediated by laser irradiation of tumours in the near-infrared window, where tissue has the lower absorption rate. Gold nanoparticles absorb radiation in this wavelength and produce enough heat to destroy the target cells [79]. Gold nanoparticles can be concentrated in tumours by passive targeting through enhanced permeation and retention (EPR) effect or by active targeting with a specific ligand. Nanobodies are excellent ligands for this therapy due to their high thermostability. A nanobody against the human epidermal growth factor receptor 2 (HER2), which is over-expressed in breast and ovarian tumour cells, was coupled to gold nanoparticles via maleimide-hydrophilic polyethylene oxide-disulfide

functionalization. Specific binding of nanobody conjugated gold nanoparticles to HER2 positive cells (ovarian carcinoma cell lines) was demonstrated by flow cytometry using a fluorescent sandwich assay. Anti-HER2 Nb conjugated gold nanoparticles were able to destroy HER2 positive cell in vitro after five minutes of laser irradiation at a wavelength of 690 nm and 38 W/cm<sup>2</sup> of intensity. Controls of untargeted gold nanoparticles (without nanobody conjugation), gold nanoparticles conjugated with a different nanobody (anti prostate-specific antigen, PSA) and laser irradiation only, did not destroy any cell. These results show that nanobody targeted photothermal therapy is an effective option for cancer treatment.

New therapeutic approaches against cancer have been focused on the ability of natural proteins to induce apoptosis, the proapoptotic proteins. One example is the use of the truncated form of the proapoptotic protein Bid (tBid) in cancer gene therapy [80]. tBid is an ideal killer transgene due to its small size and because it does not require post-transcriptional modifications. tBid transgene expression driven by specific cancerous gene promoters was highly increased and induced apoptosis in breast cancer cells, proving to be an effective approach in cancer gene therapy [80]. One of these promoters drives the overexpression of the mucin-like surface protein MUC-1 in breast and colon tumour cells. Thus, MUC-1 is considered as a tumour biomarker for breast and colon cancers [81]. In gene therapy, one of the non-viral DNA delivery system used consists in polyethylenimine -PEI- polyplex nanoparticles functionalized with PEG [82]. PEI is a polycation which forms polyplexes with high capacity for packaging DNA. Due to their high positive charge, polyplexes interact non-specifically with blood proteins and with the negatively charged surface of non target cells, delivering their cargo in undesired sites [83]. The polyplex positive charge can be masked by PEGylation [84-85]. Nanobody based technology has recently been applied to specifically deliver gene therapy. A high specific anti-MUC1 Nb was attached to PEGylated PEI conjugates loaded with a plasmid containing tBid transgene downstream of MUC1 promoter. This system selectively induced cell death in a MUC1 expressing cell line [86]. This study showed the advantage of combining Nb-based technology with gene therapy as a secure way to deliver transgenes to specific cells.

Infectious diseases are good candidates for targeted therapies. Specific molecules of the infectious agent (not present in host cells) can be used as targets, reducing the risk of cross reactions and side effects. We have developed nanobody conjugated drug delivery systems for active targeting of parasitic infectious diseases. *Trypanosoma brucei*, the causative agent of sleeping sickness, was selected as model

for its peculiar characteristics of endocytosis, capability to evade host immune response and limitations of current chemotherapy. Recently, it has been reported that the anti-*T. brucei* NbAn33 Nb (see section 2) bound to its VSG specific epitope is internalized by the classical endocytosis process into the parasites. This mechanism offers an alternative pathway to introduce trypanocidal agents into parasites, avoiding the classical drug uptake mediated by cell surface transporters [87-88]. Thus, a new drug delivery system based on  $\beta$ -cyclodextrin loaded with an anti-trypanosomatid drug and coupled to the anti-*T. brucei* NbAn33 Nb enhanced trypanocidal activity [89]. Moreover, PEGylated nanoparticles of PLGA and chitosan loaded with a trypanocidal drug and coated with NbAn33 allowed a great reduction of *in vitro* half-inhibitory drug concentration (IC<sub>50</sub>) and *in vivo* minimal full curative doses, relative to free drug [90-91]. Nanobody conjugation was essential for the effectiveness of the formulations in both *in vitro* and *in vivo* experiments. These effective and flexible targeted drug delivery systems represent alternative therapeutic approaches to the actual anti-trypanosome therapies.

## **4. Other applications of Nbs in medicine.**

In this section we collected different applications of nanobodies in medicine, mainly related with the direct blocking of their targets.

### **4.1. Viruses.**

Nbs have been used against a wide range of viruses such as Influenzavirus, Respiratory Syncytial virus (RSV), Rabies virus, Poliovirus, Foot-and-mouth disease virus (FMDV), Rotavirus, Human Immunodeficiency virus (HIV) (anti-envelope Nbs, anti-Rev Nbs, anti-Nef Nbs and anti-CXCR4 Nbs), Hepatitis B virus (HBV) (anti-HBV S domain Nbs, anti-HBV nucleocapsid Nbs and anti-Porcine endogenous retrovirus (PERV) Nbs), Vaccinia virus, Marburg virus and plant viruses as Tulip virus X (TuVX) (reviewed in Vanlandschoot *et al.* [92]). The following is an update of the employ of Nbs as anti-viral tools.

#### **4.1.1. Influenzavirus.**

Influenzavirus is the agent responsible for influenza, commonly known as flu. Each year becomes a worldwide health problem due to seasonal influenza epidemics. Different kinds of Nbs have been generated against specific virus proteins to combat this infection [92]. Influenzavirus matrix-2 (M2) proton channel is an essential virulence factor responsible for removing the viral envelope once the virus is into the endosomes. A Nb against M2 proton channel inhibited virus replication and protected mice from a lethal dose of virus [93].

Another virulence factor is the influenzavirus haemagglutinin (HA), an envelope glycoprotein responsible for host cell binding and internalization. The antigenic shift of this protein is associated with flu pandemic outbreaks. Different works reported the development of Nbs in mono, bi or trivalent formats (see Figure 3, A-F) against influenzavirus HA [92]. The combined administration of an anti HA-Nb and recombinant adenovirus expressing the same Nb prolonged up to 14 days the protective effect (passive immunization) against influenza virus in mouse model [94]. The modification of this Nb with coiled-coil sequences, such as the isoleucine zipper domain (ILZ), allowed the Nb to spontaneously adopt trimeric parallel conformations with higher virus neutralizing effects [95-96].



#### **4.1.2. HIV (Human immunodeficiency virus).**

Different Nb-based strategies have been developed in HIV therapy [47-48, 92]. HIV envelope glycoproteins (gps) bind to the CD4 receptor of T-cell surface and mediate virus entry by fusing viral and host cell membranes. Nanobodies generated against HIV gps had a potent neutralizing activity on a broad spectrum of HIV subtypes [97-98].

#### **4.1.3. Respiratory syncytial virus.**

Respiratory syncytial virus (RSV) is one of the major causes of lower respiratory tract infections. Nbs against the site II of the trimeric fusion protein of RSV avoided the propagation of the infection by inhibiting the entry of the virus in non-infected epithelial cells [99]. These neutralizing Nbs, in a bivalent monospecific format (Figure 3B), protected mice against RSV infection [100]. A trivalent monospecific anti-RSV Nb (Figure 3F), called ALX-0171, designed for delivery on the infection site through intranasal administration, has just finished the phase I clinical trials [201].

#### **4.2. Rheumatoid arthritis (RA).**

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation. Currently, RA therapy is based on symptomatic treatment and modification of the disease process using disease-modifying antirheumatic drugs. Among them, the most used are monoclonal Abs against tumour necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-6 receptor (IL-6R). On this line, specific anti-human and anti-murine TNF- $\alpha$  Nbs have shown higher effectiveness than conventional monoclonal Abs used in clinic [29]. Both, anti-human and anti-murine TNF- $\alpha$  Nb were formatted in a bivalent monospecific arrangement fused to a Nb against serum albumin. These trivalent-bispecific entities (Figure 3 D) had longer serum half-life and improved biodistribution, increasing their accumulation in inflamed joints. The anti-murine formatted Nb showed higher efficacy and lower effective dose in an arthritis mouse model than TNF- $\alpha$  antagonists currently in use. The trivalent-bispecific anti-human TNF- $\alpha$  Nb, named as ATN-0103 (Ozoralizumab<sup>®</sup>), is in phase II clinical trials [101, 201]. This anti-human TNF- $\alpha$  Nb has been formatted in different ways to increase its activity [102-104]. Another anti-TNF- $\alpha$  Nb, called ATN-192, is now in phase I clinical trials [201]. Recently, it has been announced excellent results in phase II clinical trials of an

anti-human IL-6R Nb (named ALX-0061) fused to an albumin binding Nb for RA treatment [201]. Finally, anti-TNF- $\alpha$  Nbs has been used in inflammatory bowel disease. The oral administration of *Lactococcus lactis* expressing a bivalent anti-murine TNF- $\alpha$  Nb showed high efficacy in chronic colitis mouse model [29, 105].

#### **4.3. Thrombotic thrombocytopenic purpura (TTP) and coagulation disorders.**

Thrombotic thrombocytopenic purpura (TTP) is a blood coagulation disorder caused by the absence of ADAMTS 13 protease (a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13), which cleaves and inactivates the von Willebrand factor (vWF). The deficiency of ADAMTS 13 protease may have autoimmune origin or may be caused by a genetic disorder (Upshaw-Schülman syndrome). The vWF mediates platelets aggregation after endothelial damage. This factor interacts with platelet collagen and platelet glycoprotein Ib (GPIb) complex through its A3 and A1 domains, respectively. In TTP, the absence of ADAMTS 13 produces high levels of vWF leading to uncontrolled thrombotic events. In this context an anti-vWF Nb, which recognizes the active state of vWF (bound to GPIb), allowed the detection vWF in plasma of patients of ADAMTS13 deficiency and von Willebrand disease type 2B [106]. A bivalent monospecific format of a humanized anti-vWF Nb targeting the vWF A1 domain (called ALX-0081 or Caplacizumab<sup>®</sup>) was generated. ALX-0081 showed a potent inhibitory effect on platelet aggregation in blood extracted from stable angina patients undergoing percutaneous coronary intervention [107]. In addition, ALX-0081 presented higher efficacy with an improved safety profile compared to the antiplatelets drugs currently in use for acute coronary syndrome [108-109]. This humanized bivalent anti-vWF Nb has two forms of administration: intravenous (the mentioned ALX-0081) or subcutaneous (ALX-0681). Nowadays, ALX-0081 is under international Phase II clinical trials and ALX-0681 is in pre-clinical development [110-111, 201].

#### **4.4. Alzheimer's disease (AD).**

Alzheimer Disease (AD) and other related diseases referred generically as Amyloidosis (such as Huntington, spongiform encephalopathies, systemic amyloid light chain syndrome and some diabetes type II signs), are associated with the accumulation of amyloid fibrils (AF). AFs are deposits of aggregated polypeptides with a characteristic cross- $\beta$  sheet conformation [112-113]. A bivalent monospecific Nb against the amyloid  $\beta$ 1-40 peptide was able to inhibit the formation of the matured form

of AF [114]. Another specific Nb against the amyloid  $\beta$ 42 peptide, which recognizes specifically amyloid  $\beta$  oligomers, inhibited the formation of AF and avoided the associated neurotoxicity. Moreover, this Nb allowed detection of amyloid  $\beta$  oligomers for first time into the neurons of AD patient brains, opening the possibility to be used in diagnosis [115]. Recently, it has been submitted a clinical trial application to the European regulatory authorities to initiate a Phase I clinical trial of a new Nb for AD treatment [201].

#### 4.5. Scorpion venom.

*Androctonus australis hector* (Aah) scorpion envenoming is a serious health problem responsible for frequent accidents in many parts of the world. Envenoming treatment is based on the use of anti-symptomatic drugs and, lately, on the use of therapeutic anti-sera. However, the limitations of the anti-sera therapy have forced to seek novel immunotherapeutic agents. Two novel Nbs against Aahl' and AahlII toxins, the two most abundant Aah scorpion toxins, possessed a potent scorpion venom-neutralizing capacity in an *in vivo* model [116-117]. A bivalent bispecific Nb format (Figure 3C) composed by an anti-Aahl' toxin Nb fused to anti-AahlII toxin Nb provided full protection to mice against scorpion envenoming [118]. Moreover, a pre-clinical trial of this anti-scorpion bispecific Nb has shown to prevent the envenoming fatal alterations [119]. The future of using these anti-scorpion Nbs as therapeutic agents was strengthened when the neutralizing activity of the anti-AahlII toxin Nb was increased after its humanization [120].

#### 4.6. Malaria.

The duffy antigen receptor for chemokines (DARC, also CD234) is a protein of the human erythrocyte membrane which is used by two malaria parasites, *Plasmodium vivax* and *Plasmodium knowlesi*, to internalize into red blood cells [121]. Human populations from different parts of the world present a *cis*-regulatory polymorphism that silences *DARC* gene expression in erythrocytes. This mutation offers high protection against *P. vivax* and *P. knowlesi* infection [122]. An anti-DARC Nb was able to interfere in DARC function and, as consequence, in *P. vivax* infection of red blood cells. This was the first reported Nb with modulating activity on an erythrocyte cell marker [123].

#### 4.7. Botulinum toxin.

Botulinum neurotoxin, BoNT, is produced by the bacteria *Clostridium botulinum*. BoNT ingestion or inhalation induces a severe and fatal muscle paralysis known as botulism. This toxin presents different serotypes which exert protease activity over different SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). SNARE degradation causes disabling of the synaptic neuronal vesicle exocytosis and produces the paralysis. The symptoms are irreversible once BoNT is into the neurons. Two anti-BoNT serotype A Nbs were able to neutralize the protease activity of the neurotoxin [124-125]. Moreover, a potent inhibitor Nb against BoNT serotype A was successfully expressed as intrabody in transfected neuronal cells, diminishing SNARE proteolysis after toxin addition [126]. Although no cure exists for the neuromuscular paralysis in botulism, Nbs have potential use as therapeutic agents.

#### 4.8. Bone disorders.

A variety of degenerative bone diseases are due to an alteration of the balance OPG (Osteoprotegerin)/ RANK (Receptor activator of nuclear factor- $\kappa$ B)/ RANKL (RANK- Ligand) system. RANK/RANKL interaction promotes osteoclast differentiation and bone resorption. However, OPG inhibits this process by sequestering RANKL. Hence, RANKL antagonists are suitable to treat bone disorders. A trivalent bispecific Nb, named ALX-0141, was designed from two anti-RANKL Nbs fused to an anti-serum-albumin Nb. This formatted Nb produced a potent inhibition of the RANKL/RANK interaction, blocking osteoclast differentiation and bone resorption in inflamed and cancerous joints. Nowadays, ALX-0141 is at the end of the phase I clinical trials [201].

#### 4.9. Cancer.

Several works have described the development of Nbs for cancer treatment. The first report on the production of a Nb against a tumour-associated peptide was accomplished by Rahbarizadeh *et al.* when after camel immunization with homogenized cancerous tissues obtained specific Nbs against MUC1 tumour marker [127].

Others Nbs against different tumour markers are the following:

- Endoglin (CD105) is an accessory protein of the transforming growth factor- $\beta$  receptor system expressed on activated vascular endothelial cells. Endoglin has been found up-regulated in neovascularization tumour processes. An anti-endoglin

Nb inhibited the proliferation of human endothelial cells and the associated angiogenic processes [128].

- As mentioned in section 2, EGFR is an important target molecule for cancer therapy. A bivalent monospecific anti-EGFR Nb blocked EGFR and delayed tumour proliferation [61]. The same bivalent entity fused to an anti-human serum albumin Nb showed improved pharmacokinetics and tumour growth inhibition [129]. Moreover, a bivalent biparatopic Nb entity (Figure 3E) against EGFR was more efficient than bivalent-monospecific Nbs in tumour growth inhibition [130].
- VLA-3 (very late antigen 3) also known as integrin  $\alpha 3 \beta 1$  is a member of the integrin receptor family for cell adhesion to the extracellular matrix. VLA-3 has important roles in cancer progression and metastasis. A Nb against integrin  $\alpha 3 \beta 1$  (VLA-3) was identified after llama immunization with carcinoma membrane vesicles. This anti-VLA-3 Nb inhibited VLA-3 mediated cell adhesion process in a tumour cell line model [131].
- One of the approaches in cancer therapy has been focused on the use of specific Abs to target and activate cell effectors, such as natural killer (NK) cells, which destroy tumour cells. An Nb generated against CD16 (also Known as Fc receptor Fc $\gamma$ RIII), arranged in a bivalent bispecific format, induced a strong NK cells activation. This effect opens the possibility to use this Nb to recruit Fc $\gamma$ RIII killer cells to destroy tumour cells [132]. A T cell line, transfected with a anti-MUC1 Nb as chimeric antigen receptor was activated after co-culture with a MUC-1 expressing human breast cancer cell line, producing tumour cells death [133].
- Initially, Nbs against C-X-C chemokine receptor type 4 (CXCR4), a co-receptor for HIV virus entry, was developed for HIV therapy [134]. However, the role of CXCR4 in tumour angiogenesis has led to an Ab directed therapy against this receptor in cancer treatment [135-136]. A bivalent biparatopic anti-CXCR4 Nb, named as ALX-0165, has just finished the phase I clinical trials [201].

#### **4.10. Bacteria.**

$\beta$ -lactamases are bacteria enzymes responsible for beta lactam resistance. New strategies to combat bacteria resistance are based on the inhibition of these enzymes. Specific Nbs against TEM1 and *Bacillus cereus* 569/H (BcII)  $\beta$ -lactamases

were able to inhibit the activity of this  $\beta$ -lactamases. Moreover, the presence of the anti-TEM1 Nb led to an increase of ampicillin sensitivity in a resistant TEM1-expressing *E. coli* strain [137]. A great clinical concern is the verona integron-encoded metallo- $\beta$ -lactamase 4 (VIM4), a potent enzyme produced by multiresistant gram-negative bacteria. An anti-VIM4 Nb targeted a distant residue from the active site and blocked the enzyme activity by inducing allosteric modifications [138].

#### **4.11. *Trypanosoma brucei*.**

As commented before, *T. brucei* is the causative agent of sleeping sickness. High affinity Nbs against the VSG of *T. brucei* has been showed to be potent trypanolytic agents [139]. Moreover, the affinity of these anti-VSG Nbs has been demonstrated to be determinant in their trypanolytic action [140]. However, high concentrations of these Nbs are necessary to produce trypanolysis.

## **FUTURES PERSPECTIVES**

The recent progress made in Nanobody technology has opened a wide range of possibilities for clinical and biotechnological applications. The unique features of these fully functional immune entities include their small size, stability, solubility, format flexibility, specificity, high-affinity, low cost production and potential of genetically engineered modifications. Together, these characteristics offer the possibility that in the future Nanobody technology can supplement conventional antibody technology in a number of applications. The extended half-life by different formatting options and the possibility of its humanization allows us to improve their pharmacokinetic properties and might reduce immune rejection. Furthermore, the fact that Nbs can be coupled to drug delivery systems provides an opportunity to reduce the drug doses with an improved efficiency, not only for the specific of the targeted therapy, but also by the resulting reduction of adverse effects associated with these drugs. These improvements, together with the ease of generation and low cost of production will lead the way into future clinical application, as evidenced by current clinical trials.

**Summary table**

<b>DISEASE/ PATHOGEN</b>	<b>TARGET</b>	<b>FORMAT</b>	<b>ACTION</b>	<b>REFERENCE</b>
Cancer	Carcinoembryonic antigen (CEA)	Monovalent anti-CEA Nb fused to $\beta$ -lactamase enzyme (Figure 3I).	<ul style="list-style-type: none"> <li>Efficient Ab-directed enzyme prodrug therapy.</li> <li>Cures established xenograft tumour.</li> </ul>	[25]
Cancer	Mucin-like surface protein 1 (MUC1)	Monovalent anti-MUC1 Nb. (Figure 3A)	<ul style="list-style-type: none"> <li>First anti-peptide Nb.</li> <li>Reacts with 17 cancerous cell lines.</li> </ul>	[127]
Cancer	MUC1	Monovalent anti-MUC1 Nb as chimeric antigen receptor.	<ul style="list-style-type: none"> <li>Induces the T cell activation and the consequent cell death of tumour cells.</li> </ul>	[133]
Cancer	MUC1	Anti-MUC1 Nb coupled to polyplex carrying plasmid containing truncated <i>Bid</i> transgene downstream of MUC1 promoter. (Figure 3P)	<ul style="list-style-type: none"> <li>Effective gene therapy combined with Nb-based technology.</li> <li>Induces selectively cell death in a MUC1 expressing cell line.</li> </ul>	[86]
Cancer	Epidermal growth factor receptor (EGFR)	Bivalent anti-EGFR Nb. (Figure 3B)	<ul style="list-style-type: none"> <li>Blocks EGF-induced cell proliferation.</li> <li>Delays tumour growth.</li> </ul>	[61]
Cancer	EGFR	Trivalent Nb entity (two anti-EGFR Nbs fused to anti-human serum albumin (HSA) Nb). (Figure 3D)	<ul style="list-style-type: none"> <li>Efficient pharmacokinetics properties and tumour deposition and uptake.</li> </ul>	[129]
Cancer	EGFR	Biparatopic anti-EGFR Nb entity (two Nbs which recognize different epitopes of EGFR). (Figure 3E)	<ul style="list-style-type: none"> <li>Efficient inhibition of EGFR signalling and tumour growth.</li> </ul>	[130]
Cancer	EFGR	Anti-EFGR Nb, called EGa1 [60], coupled to PEG-liposomes. (Figure 3K)	<ul style="list-style-type: none"> <li>EGFR downregulation.</li> <li>Inhibitory effect on tumour cell proliferation.</li> </ul>	[57]
Cancer	EGFR	EGa1 coupled to rhodamine loaded micelles. (Figure 3L)	<ul style="list-style-type: none"> <li>High binding capacity and uptake by EGFR expressing tumoral cells.</li> </ul>	[72]
Cancer	EGFR	EGa1 coupled to PEG-liposomes loaded with IGF-1R kinase inhibitor.	<ul style="list-style-type: none"> <li>Blocks both EGFR and IGF-1R activation.</li> <li>EFGR downregulation.</li> <li>Inhibition of tumour cell proliferation.</li> </ul>	[64]
Cancer	EGFR	EGa1 coupled to albumin nanoparticles loaded with multikinase inhibitor 17864. (Figure 3J)	<ul style="list-style-type: none"> <li>Complete inhibition of tumour cell growth.</li> </ul>	[74]
Cancer	CD16 (also Know as Fc receptor Fc $\gamma$ RIII)	Bispecific Anti-CD16 Nb (two Nbs which recognize different CD16 receptor isoforms). (Figure 3C)	<ul style="list-style-type: none"> <li>Induces the activation of specific natural killer cells.</li> </ul>	[132]
Cancer	Endoglin (CD105)	Monovalent Anti-CD105 Nb.	<ul style="list-style-type: none"> <li>Inhibits endothelial cell proliferation and the associated angiogenic processes.</li> </ul>	[128]
Cancer	Integrin $\alpha 3\beta 1$ (VLA-3)	Monovalent anti-VLA-3 Nb.	<ul style="list-style-type: none"> <li>Inhibits VLA-3 mediated cell adhesion process in a tumour cell line.</li> </ul>	[131]
Cancer	Human epidermal growth factor receptor 2 (HER2)	Anti-HER2 Nb coupled to gold nanoparticles. (Figure 3N)	<ul style="list-style-type: none"> <li>Effective photothermal anti-cancer therapy.</li> </ul>	[78]



Cancer	Vascular endothelial growth factor receptor 2 (VEGFR2)	Anti VEGFR2-specific Nb fused to truncated form of PE. (Figure 3G)	<ul style="list-style-type: none"> <li>Inhibits the spread of VEGFR2-expressing cells.</li> </ul>	[55]
Cancer	Vascular Cell Adhesion Molecule-1 (VCAM-1)	Anti-VCAM1 Nb coupled to microbubbles-based system. (Figure 3M)	<ul style="list-style-type: none"> <li>Tumours detection.</li> </ul>	[77]
Cancer	EGFR	EGa1 coupled to doxorubicin loaded micelles.	<ul style="list-style-type: none"> <li>Almost complete suppressing tumour growth.</li> </ul>	[73]
Bone disorders	RANKL (Receptor activator of nuclear factor- $\kappa$ B ligand)	Trivalent Nb entity (two anti-RANKL Nbs fused to anti-HSA Nb) (ALX-0141). (Figure 3F)	<ul style="list-style-type: none"> <li>Inhibits the RANKL/RANK interaction, blocking the osteoclast differentiation and avoiding the bone resorption in inflamed and cancer regions.</li> </ul>	[201]
Influenza A virus	Matrix-2 (M2) proton channel protein	Monovalent anti-M2 Nb.	<ul style="list-style-type: none"> <li>Potent neutralizing activity for both wild-type and amantadine-resistant influenza A viruses.</li> <li>Protects mice from a lethal dose of viral infection.</li> </ul>	[93]
Influenza A virus	Haemagglutinin (HA)	Monovalent Anti-HA Nb and recombinant adenovirus expressing the anti-HA Nb.	<ul style="list-style-type: none"> <li>Passive immunization against influenza virus infections.</li> </ul>	[94]
Influenza A virus	HA	Trivalent anti-HA Nb.	<ul style="list-style-type: none"> <li>Protects mice from H5N2 influenza A virus strain.</li> </ul>	[96]
HIV	C-X-C chemokine receptor type 4 (CXCR4)	Biparatopic anti-CXCR4 Nb.	<ul style="list-style-type: none"> <li>Antiretroviral activity against T cell-tropic and dual-tropic HIV-1 strains.</li> </ul>	[134]
HIV	Envelope glycoproteins	Monovalent anti-gp140/120 Nb.	<ul style="list-style-type: none"> <li>Neutralizes HIV A, B and C subtypes.</li> </ul>	[97]
HIV	Negative regulatory factor (Nef)	Anti-Nef Nb fused to SH3 domain of Hck.	<ul style="list-style-type: none"> <li>Inhibitory activity of Nef functions.</li> </ul>	[47-48]
HIV	Envelope glycoprotein (gp120) binding sites of soluble CD4 and co-receptors	Monovalent anti-gp140/120 Nb, bivalent anti-gp140/120 Nb and trivalent anti-gp140/120 Nb.	<ul style="list-style-type: none"> <li>Neutralize a broad spectrum of HIV-1 subtypes.</li> </ul>	[98]
Respiratory syncytial virus (RSV)	RSV fusion protein (F) site II	Biparatopic anti-F RSV Nb.	<ul style="list-style-type: none"> <li>Inhibits the fusion of the virus with non-infected epithelial cells, avoiding the propagation of the infection.</li> </ul>	[99]
RSV	RSV F protein site II	Bivalent anti-F RSV Nb.	<ul style="list-style-type: none"> <li>Reduces virus propagation and immunizes mice against RSV infection</li> </ul>	[100]
RSV	RSV F protein site II	Trivalent Nb entity (two Anti-F RSV Nb fused to anti-HSA Nb) (ALX-0171)	<ul style="list-style-type: none"> <li>Phase I clinical trials finished.</li> </ul>	[201]
Rheumatoid arthritis	Human and murine tumour necrosis factor- $\alpha$ (TNF- $\alpha$ )	Anti-human and anti-murine trivalent Nb entities (two Anti-TNF- $\alpha$ Nbs fused to anti-serum albumin Nb)	<ul style="list-style-type: none"> <li>Improve <i>in vivo</i> biodistribution properties.</li> <li>Anti-murine trivalent entity improves efficacy versus current therapeutic agents.</li> </ul>	[29]
Rheumatoid arthritis	Human TNF- $\alpha$	Multivalent anti-human TNF- $\alpha$ Nb entity	<ul style="list-style-type: none"> <li>Improves TNF-<math>\alpha</math> neutralizing activity.</li> </ul>	[103]
Rheumatoid arthritis	Human TNF- $\alpha$	Bivalent Nb-based molecules (dimers of molecules)	<ul style="list-style-type: none"> <li>Increases binding activity.</li> </ul>	[103]

		composed by an anti-TNF- $\alpha$ Nb fused to k light chain domain)		
Rheumatoid arthritis	Human TNF- $\alpha$	Bivalent Nb entity molecules (dimers of molecules composed by an anti-TNF- $\alpha$ Nb fused to truncated human IgG1 Fc region)	<ul style="list-style-type: none"> <li>Presents high activity against TNF-<math>\alpha</math></li> </ul>	[104]
Rheumatoid arthritis	Human TNF- $\alpha$	Trivalent Nb entity (two Anti-human TNF- $\alpha$ Nbs fused to anti-HSA Nb) (ATN-0103 or Ozoralizumab®)	<ul style="list-style-type: none"> <li>Phase II clinical trial</li> </ul>	[101] [201]
Rheumatoid arthritis	Human TNF- $\alpha$	ATN-0192	<ul style="list-style-type: none"> <li>Phase I clinical trial.</li> </ul>	[201]
Rheumatoid arthritis	Interleukin 6 receptor (IL-6R)	Trivalent Nb entity (two Anti-IL-6R Nbs fused to anti-HSA Nb) (ALX-0061)	<ul style="list-style-type: none"> <li>Excellent efficacy results in phase II clinical trial.</li> </ul>	[201]
Inflammatory bowel disease.	Murine TNF- $\alpha$	<i>Lactococcus lactis</i> expressing a bivalent anti-murine TNF- $\alpha$ Nb.	<ul style="list-style-type: none"> <li>High efficacy in induced chronic colitis mouse model.</li> </ul>	[105]
Thrombotic thrombocytopenic purpura (TTP)	von Willebrand factor (vWF)	Monovalent anti-vWF Nb.	<ul style="list-style-type: none"> <li>Detects vWF in plasma of TTP patients.</li> </ul>	[106]
Thrombotic thrombocytopenic purpura (TTP)	vWF	Bivalent humanized anti-vWF Nb (ALX-0081 or Caplacizumab®).	<ul style="list-style-type: none"> <li>Presents high efficacy with an improved safety profile compared to the currently marketed antiplatelets drugs in acute coronary syndrome.</li> </ul>	[107-109]
Thrombotic thrombocytopenic purpura (TTP)	von Willebrand factor (vWF)	Bivalent humanized anti-vWF Nb (ALX-0081 or Caplacizumab® intravenous administration, or ALX-0681 to subcutaneous administration).	<ul style="list-style-type: none"> <li>ALX-0081 phase II clinical trial.</li> <li>ALX-0681 preclinical development.</li> </ul>	[110-111]
Alzheimer's disease	Amyloid $\beta$ (A $\beta$ ) (1-40) peptide	Bivalent anti-A $\beta$ Nb.	<ul style="list-style-type: none"> <li>Inhibits the formation of matured form of amyloid fibrils by stabilization of its precursors, the protofibrils.</li> </ul>	[114]
Alzheimer's disease	A $\beta$ 42 peptide	Monovalent anti-A $\beta$ Nb.	<ul style="list-style-type: none"> <li>Recognizes specifically A<math>\beta</math> oligomers avoiding A<math>\beta</math> associated-neurotoxicity and the formation of AF.</li> <li>Detects A<math>\beta</math> oligomers into the neurons of AD patient brains.</li> </ul>	[115]
Alzheimer's disease	-	-	<ul style="list-style-type: none"> <li>Clinical Trials Application (CTA) to the European regulatory submitted by Ablynx and Boehringer Ingelheim.</li> </ul>	[201]
<i>Androctonus australis hector</i> (Aah) Scorpion venom	Aahl' toxin	Bivalent anti-Aahl' toxin Nb. Chimeric HcAb (Anti-Aahl' toxin Nb assembled with Fc of HcAb).	<ul style="list-style-type: none"> <li>Neutralizing capacity in an in vivo model</li> </ul>	[116]
Aah Scorpion venom	AahlII toxin	Monovalent anti-AahlII toxin Nb.	<ul style="list-style-type: none"> <li>Scorpion toxin-neutralizing activity in mice.</li> </ul>	[117]
Aah Scorpion venom	Aahl' and AahlII toxins	Bispecific anti-Aah toxins Nb (anti-Aahl' Nb fused to anti-AahlII Nb)	<ul style="list-style-type: none"> <li>Provides full protection against scorpion envenoming in mouse model and shows</li> </ul>	[118]

			excellent venom neutralizing capacity.	
Aah Scorpion venom	Aahl toxin	Humanized monovalent anti-Aahl toxin Nb.	<ul style="list-style-type: none"> <li>Scorpion toxin-neutralizing activity in mice without loss of its antigen binding and stability properties.</li> </ul>	[120]
Aah Scorpion venom	Aahl' and Aahl toxins	Bispecific anti-Aah toxins Nb (anti-Aahl' Nb fused to anti-Aahl Nb)	<ul style="list-style-type: none"> <li>Prevents effectively the fatal disturbances induced by Aah venom.</li> </ul>	[119]
Malaria	Duffy antigen receptor for chemokines (DARC or CD234)	Monovalent anti-DARC Nb.	<ul style="list-style-type: none"> <li>Interferes in DARC function and in Plasmodium vivax infection.</li> </ul>	[123]
Botulinum neurotoxin BoNT	BoNT serotype A	Monovalent anti-BoNT serotype A Nb.	<ul style="list-style-type: none"> <li>Shows neutralizing capabilities on protease activity inhibiting the cleave of its specific SNARE substrate, SNAP25</li> </ul>	[124]
BoNT	BoNT serotype A	Monovalent anti-BoNT serotype A Nb.	<ul style="list-style-type: none"> <li>Expressed as intrabody in transfected neuronal cells, diminishes SNAP25 cleavage after BoNT A addition.</li> </ul>	[126]
BoNT	BoNT serotype A light chain (BoNT/A Lc)	Monovalent anti-BoNT serotype A light chain (BoNT/A Lc) Nb.	<ul style="list-style-type: none"> <li>Potent inhibitor of the toxin enzymatic activity.</li> </ul>	[125]
Bacteria	TEM1 and <i>Bacillus cereus</i> 569/H (BclI) $\beta$ -lactamases	Monovalent anti-TEM1 Nb. Monovalent anti-BclI Nb.	<ul style="list-style-type: none"> <li>Inhibit TEM1 and BclI activities.</li> <li>The presence of anti-TEM1 Nb increases the ampicillin sensitivity of TEM1 expressing <i>E. coli</i> strain</li> </ul>	[137]
Bacteria	Metallo $\beta$ -lactamase VIM4	Monovalent anti-VIM4 Nb.	<ul style="list-style-type: none"> <li>Blocks the enzyme activity.</li> </ul>	[138]
Sleeping sickness	Surface invariant epitope of <i>Trypanosoma brucei</i>	Monovalent anti- <i>T. brucei</i> Nb	<ul style="list-style-type: none"> <li>Detection of parasites in blood smears.</li> </ul>	[42]
Sleeping sickness	Surface invariant epitope of <i>Trypanosoma brucei</i>	Monovalent anti- <i>T. brucei</i> Nb fused to truncated human trypanolytic factor. (Figure 3H)	<ul style="list-style-type: none"> <li>Immunotoxin with lytic activity against a wide range of African trypanosomes</li> </ul>	[43]
Sleeping sickness	Surface invariant epitope of <i>Trypanosoma brucei</i>	Anti- <i>T. brucei</i> Nbs.	<ul style="list-style-type: none"> <li>High affinity Nbs against the VSG of <i>T. brucei</i> are potent trypanolytic agents.</li> </ul>	[139]
Sleeping sickness	Surface invariant epitope of <i>Trypanosoma brucei</i>	Anti- <i>T. brucei</i> Nbs.	<ul style="list-style-type: none"> <li>Affinity is determinant in the trypanolytic action of the Anti-<i>T. brucei</i> Nbs.</li> </ul>	[140]
Sleeping sickness	Surface invariant epitope of <i>Trypanosoma brucei</i>	Anti- <i>T. brucei</i> Nb coupled to $\beta$ -cyclodextrins and loaded with anti-trypanosomatid drug.	<ul style="list-style-type: none"> <li>Enhances trypanocidal activity.</li> </ul>	[89]
Sleeping sickness	Surface invariant epitope of <i>Trypanosoma brucei</i>	Anti- <i>T. brucei</i> Nb coupled to trypanocidal drug loaded polymeric nanoparticles. (Figure 3O)	<ul style="list-style-type: none"> <li>Reduce <i>in vitro</i> half-inhibitory concentration (IC50) and <i>in vivo</i> minimal full curative doses, relative to free drug.</li> </ul>	[90-91]

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\* of interest

\*\* of considerable interest

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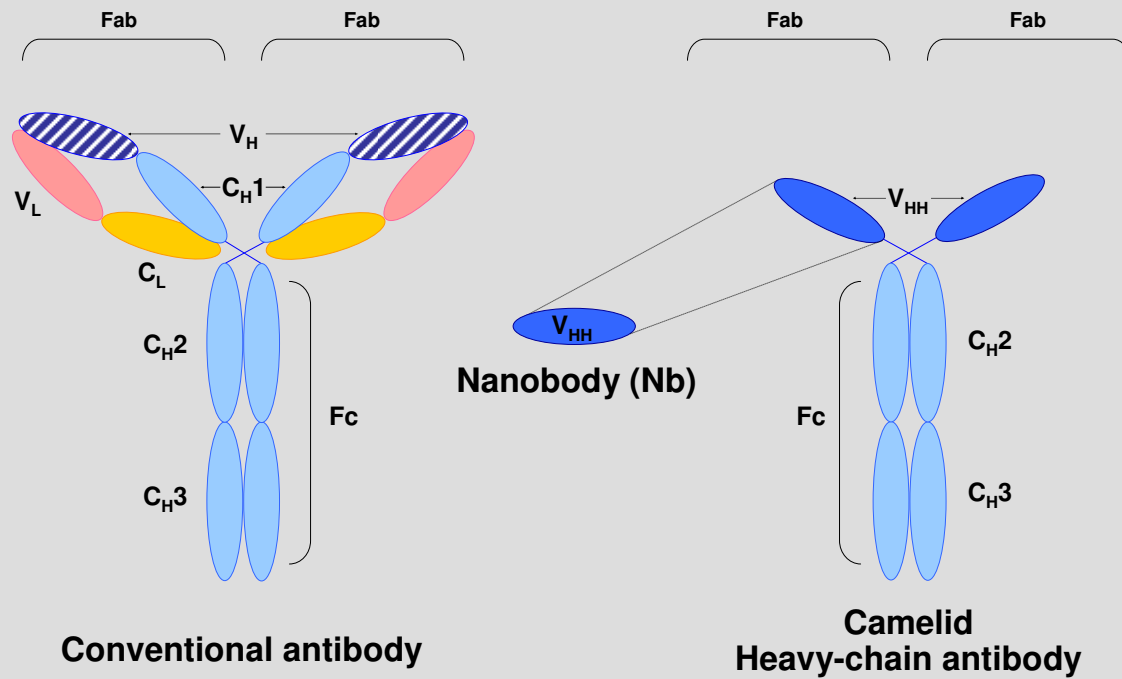
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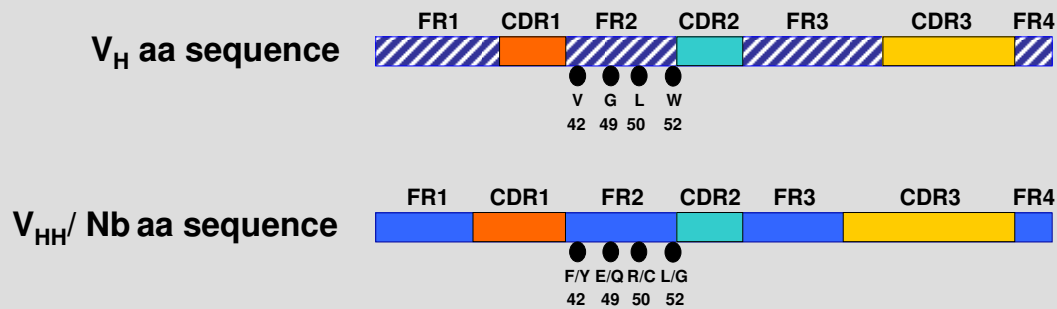
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