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REVIEW ARTICLE

Single Domain Antibodies: A New Approach in Therapeutics

Narkhede Mahesh *, Narkhede Rohan, Mapari Mangesh

Department of Pharmacology, Dr. Rajendra Gode College of Pharmacy, Malkapur-443101, India

Correspondence
Rohan Narkhede
Rajendra Gode college of pharmacy, malkapur, MH India.
rohannarkhede208@gmail.com

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ABSTRACT

Single domain antibodies are rapidly comes into therapeutics in last two decades due to their therapeutic advantages. Most of drugs are derived from antibodies based proteins. Single domain antibodies having advantages over the monoclonal antibodies such as small size, heat resistant, stability, hydrophobicity, low immunogenicity, high solubility. These are small in size hence called nobodies. Their production is carried outing mammalian cells for therapeutic uses. These single domain antibodies are now employed in drug delivery system. They are also utilized in identification of toxin. Single domain antibodies are recently employed in treatment diseases like cancer, Alzheimer's disease, Parkinson's disease etc. They are also employed in virus detection.

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INTRODUCTION

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. Approximately 30% of drugs in development are biologics, most of which are Ab-based proteins used as treatment for inflammatory diseases, cancer, and allergies [1]. The success of the anti-tumor necrosis factor (TNF) Abs has boosted the search for other Abs. In 2013, 22% of the sales of large pharmaceutical companies were biologics and this figure is expected to rise still further [2]. Monoclonal Abs (mAbs) have become indispensable therapeutic and research tools. Given that they are difficult and expensive to produce, they impose a heavy burden on healthcare and research budgets. Moreover, they are not suitable for some applications. First, they are large molecules (150 kDa), which limits their tissue and/or tumor penetration and bio distribution. Second, they can elicit immune reactions that neutralize their activities, which sometimes limits the long-term use of chimeric and humanized Abs available on the market. Third, mAbs typically have a half-life of several days and this limits their use in molecular imaging because of the intense background signal [3, 4]. The disadvantages of Abs are related to their large size, efforts have been made to minimize them. This leads to the development of antigen-antibody fragments (Fab fragments), variable fragments (Fv fragments), and single-chain variable fragments (scFv fragments) [5]. The stability of these newer antibodies also enhanced.

During the early 1990s, Hamers, Casterman and her team discovered a new antibody in Camelidae members. Compared to older conventional antibody immunoglobulin G (IgG) Antibodies, camelid antibodies found to express antibodies devoid of light chains, called "heavy-chain-only antibodies (HcAbs) [6]. Although single-domain antibodies later were also identified in particular cartilaginous fish, most research on the biotechnological application of single-domain antibodies was done using camelids because of their ease of handling, including immunization [7].

Although they do not originate from humans, Nbs have a low immunogenicity because of a large sequence identity with the human VH gene family III, making them suitable for chronic indications. In nine clinical studies with incidence of antidrug antibodies was low (3%) and their presence mainly transient [8].

"A single domain antibody called Nano body is an antibody fragment costing of single monomeric variable antibody domain."

PROPERTIES OF SINGLE DOMAIN ANTIBODIES:

These are much smaller (12-15 kDa) than common antibodies (150-160kDa) [9]. A single domain antibody is a peptide chain of about 120 amino acids long comprising of one variable heavy chain domain (VHH) of a heavy chain antibody or of a common IgG [6]. Single domain antibodies have same affinity towards antigen as whole antibodies but, are more heat resistant and stable towards detergent and high concentrations of Urea [10]. Nano bodies derived from camelid are less lipophilic and more water soluble because several of the hallmark hydrophilic amino acid residues of VHHs was more stable than the original VH fragment [11, 12]. In contrast to common antibodies, two out of six single-domain antibodies survived a temperature of 90 degree Celsius without losing their ability to bind antigens in 1999 study [13, 14].

Stability towards gastric acid and protease [15-19]. The comparatively low molecular mass leads to a better permeability in tissues [20]. Unlike whole antibodies, they do not show complement system triggered cytotoxicity because they lack an Fc region [21]. These are potentially weak immunogenic [22].

STRUCTURE OF SINGLE DOMAIN ANTIBODIES:

Variable domains derived from the anti-body heavy (VH) and light (VL) chains are shaded dark gray and light gray, respectively, whereas constant domains (CH and CL) are not shaded. Note the absence of the light chain and CH1 domain in heavy-chain antibodies. Anti-body domains that pair by non-covalent interactions are indicated by overlaying them.

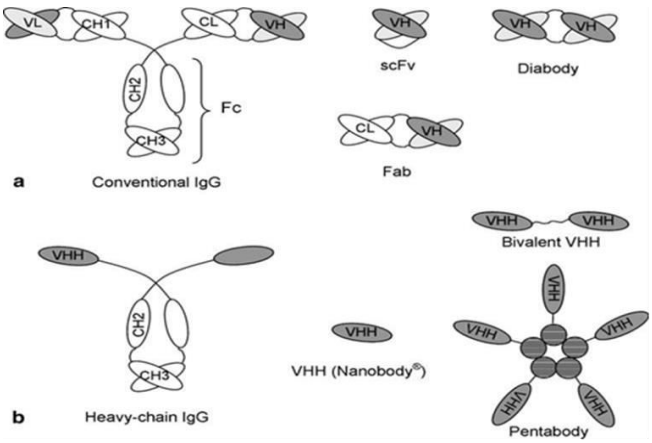

Figure: Schematic diagram of conventional antibodies (b): Heavy chain antibodies and fragments.

These contains two constant domains (CH2 and CH3), a hinge region, and an antigen-binding or variable heavy chain domain (VHH) called the Nb, which retains full antigen-binding capacity [6]. The framework regions present surrounding to complementarity determining regions (CDR). It might participate in Ag identification and binding. [6]. about 61-80% of contact with Ag is through this regions [23, 24]. Nbs are small (15 kDa) and can have a long protruding CDR3 loop. Their prolate shape exposes a convex paratope and both these features help them to access receptor clefts or binding pockets more easily that are inaccessible to Abs [8, 9].

Also the CDRs of VHHs contain some characteristic features. The N-terminal part of CDR1 is more variable. Secondly, many dromedary VHHs have an extended CDR3 that is often stabilized by an additional disulfide bond with a cysteine in CDR1 or resulting in the folding of the CDR3 loop across the former VL interface [25, 26, and 27].

ADVANTAGES OF SINGLE DOMAIN ANTIBODIES:

(1) Fast clearance

(2) Single-domain nature

(3) No decrease in library size because of reshuffling of VL and vh domains

(4) Efficient refolding due to increased hydrophobicity and single-domain nature increased hydrophobicity

(5) Small size and extended flexible CDR3 small size

(6) Efficient folding due to increased hydrophobicity and single-domain nature

(7) High solubility

(8) Increased functional size of immune libraries

PRODUCTION OF SINGLE DOMAIN ANTIBODIES:

Most functional complete antibodies can be efficiently produced using mammalian cells, especially when their appropriate glycosylation is required for therapeutic applications. The antibody fragments having absence of Fc with its N-linked oligosaccharide are preferably produced in microbes [28]. These have a shorter development time from gene to product and require simple well-established fermentation conditions that can be performed on large-scale resulting in low cost [29]. The microbial production is mainly based on E. coli, yeasts, or filamentous fungi. In E. coli they can be produced by secretion into the oxidizing periplasmic space or expression in the reducing cytosol. In fungi the production can be done by cumbersome refolding of antibody fragments [30].

VHHs are mostly produced in E. coli [31, 32]. There is only one example of VHH production that can be produced in filamentous fungi [33], which results in limited proteolytic degradation of the secreted product due to high levels of proteases secreted by filamentous fungi [34]. The yeast Saccharomyces are also capable of producing VHHs [35, 36]. The N-glycosylated VHHs can also be produced by yeast [35]. This can affect antigen binding [37]. Furthermore, it could complicate their therapeutic use because the addition of yeast specific high-mannose oligosaccharides results in a high immunogenicity and decreased serum half-life because of binding to specific mannose receptors on cells of the reticulo-endothelial system [38].

Several VHH sequence pattern can be associated with their production level. First his presence of a potential N-linked glycosylation site increases production levels in yeast [39]. Second, the presence of unpaired C-terminal cysteines reduces expression levels [40]. Third, replacement of hydrophobic residues of conventional VH domains normally interacting with CH1 increased scFv production in E. coli suggesting that the hydrophilic mutations that naturally occur at these positions in VHHs also contributes to their high expression level [41]. There are many examples of VHHs that differ by only a few amino acids and are produced at highly variable levels where the exact amino acid change responsible for the difference in production level is difficult to predict [26,42]. Furthermore, without such knowledge, VHH production can be enhanced by random molecular evolution using deoxyribonucleic acid shuffling [43] , as has often been done for conventional antibody fragments [44]. In baker's yeast, the specific VHH production rate is correlated with growth rate [45], and can be up to fivefold increased by growing on carbon source like ethyl alcohol [46]. The medium is provided with supplements like sorbitol, casamino acids, or ethylenediamine tetra acetic acid improves VHH production by P. pastoris [47].

VHHs cannot recruit effector functions such as ADCC and CDC on their own. This limits their therapeutic application. Although such effector functions can be indirectly recruited using conventional antibody fragments binding to host immunoglobulin [48] , it may be more efficient to recruit these functions by fusing VHHs to host Fc domains. Production of such functional

antibodies requires the correct glycosylation of the CH2 domain, which until recently could only be accomplished using higher eukaryotic cells [49] but not by microbial production. But, this may now be feasible using P. pastoris strains with an engineered glycosylation machinery that are capable to produce proteins with a specific human glycoform [50]. Furthermore, transgenic mice containing hybrid llama/ human antibody loci that contain llama V regions and human D, J, and C regions have recently been used to generate human heavy-chain antibodies in mice [51].

In addition to monovalent VHHs, several expression formats for the production of VHH multimers have been described. These include genetic fusions of two [52, 53] or three VHHs [54, 55] that either recognize different antigens or the same repeating antigen to increase functional affinity. Although such VHH fusions are less efficiently produced than their monovalent versions, their production level exceeds that of their conventional-antibody-based fusion counterparts without aggregation or low solubility. However, antigen binding by the C-terminal VHH in such fusions can be compromised [52] presumably because of steric hindrance by the N-terminal VHH.

APPLICATIONS OF SINGLE DOMAIN ANTIBODIES:

(1) Nbs incorporated into drug delivery systems:

Nbs can also be chemically attached to the surface of other drug delivery systems, such as Nano sized drug carriers or NPs, which can then be encapsulated with nonspecific drugs for active delivery to the site of interest. This is an attractive approach because it protects the body against systemic toxicity and allows solubilization of hydrophobic drug in hydrophilic structures, such as liposomes or micelles. Additionally, it permits administration of larger drug doses simultaneously, which could reduce the administration frequency and immunogenicity [56].

Important advances have been made with an Nb coupled to a drug delivery system.

A new interesting class of carrier systems is the polymer some, which architecturally resembles liposomes but is highly stable and can encapsulate larger amounts of hydrophilic drugs compared with micelles. This makes them particularly interesting for the delivery of cargo intracellular or for the controlled release of drugs. As an example, tumor vessel-targeting polymerases decorated with Nbs that target PlexinD1 a Tran's membrane protein overexpressed in tumor vasculature [57, 58]

(2) Toxin identification and detoxification:

The use of Nbs could further enhance toxin neutralization. Given their good tissue distribution, Nbs can more easily reach and neutralize toxins. However, some toxins are small, no immunogenic polypeptides, so obtaining a proper immune response can be problematic. Nbs have been raised against different toxic venom fractions of the Androctonus australis hector scorpion venom (AahG50, AahII, and AahI') [59, 60, 61, and 62]. Multiagency induced by polymerization of two Nbs against the two most toxic venom fractions improved their affinity. Also, proof-of-principle was obtained from experiments on rats, in which the specific Nbs had good

pharmacodynamics properties and effectively protected against envenoming [63, 64]. Hemiscorpius lepturus is another scorpion and the most dangerous in Iraq; heminenecrolysin (HNc) is the major hemolytic and dermo-necrotic venom fraction known from this species. Anti-HNc Nbs were raised and completely protected against HNc-induced envenoming [65]. Neutralizing antivenom Nbs against a-cobratoxin were also generated and fused to a human Fc fragment, thus retaining their high binding affinity to the toxin via the Nb but exerting the immunological properties of conventional Abs [66].

(3) Treatment of cancer:

As monoclonal antibodies single domain antibodies (nanobodie) are distributed homogeneously in tumor tissue [67]. As anti-cancer biological agents, Nbs can be used as antagonistic drugs, but due to the absence of an Fc-effector domain, their efficacy as a pure immunotherapeutic is inferior to that of mAbs [68]. Nevertheless, the absence of the Fc-domain in Nbs can reduce the number of unwanted immune-mediated adverse effects that are elicited by this domain. However, more promising approaches were recently intro-duced, such as their use as targeting moieties linked to effector domains and radionuclides. Additionally, they can be decorated on nanoparticles (NPs) that can be filled with other (small-molecule) anticancer drugs for active targeting to the specified tumor cells [69]. Two aspects need to be considered when such Nb – effector domain complexes are generated. First, the stability of the Nbs, which are reportedly very stable, might be attenuated [70] and, second, a change in Nb-binding affinity has been reported [68].

(4) Targeting bacteria and phages:

Nbs to combat bacteria can be raised against bacterial surface proteins to block bacterial attachment to host cells. Based on this principle, Nbs against the lactin domain of F18 fimbrial adhesion of the entero toxigenic E. coli and Shiga toxin-producing E. coli prevented attachment in vitro [71]. Pentameric Nbs enhance antigen agglutination, and pentavalency of Nbs can be conferred by exploiting the homo penta merization properties of nontoxic verotoxin B via linking the Nbs to that toxin [72]. Subsequently, the high-avidity pentabodies that bound the flagella of Campylobacter jejuni and other specific protease-resistant antiflagella Nbs demonstrated remarkable stability and potently inhibited the motility of C. jejuni [73]. The pentabodies was also potent in vivo, reducing C. jejuni colonization in the ceca of infected chickens [74]. By targeting the flagella, both bacterial motility and biofilm formation can be inhibited, as in the use of antiflagella Nbs against Pseudomonas aeruginosa [75]. Another protein that is important during biofilm formation is the biofilm-associated protein (Bap) and, consequently, anti-bap Nbs were developed as a strategy to combat Acinetobacter bauman-nii [186]. Nbs that prevent bacterial secretion of toxins can also be designed, such as Nbs against the type VI secretion system of Gram-negative bacteria [76].

(5) Single-domain antibodies against fungi and protozoans:

The potentials of single-domain antibodies have been only scarcely applied to fungi and protozoans. It is the case of the antibodies raised against a cell wall protein of *Malassezia furfur*, a fungus implicated in dandruff. Since the selected VHH antibodies should be potentially included in a shampoo formulation, they had to resist to the harsh

chemical conditions brought about by elevated concentrations of anionic and nonionic surfactants. Therefore, the panning washing conditions were adapted to represent the high-detergent content of shampoos [77]. This approach enabled the recovery of VHHs with specifically increased stability under denaturing. Nbs that target the paraflagellar rod protein of different trypano-somes have been described, but they are mainly useful as diagnostic markers of trypanosomiasis [78].

(6) Strategies for virus detection and neutralization:

Nbs can interfere at different levels of the viral replication cycle, such as by preventing virus–cell attachment, viral entry, and viral uncoating [79, 80]. An Nb directed against hepatitis C virus (HCV) specifically prevents viral cell entry and cell–cell transmission [81]. An intracellular expressed Nb that interferes with Rev Multimerization of HIV-1, a protein that is involved in nuclear trafficking of viral mRNAs, efficiently inhibited this crucial step in virus replication [82, 83]. Additionally, Nbs are useful in the study of the mechanisms of oligomer assembly of HIV [84]. In other studies, an Nb expressed in the cytosol that targets the nucleoprotein of influenza virus potently inhibited nuclear translocation [85] and another Nb expressed in the cytoplasm blocked the replication of porcine reproductive and respiratory syndrome virus (PRRSV) [86]. Prophylactic Nbs can be generated too, for example by generating modified lactobacilli that produce VHH antibody fragments, called 'lacto bodies'. Oral administration of lacto bodies expressing surface-anchored anti-rotavirus (RV) Nbs might be prophylactic against RV-induced diarrhea [87, 88, and 89]. Lactobacillus paracasei expression of bivalent Nbs or co-expression of two individual Nbs even led to protection against escape mutants and can also be used therapeutically [90,91].

(7) Nbs in neurodegenerative and other amyloid disorders:

There are currently only symptomatic treatments for neurodegenerative disorders; no disease-modifying or neuroprotective therapies that alter the natural disease course are available. Consequently, new and affordable therapies are needed.

(a) Alzheimer's disease:

AD is the most common neurodegenerative disease. It is characterized by the cerebral deposit of aggregated amyloid-b (Ab) peptide plaques and formation of neurofibrillary tangles [92], resulting in dementia and loss of cognitive functions. Ab plaques are formed via proteolytic cleavage of a large precursor protein, amyloid precursor protein (APP), by enzymes such as Beta-site APP-cleavage enzyme (BACE-1). Nbs that are selective for different amyloid (precursor) peptides have been produced, and Nbs that can prevent the formation of mature Ab fibrils by stabilizing Ab protofibrils have been identified [93, 94]. For diagnostic pur-poses, Ab-specific Nbs coupled to 99tmTc enabled the in vivo detection of vascular and parenchymal Ab deposits because they could cross the disrupted BBB, making them promising tools for such applications [95].

(a) Parkinson's disease:

Some attempts have been made to use Nbs to tackle the second most common

neurodegenerative disease, Parkinson's disease (PD). PD is characterized by the loss of dopaminergic neurons in the substantia nigra, and misfolding of a-synuclein (a-syn) into fibrillar aggregates seems to have a prominent role in the pathogenesis of this disease [96]. Consequently, reduction of the intracellular levels of a-syn is a logical therapeutic approach, the aim being the prevention of misfolding, aggregation, and toxicity [97]. NbSyn2 is an Nb directed against the C-terminal part of monomeric a-syn, but the Nb could not prevent aggregation because it bound both monomeric and aggregated protein [98, 99]. Nevertheless, those Nbs provide information about possible conformational rearrangements during fibrillar maturation and, therefore, are valuable for gaining knowledge about the structure of a-syn.

REFERENCES:

(1) Mullard, A, 2015. 2014 FDA drug approvals. Nat. Rev. Drug Discov. 14, 77–81 ss (2) Anon (2015) Pharmaceuticals going large. The Economist.

(2) Lipman, N.S., 2005. Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. ILAR J. 46, 258–268

(3) Olafsen, T., Wu, A.M., 2010. Antibody vectors for imaging. Semin. Nucl. Med. 40, 167–181

(4) Gong, R, 2012. Expression, purification, and characterization of engineered antibody CH2 and VH domains. Methods Mol. Biol. 899, 85–102

(5) Hamers Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R, 1993. Naturally occurring antibodies devoid of light chains. Nature 363:446–448

(6) Greenberg AS, Avila D, Hughes M, Hughes A, McKinney EC, Flajnik MF, 1995. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. Nature 374:168–173

(7) Muyldermans, S., 2013. Nano bodies' natural single-domain antibodies. Annu. Rev. Biochem. 82, 775–797

(8) Muyldermans, S., 2009. Camelid immune globulins and Nano body technology. Vet. Immunol. Immunopathology. 128, 178–183

(9) Dolk, E. 2005. Isolation of llama antibody fragments for prevention of dandruff by phage display in shampoo. Appl. Environ. Microbial. 71, 442–450

(10) Davies J, Reichmann L, 1995. Antibody VH domains as small recognition units. Biotechnology (NY) 13:475–479

(11) Davies J, Reichmann L, 1996. Single antibody domains as small recognition units: design and in vitro antigen selection of camelized, human VH domains with improved protein stability. Protein Eng. 9:531–537

(12) Van der Linden RH, Franken LG, De Geus B, Harmsen MM, Ruuls RC, Stok W, de Ron L, Wilson S, Davis P, Verrips CT ,1999. Comparison of physical chemical properties of llama VHH antibody fragments

and mouse monoclonal antibodies. Biochem Biopsy's Acta 1431:37–46

(13) Perez JM, Renisio JG, Prompers JJ, van Platerink CJ, Cambillau C, Darbon H, Franken LG, 2001. Thermal unfolding of a llama antibody fragment: a two-state reversible process. Biochemistry 40:74–83

(14) Perez, J.M, 2001. Thermal unfolding of a llama antibody fragment: a two-state reversible process. Biochemistry 40, 74–83

(15) Lauwereys, M., 1998. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. EMBO J. 17, 3512–3520

(16) Desmoulins, M., 2002. Single-domain antibody fragments with high conformational stability. Protein Sci. 11, 500–515

(17) Dolk, E., 2005. Isolation of llama antibody fragments for prevention of dandruff by phage display in shampoo. Appl. Environ. Microbial. 71, 442–450

(18) Hussack, G., 2011. Engineered single-domain antibodies with high protease resistance and thermal stability. PLoS ONE 6, e28218

(19) Van Bockstaele, F., 2009. The development of Nano bodies for therapeutic applications. Curr. Opin. Investing. Drugs 10, 1212–1224

(20) Behar, G., 2008 Isolation and characterization of anti-Fcgamma RIII (CD16) llama single-domain antibodies that activate natural killer cells. Protein Eng. Des. Sel. 21, 1–10

(21) Coppieters K, Dreier T, Silence K, de Haard H, Lauwereys M, Casteels P, Beirnaert E, Jonckheere H, Van de Wiele C, Staelens L, Hostens J, Revets H, Remaut E, Elewaut D, Rottiers P, 2006. Formatted anti-tumor necrosis factor alpha VHH proteins derived from camelids show superior potency and targeting to inflamed joints in a murine model of collagen-induced arthritis. Arthritis Rheum 54:1856–1866

(22) Transue, T.R., 1998. Camel single-domain antibody inhibits enzyme by mimicking carbohydrate substrate. Proteins 32, 515–522

(23) De Genst, E., 2006. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. Proc. Natl. Acad. Sci. U. S. A. 103, 4586–4591

(24) Vu KB, Ghahroudi MA, Wyns L, Muyldermans S, 1997. Comparison of llama VH sequences from conventional and heavy chain antibodies. Mol Immunol 34:1121–1131

(25) Harmsen MM, Ruuls RC, Nijman IJ, Niewold TA, Frenken LGJ, de Geus B ,2000. Llama heavy-chain V regions consist of at least four distinct subfamilies revealing novel sequence features. Mol Immunol 37:579–590

(26) Nguyen VK, Hamers R, Wyns L, Muyldermans S, 2000. Camel heavy-chain antibodies: diverse germline V (H) H and specific mechanisms enlarge the antigen-binding repertoire. Embo J 19:921–930

(27) Arbabi-Ghahroudi M, Tanha J, MacKenzie R, 2005. Prokaryotic expression

of antibodies. Cancer Metastasis Rev 24:501–519

(28) Estell D, 2006 Adapting industry practices for the rapid, large-scale manufacture of pharmaceutical proteins. Bridge 36:39–44

(29) Arbabi-Ghahroudi M, Tanha J, MacKenzie R, 2005. Prokaryotic expression of antibodies. Cancer Metastasis Rev 24:501–519

(30) Arbabi-Ghahroudi M, Desmyter A, Wyns L, Hamers R, Muyldermans S, 1997. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. FEBS Lett 414:521–526

(31) Rahbarizadeh F, Rasaee MJ, Forouzandeh Moghadam M, Allameh AA, 2005. High expression and purification of the recombinant camelid anti-MUC1 single domain antibodies in Escherichia coli. Protein Expr Purif 44:32–38

(32) Joosten V, Roelofs MS, van den Dries N, Goosen T, Verrips CT, van den Hondel CA, Lokman BC, 2005. Production of bifunctional proteins by Aspergillus awamori: llama variable heavy chain antibody fragment (V(HH)) R9 coupled to Arthromyces ramosus peroxidase (ARP). J Biotechnol 120:347–359

(33) Gerngross TU, 2004. Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. Nat Biotechnol 22:1409–1414

(34) Frenken LGJ, van der Linden RH, Hermans PW, Bos JW, Ruuls RC, de Geus B, Verrips CT, 2000. Isolation of antigen specific llama VHH antibody fragments and their high level secretion by Saccharomyces cerevisiae. J Biotechnol 78:11–21

(35) Thomassen YE, Meijer W, Sierkstra L, Verrips CT, 2002. Large-scale production of VHH antibody fragments by Saccharomyces cerevisiae. Enzyme Microb Technol 30:273–278

(36) Van der Vaart JM, Pant N, Wolvers D, Bezemer S, Hermans PW, Bellamy K, Sarker SA, van der Logt CP, Svensson L, Verrips CT, Hammarstrom L, van Klinken BJ, 2006. Reduction in morbidity of rotavirus induced diarrhoea in mice by yeast produced monovalent llama-derived antibody fragments. Vaccine 24:4130–4137

(37) Sethuraman N, Stadheim TA, 2006. Challenges in therapeutic glycoprotein production. Curr Opin Biotechnol 17:341–346

(38) Sagt CM, Kleizen B, Verwaal R, de Jong MD, Muller WH, Smits A, Visser C, Boonstra J, Verkleij AJ, Verrips CT, 2000. Introduction of an N-glycosylation site increases secretion of heterologous proteins in yeasts. Appl Environ Microbiol 66:4940–4944

(39) Simmons DP, Abregu FA, Krishnan UV, Proll DF, Streltsov VA,Doughty L, Hattarki MK, Nuttall SD, 2006. Dimerisation strategies for shark IgNAR single domain antibody fragments. J Immunol Methods 315:171–184

(40) Nieba L, Honegger A, Krebber C, Pluckthun A, 1997. Disrupting the hydrophobic patches at the antibody variable/constant domain interface:

improved in vivo folding and physical characterization of an engineered scFv fragment. Protein Eng. 10:435–444

(41) Harmsen MM, Van Solt CB, Fijten HPD, Van Setten MC, 2005. Prolonged in vivo residence times of llama single-domain antibody fragments in pigs by binding to porcine immunoglobulins. Vaccine 23:4926–4934

(42) Van der Linden RH, De Geus B, Frenken GJ, Peters H, Verrips CT, 2000. Improved production and function of llama heavy chain antibody fragments by molecular evolution. J Biotechnol 80:261–270

(43) Dufner P, Jermutus L, Minter RR, 2006. Harnessing phage and ribosome display for antibody optimisation. Trends Biotechnol 24:523–529

(44) Thomassen YE, Verkleij AJ, Boonstra J, Verrips CT, 2005. Specific production rate of VHH antibody fragments by Saccharomyces cerevisiae is correlated with growth rate, independent of nutrient limitation. J Biotechnol 118:270–277

(45) Van de Laar T, Visser C, Holster M, Lopez CG, Kreuning D, Sierkstra L, Lindner N, Verrips T, 2007. Increased heterologous protein production by Saccharomyces cerevisiae growing on ethanol as sole carbon source. Biotechnol Bioeng 96:483–494

(46) Rahbarizadeh F, Rasaee MJ, Forouzandeh M, Allameh AA, 2006. Over expression of anti-MUC1 single-domain antibody frag-ments in the yeast Pichia pastoris. Mol Immunol 43:426–435

(47) Nguyen VK, Zou X, Lauwereys M, Brys L, Bruggemann M, Muyldermans S ,2003. Heavy-chain only antibodies derived from dromedary are secreted and displayed by mouse B cells. Immunology 109:93–101

(48) Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi BK, Hopkins D, Wischnewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S, Gerngross TU, 2006. Humanization of yeast to produce complex terminally sialylated glycoproteins. Science 313:1441–1443

(49) Janssens R, Dekker S, Hendriks RW, Panayotou G, van Remoortere A, San JK, Grosveld F, Drabek D, 2006. Generation of heavy-chain-only antibodies in mice. Proc Natl Acad Sci USA 103:15130–15135

(50) Conrath KE, Lauwereys M, Wyns L, Muyldermans S, 2001. Camel single-domain antibodies as modular building units in bispecific and bivalent antibody constructs. J Biol Chem 276:7346–7350

(51) Harmsen MM, Van Solt CB, Fijten HPD, Van Setten MC, 2005. Prolonged in vivo residence times of llama single-domain antibody fragments in pigs by binding to porcine immunoglobu-lins. Vaccine 23:4926–4934

(52) Coppieters K, Dreier T, Silence K, de Haard H, Lauwereys M, Casteels P, Beirnaert E, Jonckheere H, Van de Wiele C, Staelens L, Hostens J, Revets H, Remaut E, Elewaut D, Rottiers P, 2006. Formatted anti-tumor necrosis factor alpha VHH proteins derived from camelids show superior

potency and targeting to inflamed joints in a murine model of collagen-induced arthritis. Arthritis Rheum 54:1856–1866

(53) Roovers RC, Laeremans T, Huang L, De Taeye S, Verkleij AJ, Revets H, De Haard HJ, Van Bergen en Henegouwen PM , 2007. Efficient inhibition of EGFR signaling and of tumour growth by antagonistic anti-EFGR Nanobodies. Cancer Immunol Immun-other 56:303–317

(54) Oliveira, S., 2013. Targeting tumors with nanobodies for cancer imaging and therapy. J. Control Release 172, 607–617

(55) Debets, M.F., 2013 Nanobody-functionalized polymersomes for tumor-vessel targeting. Macromol. Biosci. 13, 938–945

(56) Zou, T., 2015. Nanobody-functionalized PEG-b-PCL polymersomes and their targeting study. J. Biotechnol. 214, 147–155

(57) Meddeb Mouelhi, F., 2003. Immunized camel sera and derived immunoglobulin subclasses neutralizing Androctonus australis hector scorpion toxins. Toxicon 42, 785–791

(58) Abderrazek, R.B., 2009. Identification of potent nanobodies to neutralize the most poisonous polypeptide from scorpion venom. Biochem. J. 424, 263–272

(59) Hmila, I, 2008. VHH, bivalent domains and chimeric heavy chain-only antibodies with high neutralizing efficacy for scorpion toxin AahI'. Mol. Immunol. 45, 3847–3856

(60) Ezzine, A. 2012. Efficient expression of the anti-AahI' scorpion toxin nanobody under a new functional form in a Pichia pastoris system. Biotechnol. Appl. Biochem. 59, 15–21

(61) Hmila, I., 2010. A bispecific nanobody to provide full protection against lethal scorpion envenoming. FASEB J. 24, 3479–3489

(62) Hmila, I., 2012. Pre-clinical studies of toxin-specific nanobodies: evidence of in vivo efficacy to prevent fatal disturbances provoked by scorpion envenoming. Toxicol. Appl. Pharmacol. 264, 222–231

(63) Yardehnavi, N. 2014. A camelid antibody candidate for development of a therapeutic agent against Hemiscorpius lepturus envenomation. FASEB J. 28, 4004–4014

(64) Richard, G, 2013. In vivo neutralization of alpha-cobratoxin with high-affinity llama single-domain antibodies (VHHs) VHH-Fc antibody. PLoS ONE 8, e69495

(65) Oliveira, S., 2012. Rapid visualization of human tumor xenografts through optical imaging with a near-infrared fluorescent anti epidermal growth factor receptor nanobody. Mol. Imaging 11, 33–46

(66) Kijanka, M, 2013. Rapid optical imaging of human breast tumour xenografts using anti-HER2 VHHs site-directly conjugated to IRDye 800CW for image-guided surgery. Eur. J. Nucl. Med. Mol. Imaging 40, 1718–1729

(67) Oliveira, S, 2013. Targeting tumors with Nano bodies for cancer imaging and therapy. J. Control Release 172, 607–617

(68) Kijanka, M., 2015. Nanobody-based cancer therapy of solid tumors. Nano medicine 10, 161–174

(69) Moonens, K, 2014. Nanobody mediated inhibition of attachment of F18 Fimbriae expressing Escherichia coli. PLoS ONE 9, e114691

(70) Zhang, J., 2004. A pentavalent single-domain antibody approach to tumor antigen discovery and the development of novel proteomics reagents. J. Mol. Biol. 341, 161–169

(71) Hussack G, 2014. Protease-resistant single-domain antibodies inhibit Campy lobacter jejuni motility. Protein Eng. Des. Sel. 27, 191–198

(72) Riazi, A, 2013. Pentavalent single-domain antibodies reduce Campylobacter jejuni motility and colonization in chickens. PLoS ONE 8, e83928

(73) Adams, H, 2014. Inhibition of biofilm formation by Camelid single-domain antibodies against the flagellum of Pseudomonas aeruginosa. J. Biotechnol. 186, 66–73

(74) Nguyen, V.S. et al. (2015) Inhibition of type VI secretion by an anti TssMllama nanobody. PLoS ONE 10, e0122187

(75) Dolk, E., 2005. Isolation of llama antibody fragments for prevention of dandruff by phage display in shampoo. Appl. Environ. microbial. 71, 442–450

(76) Obishakin, E. 2014. Generation of a nanobody targeting the paraflagellar rod protein of trypanosomes. PLoS ONE 9, e115893

(78) Schotte, L. 2014. Mechanism of action and capsid-stabilizing properties of VHHs with an in vitro antipolioviral activity. J. Virol. 88, 4403–4413

(79) Jahnichen, S. 2010. CXCR4 nanobodies (VHH-based single variable domains) potently inhibit chemotaxis and HIV-1 replication and mobilize stem cells. Proc. Natl. Acad. Sci. U. S. A. 107, 20565–20570

(80) Tarr, A.W., 2013. An alpaca nanobody inhibits hepatitis C virus entry and cell-to-cell transmission. Hepatology 58, 932–939

(81) Ibanez, L.I., 2011. Nanobodies with in vitro neutralizing activity protect mice against H5N1 influenza virus infection. J. Infect. Dis. 203, 1063–1072

(82) Tillib, S.V, 2013. Formatted single-domain antibodies can protect mice against infection with influenza virus (H5N2). Antiviral Res. 97, 245–254

(83) Boons, E., 2014. A stably expressed llama single-domain intrabody targeting Rev displays broad-spectrum anti-HIV activity. Antiviral Res. 112, 91–102

(84) Ashour, J., 2015. Intracellular expression of camelid single-domain antibodies specific for influenza virus nucleoprotein uncovers distinct features of its nuclear localization. J. Virol. 89, 2792–2800

(85) Liu, H., 2015. An intracellularly expressed Nsp9-specific nanobody in MARC-145 cells inhibits porcine reproductive and respiratory syndrome virus replication. Vet. Microbiol. 181, 252–260

(86) Pant, N., 2006. Lactobacilli expressing variable domain of llama heavy-chain antibody fragments (lactobodies) confer protection against rotavirus-induced diarrhea. J. Infect. Dis. 194, 1580–1588

(87) van der Vaart, J.M., 2006. Reduction in morbidity of rotavirus induced diarrhoea in mice by yeast produced monovalent llama-derived antibody fragments. Vaccine 24, 4130–4137

(88) Martin, M.C., 2011. Integrative expression system for delivery of antibody fragments by lactobacilli. Appl. Environ. Microbiol. 77, 2174–2179

(89) Pant, N. 2011. Lactobacilli producing bispecific llama-derived anti-rotavirus proteins in vivo for rotavirus-induced diarrhea. Future Microbiol. 6, 583–593

(90) Gunaydin, G. 2014. Co-expression of anti-rotavirus proteins (llama VHH antibody fragments) in Lactobacillus: development and functionality of vectors containing two expression cassettes in tandem. PLoS ONE 9, e96409

(91) Waldmeier, P.C. and Tatton, W.G. ,2004. Interrupting apoptosis in neurodegenerative disease: potential for effective therapy? Drug Discov. Today 9, 210–218

(92) Habicht, G., 2007. Directed selection of a conformational antibody domain that prevents mature amyloid fibril formation by stabilizing Abeta protofibrils. Proc. Natl. Acad. Sci. U. S. A. 104, 19232–19237

(93) Lafaye, P., 2009. Single-domain antibodies recognize selectively small oligomeric forms of amyloid beta, prevent Abeta-induced neurotoxicity and inhibit fibril formation. Mol. Immunol. 46, 695–704

(94) Nabuurs, R.J., 2012. In vivo detection of amyloid-b deposits using heavy chain antibody fragments in a transgenic mouse model for Alzheimer's disease. PLoS ONE 7, e38284

(95) Goedert, M., 2015. Neurodegeneration. Alzheimer's and Parkinson's diseases: the prion concept in relation to assembled Ab, tau, and a-synuclein. Science 349, 1255555

(96) Messer, A. and Joshi, S.N., 2013. Intrabodies as neuroprotective therapeutics. Neurotherapeutics 10, 447–458

(97) Vuchelen, A., 2009. (1)H, (13) C (15) N assignments of a camelid nanobody directed against human alpha-synuclein. Biomol. NMR Assign. 3, 231–233

(98) De Genst, E.J., 2010. Structure and properties of a complex of alpha-synuclein and a single-domain camelid antibody. J. Mol. Biol. 402, 326–343

(99) Guilliams, T., 2013. Nanobodies raised against monomeric alpha-synuclein distinguish between fibrils at different maturation stages. J. Mol. Biol. 425, 2397–2411.