www.phmd.pl
Review

**Received:** 2012.04.12
**Accepted:** 2012.05.18
**Published:** 2012.06.12

# Variable fragments of heavy chain antibodies (VHHs): a new magic bullet molecule of medicine?*

## Zmienne fragmenty przeciwciał ciężkołańcuchowych (VHH): nowa magiczna kula medycyny?

**Dorota Smolarek[1,2,3,4], Olivier Bertrand[2,3,4], Marcin Czerwinski[1,5]**

[1] Laboratory of Glycoconjugate Immunochemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland
[2] INSERM, UMR_S 665, F-75015 Paris, France
[3] Institut National de la Transfusion Sanguine, F-75015 Paris, France
[4] University Paris 7-Denis Diderot, 75013 Paris, France
[5] Institute of Physiotherapy, Faculty of Physical Education and Physiotherapy, Opole University of Technology, Opole, Poland

## Summary

Serum of animals belonging to the *Camelidae* family (camels and llamas) contains fully active antibodies that are naturally devoid of light chains. Variable domains derived from heavy chain antibodies (hcAb) called VHHs or nanobodies™ can bind antigens as effectively as full-length antibodies and are easy to clone and express. Because of their potential, VHHs are being intensively studied as potential therapeutic, diagnostic and imaging tools. The paper reviews the molecular background of heavy chain antibodies and describes methods of obtaining recombinant fragments of heavy chain antibodies as well as their therapeutic, diagnostic and other applications.

**Key words:** **heavy chain antibodies • VHH • Nanobody**

## Streszczenie

Zwierzęta należące do rodziny *Camelidae* (wielbłądy i lamy) wytwarzają funkcjonalne przeciwciała pozbawione lekkich łańcuchów, nazywane przeciwciałami ciężkołańcuchowymi (hcAb). Zmienne regiony tych przeciwciał (VHH lub Nanobody™) wykazują wysokie powinowactwo wobec antygenów, a jednocześnie mogą być wytwarzane z dużą wydajnością w systemach bakteryjnych. Ze względu na ich zalety w porównaniu z rekombinowanymi fragmentami klasycznych przeciwciał, prowadzi się intensywne badania nad zastosowaniem VHH w terapii, diagnostyce, obrazowaniu *in vivo* czy do oczyszczania białek.

**Słowa kluczowe:** **przeciwciała ciężkołańcuchowe • VHH • Nanobody**

**Full-text PDF:** http://www.phmd.pl/fulltxt.php?ICID=1000334

* This study was supported by grant no. N N302 118835 from the Ministry of Science and Higher Education of Poland.

**Word count:** 4491
**Tables:** –
**Figures:** 8
**References:** 90

**Author's address:** Marcin Czerwiński, Laboratory of Glycoconjugate Immunochemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, 53-114 Wrocław; e-mail: czerwins@iitd.pan.wroc.pl

**Abbreviations:** **BiP** – heavy chain binding protein; **ER** – endoplasmic reticulum; **Fab** – antigen-binding fragment of antibody; **hcAb** – camel heavy chain antibody; **scFv** – single chain variable fragment of antibody; **VHH** – variable fragment of camel heavy chain antibodies.

## Introduction

Discovery of the technique of monoclonal antibody production triggered a revolution in biotechnology. The procedure, developed by Kohler and Milstein, is based on a chemical fusion of murine spleen cells that produce antibodies with myeloma cells that give the new cells immortality [46]. Monoclonal antibodies usually are monospecific (they recognize only one type of antigen), and once cloned, they can be produced for ever. Therapeutic monoclonal antibodies belong to the fastest-growing branch of biotechnology, but they have some limitations such as high cost of production in eukaryotic systems and large size that may disturb efficient tissue penetration. A solution for these problems can be obtaining Fab fragments, since they are three times smaller than full-size antibodies and can be expressed in a bacterial system. However, since Fab fragments are heterodimers consisting of variable regions of heavy and light chains linked by disulfide bridges, their cloning may encounter several difficulties and expression is not always efficient. Expression of single-chain variable fragments (scFv) of antibody can also create problems, since such fragments are less soluble and they require a linker to keep both domains together (Fig. 1). In addition, scFv usually have lower affinity than Fab fragments or antibodies and show a tendency to aggregate [87].

A sensible approach to avoid such problems is expression of functional fragments of heavy chain antibodies (hcAb), which are present in serum of animals belonging to the *Camelidae* family. Variable regions of heavy chain antibodies called VHHs or nanobodiesTM consist of single polypeptide chains and have the following advantages over fragments of full-size antibodies and their fragments: easy, one-step cloning, small size that enables efficient penetration of tissues, and high efficiency of expression of relatively stable protein in bacteria [13,54].

## Family of Camelidae

The *Camelidae* family (order: Artiodactyla) consists of six species: dromedary (*Camelus dromedarius*), Bactrian camel (*Camelus bactrianus*), llama (*Lama glama*), guanaco (*Lama guanicoe*), alpaca (*Lama pacos*) and vicuna (*Lama vicugna*). Animals belonging to this family are well adapted to live in demanding environments such as deserts or high altitudes. They can lose water up to 30% of body mass, while for other mammals losing 12% is lethal. The high tolerance for dehydration is possible due to their ability to regulate body temperature, which allows them to decrease transpiration, and elliptic erythrocytes that are resistant to changes of ionic strength [60].

## 1. Structure and sequence of camel heavy chain antibodies

Camel heavy chain antibodies were discovered by Ungar-Waroni et al., who isolated low molecular weight Ig-like proteins from dromedary serum and showed their significance in transmission of passive immunity from dam to calf by colostrum [79]. However, the detailed characterization and demonstration of potential usefulness of heavy chain antibodies stemmed from the work of the Hamers group in Brussels [33]. When analyzing antibodies isolated from camel serum on protein A or protein G on SDS-PAGE, they found that the unusual proteins belong to a new subclass of antibodies with lower molecular weight. In the absence of a reducing agent, a band of 94 kDa was visible on the gel in addition to the expected 150 kDa band, while in the presence of a reducing agent, only one 43 kDa band was found and no light chain was observed [33]. The authors assumed that camel serum contains antibodies that are homodimers and consist of heavy chains only.

There are three subclasses of functional IgGs present in serum of *Camelidae*: IgG1 is a heterodimer consisting of heavy and light chain homodimers, while IgG2 and IgG3 consist only of heavy chains and thus are called heavy chain antibodies (hcAbs). Heavy chains of hcAbs consist of variable domains called VHHs, followed by constant domains CH2 and CH3 (Fig. 1). Contrasting with regular antibody, there is no CH1 domain [55] and the hinge region linking VHH and CH2 regions is usually elongated, composed of repeated residues of proline, lysine, glutamine or glutamic acid. In consequence, the structure of the hinge region is more rigid and the distance between two binding domains is increased in comparison to regular IgGs [56]. Antigen-binding fragments of regular antibodies can be expressed in the form of Fab or scFv fragments, and in both cases heavy and light chains have to be cloned separately. In contrast, the active antigen-binding fragment of heavy chain antibodies can be cloned and expressed in the form of VHH, which consists of only one polypeptide chain (Fig. 1).

Why are light chains not present in heavy chain antibodies? In most mammalian cells, heavy and light chains combine together in the endoplasmic reticulum immediately after translation. The major ER protein involved in translocation, secretion, quality control and (if necessary) degradation of secreted proteins is called heavy chain binding protein (BiP) [24,51]. A nascent heavy chain

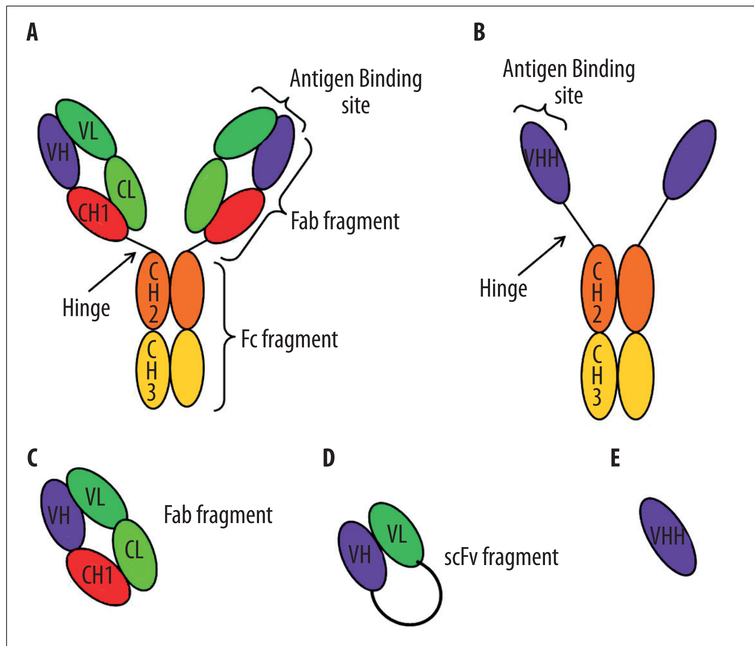


Fig. 1. Schematic representation of regular (**A**) and heavy chain antibodies (**B**) and their fragments. The antigen binding site of a hcAb is composed of only one domain, called VHH, in contrast to regular antibodies in which that region is composed of VH and VL. The hcAbs also lack the CH1 domain of a heavy chain, and the hinge region is elongated in comparison to regular IgG. Fragments that can be derived from regular antibodies: Fab (**C**) and scFv (**D**) fragments and from heavy chain antibodies: VHH (**E**)

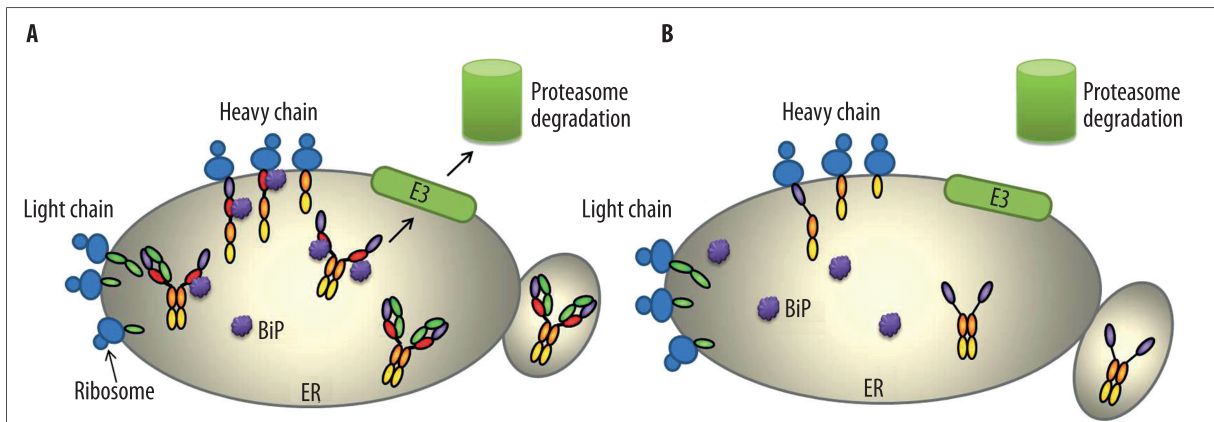


Fig. 2. Expression of heavy and light chains in endoplasmic reticulum (ER). Heavy and light chains are produced by ribosomes (R) and combine together in the lumen of the ER. (**A**) translation, secretion or degradation of conventional antibodies in the ER: the heavy chain is bound by BiP and released only when replaced by the light chain; if the light chain is not present, the heavy chain homodimer is bound by the E3 ubiquitin ligase complex and transported across the membrane to the proteasome. (**B**) translation and secretion of antibodies that lack CH1 domain in ER. BiP cannot bind hcAbs and the protein is secreted [36]

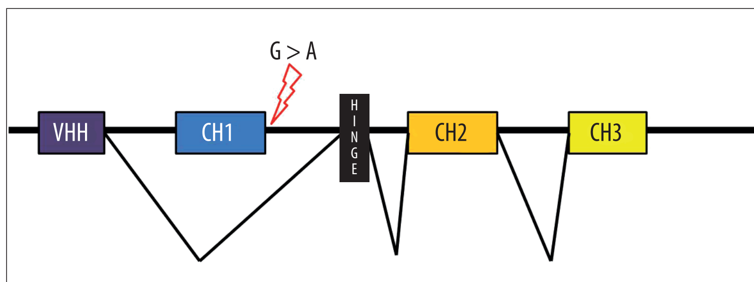


Fig. 3. Mechanism of removal of exon encoding CH1 during mRNA processing [57]. Mutation G>A that occurs at the 3'-border of the exon encoding the CH1 domain in the splicing site leads to its removal during splicing together with downstream and upstream introns

is bound by BiP immediately after translation and is retained until a light chain can replace BiP. If a light chain is absent, the heavy chain-BiP complex is bound by E3 ubiquitin ligase complex and transported across the membrane to the proteasome [70] (Fig. 2A). BiP binds specifically to the CH1 segment of the heavy chain [36] and retains the heavy chain in the endoplasmic reticulum until BiP is exchanged for a light chain. Since CH1 regions are not present in antibodies belonging to the camel class IgG2 and IgG3, such antibodies cannot be retained by BiP and are exported (Fig. 2B). CH1 regions are missing in mature heavy chain antibodies because of the mutation G>A at the 3'-border between CH1 exon and the downstream intron. The mutation does not allow the spliceosome to recognize the splicing site, so the exon encoding CH1 is removed together with introns (Fig. 3.) [57,86].

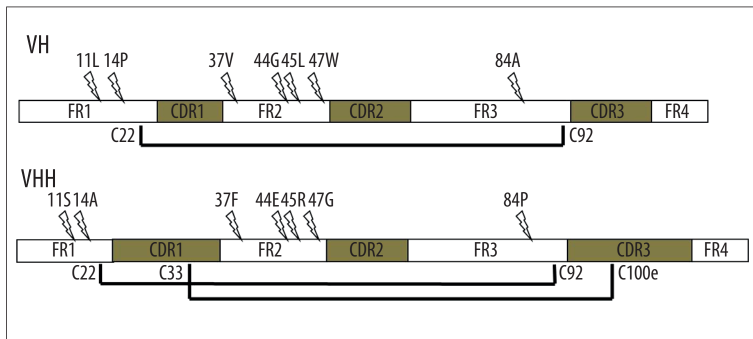


Fig. 4. Schematic representation of differences in amino acid sequence between cDNA encoding variable regions of regular and heavy chain antibodies [13]. The critical amino acid substitutions in VHH are marked by lightning symbols. Thick black lines connecting FR1-FR3 and CDR1-CDR3 represent disulfide bridges that stabilize the structure of the heavy chain

Variable domains of hcAbs contain several amino acid substitutions in comparison with heavy chains of regular antibodies. Most of these amino acid changes are found in the region that is engaged in hydrophobic interactions with a light chain: Val-37, Gly-44, Leu-45 and Trp-47 are usually replaced in hcAbs by Phe, Glu, Arg and Gly, respectively (Fig. 4) [55]. Since a light chain is not present, these mutations provide higher solubility and stability to VHH (and exclude the possibility of pairing to a light chain). Other mutations may occur within framework regions and can make VHHs even more stable.

There are several additional features that make VHHs different to regular antibodies and determine their unique properties. The CDR3 loops of hcAbs are usually longer than CDR3s in regular IgGs, and it is believed that such long loops may compensate for the smaller antigen binding site. Thus, the average length of CDR3 in heavy-chain antibody is 16-18 amino acids [55] in comparison to 9-12 amino acids in human antibody [88]. An extremely long CDR3 with a 24 amino acid loop was found in a camel VHH that recognizes lysozyme: it was shown that the VHH can reach the active site of an enzyme and act as its competitive inhibitor [19]. However, it should be noted that elongated CDR3 regions are not always found in heavy chain antibodies, because CDR3s as short as 3 amino acids have also been described, particularly in llama VHHs [82]. It was also found that the CDR1 region can be elongated in VHHs and may show high variability [58,82].

Another distinctive feature of VHHs is a second disulfide bridge linking CDR1 and CDR3 (usually between amino acids 33 and 100e, Fig. 4.). The additional disulfide bridge can stabilize the protein, but it may also enable formation of a new kind of loop that may recognize increased variety of epitopes [58].

It should be noted that heavy chain antibodies may also occur in humans or animals as a rare pathological event. Lack of a light chain is most often caused by mutations that lead to removal or shortening of the N-terminus of the heavy chain, which makes joining of light and heavy chains impossible. However, such abnormal proteins do not behave as antibodies (i.e. do not recognize a peculiar antigen) and tend to form aggregates that accumulate in different body compartments, causing the syndrome of heavy chain disease [83].

## 2. Heavy chain antibodies in other species

In addition to the members of the *Camelidae* family, heavy chain antibodies have been found in the adaptive immune system of cartilaginous fishes (sharks, rays and ratfish). Such antibodies, called immunoglobulin new antigen receptors (IgNARs), consist of five constant domains and one variable domain, which is responsible for antigen binding [30,66]. Interestingly, presence of the elongated CDR3 domain, increased occurrence of somatic mutations and an additional pair of cysteines that stabilize the structure are features that resemble antibodies found in animals belonging to the *Camelidae* family [20,21,30].

The occurrence of hcAbs in evolutionarily distinct animal was reviewed by Flajnik et al. [25]. The authors suggest several possible explanations of this phenomenon: the light chain could have been used by a virus as a co-receptor for infection of B lymphocytes and that was the cause of light chain deletion. When the virus disappeared, the light chain could be used again; however, it was not restored to all kinds of IgGs. Alternatively, light chains could cause amyloidosis, and so their disappearance could serve as a positive evolutionary factor. However, the most probable possibility is that hcAbs have arisen as a means to reach less accessible epitopes.

## 3. Recombination and rearrangement

Shuffling of genes encoding VH and VL domains, which is one of the sources of imunoglobulin diversity, obviously does not exist in hcAbs. Nevertheless, the diversity of heavy chain antibodies is similar to the diversity of regular antibodies. Variable regions of antibodies belonging to all classes of *Camelidae* IgG are encoded by the same pool of D and J genes, but V gene segments that encode VHs and VHHs are usually distinct. Approximately 40 germline V genes used exclusively by heavy chain antibodies are present in the camel genome, and all are homologous to the genes of the human VH3 family [14]. Four additional germline genes used by both VHs and VHHs share homology with genes belonging to the human VH4 family [16]. It is noteworthy that camel VHH fragments are more diverse than VH fragments [58]. The increased diversity of hcAbs and high number of off-sized VHHs in comparison with regular antibodies may be caused by the following mechanisms:

- increased number of hotspots in the regions that usually do not show high hypervariability (e. g. elongated CDR1, aa 27–30 [58]);
- more frequent insertions and deletions that increase variability of CDR3 length [58];
- elongation of CDR3 that may be caused by use of more than one D gene segments during V-D-J recombination, or enhanced activity of terminal deoxynucleotidyl transferase [56].

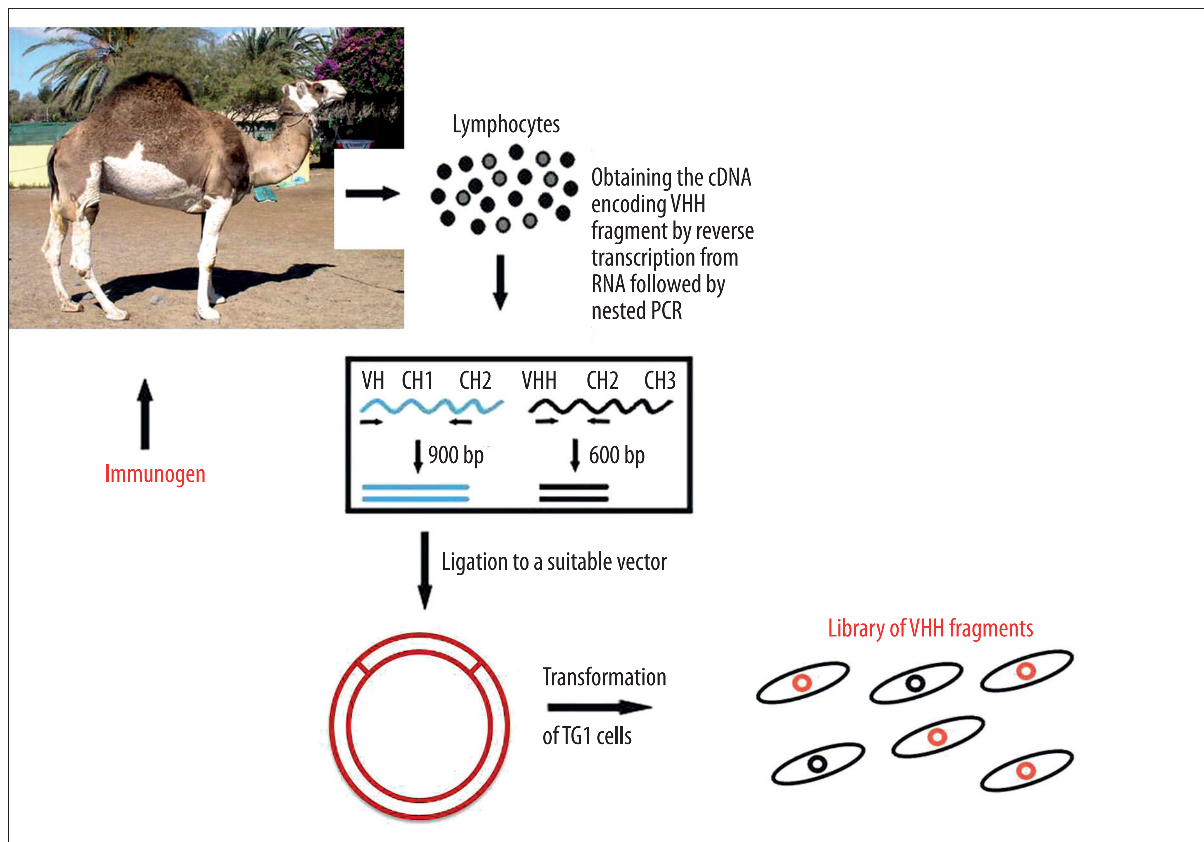


Fig. 5. Procedure of obtaning a library of heavy chain antibody fragments. An animal is immunized with antigen, mRNA is isolated and cDNA encoding VHH fragments is cloned into the phage expression vector. Antigen-binding VHH fragments are then selected from the library by a panning procedure

## 4. Unique properties of VHH fragments

VHHs have the following advantages over murine antibodies and their recombinant fragments:

- VHHs are weakly immunogenic in humans because genes encoding them share high sequence homology with genes belonging to the human VH families 3 and 4 [16,20];
- since VHHs consist of only one domain, they are easy to clone and can be expressed with high yields using different expression systems (described in section 7) [34];
- the high variability of length and sequence of VHHs makes them capable of recognizing a variety of epitopes, located not only on the surface of a protein [17], but also buried deep in the clefts [4]. VHHs have been shown to recognize a wide range of epitopes, ranging from small haptens [47,72,73] to binding sites of enzymes [18,19];
- the small size of VHHs allows them to penetrate tissues, pass through barriers such as the blood-brain barrier and bind epitopes that cannot be reached by conventional antibodies [2,48];
- VHHs show high solubility and stability even in denaturing conditions or high temperatures [47].

## 5. Methods of VHH preparation

Heavy chain antibodies can be produced in animals belonging to all species of the *Camelidae* family. Llamas are easier to raise and breed (because they are smaller), but camels have a better ratio of heavy chain antibodies to regular antibodies [54]. VHHs are usually obtained from libraries that can be prepared from RNA of a naive or immunized animal (a general scheme of obtaining a heavy chain library is shown in Fig. 5). Usually, an animal is immunized with 1 mg or less of antigen (several antigens can be injected simultaneously) once a week for five consecutive weeks (but obviously other regimens may be used). Complete Freund adjuvant may be used for the first injection and incomplete Freund adjuvant for the following injections. Lymphocytes that are the source of mRNA can be isolated either from peripheral blood or from lymph nodes. Alternatively, libraries can be prepared as a synthetic pool obtained by grafting randomized CDR regions into heavy chain scaffolds [54,67]. The first method has an obvious advantage: VHHs that are matured *in vivo* usually have higher affinity and are more stable than those from non-immune or synthetic pools. On the other hand, non-immune (from naive animal or synthetic) libraries may be the only solution if the antigen is toxic or cannot be used for immunization. Such VHHs may have lower kinetic constants, but sometimes high affinity is not required. If a high affinity is desired, one may use *in vitro* maturation (e.g. by error-prone PCR), but it can be a long and tedious process.

When a library of appropriate size is obtained, VHHs can be selected. The most frequently used method is solid-phase phage display (Fig. 6), in which amplified cDNAs encoding variable regions are expressed on the surface of filamentous phages. Phages presenting variable regions that bind to the antigen are isolated by a procedure called panning and can be used in the next round of panning or to clone variable regions [13,85].

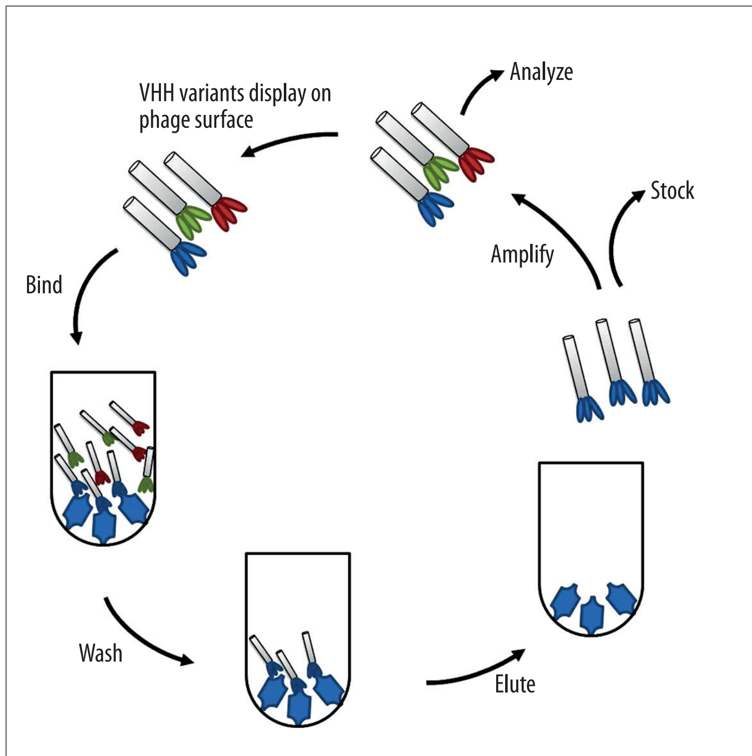


Fig. 6. Procedure of solid phase panning of proteins using phage display. The phages expressing VHH fragments on the surface are incubated with an antigen coated on a solid phase and the unbound phages are washed out. The phages bound to the antigen are eluted using high pH solution. Obtained phages can be used to infect bacteria for the next round of panning or to characterize the VHHs encoded by phages

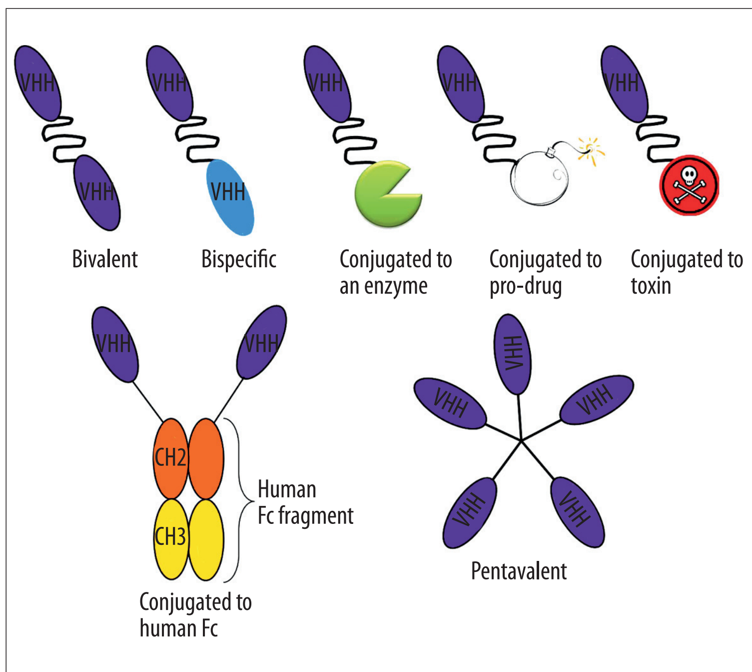


Fig. 7. Different kinds of VHH multimerization or tagging

There are several methods of expression of soluble VHH fragments. The first choice is usually production in *Escherichia coli*, where depending on the selected vector and bacterial strain, expressed protein can be directed to the periplasm or remain in the cytoplasm as a soluble product or inclusion bodies, with yields reaching 5–10 mg of protein from 1 l of culture [27,32]. The other option is expression in yeast; yields of VHHs expressed in *Saccharomyces cerevisiae* can reach 250 mg of pure protein secreted into one liter of culture [77]. The yields of protein can be even higher if *Pichia pastoris* yeast is selected for protein expression [62]. Production of VHHs is also possible in tobacco [43,63].

VHHs can be fused to various partners, either in order to increase their size or to confer new properties or effector functions. Thus the VHHs can be (Fig. 7):

- bivalent
  – fused by a linker [8];
  – fused to the IgG Fc domain, which can in addition confer antibody-dependent cell cytotoxicity [37];
- bispecific [10,42];

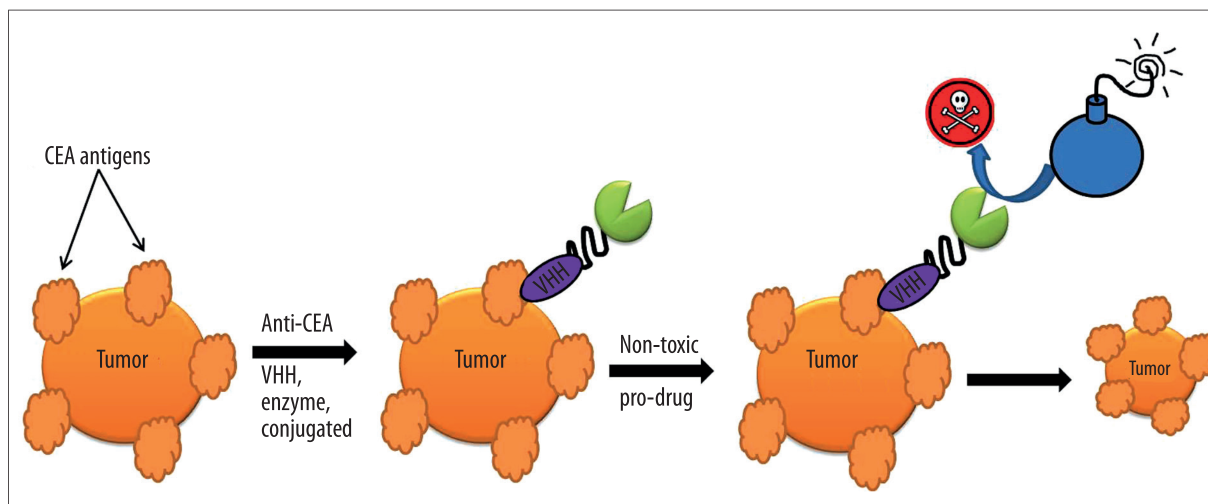


Fig. 8. Application of VHH in ADEPT therapy. VHH fused to an enzyme (green pacman) cleaves pro-drug (cephalosporin mustard marked as a bomb) into its active form phenylenediamine mustard (skull), causing decrease of tumor mass. Generation of a toxic pro-drug in the close vicinity of the tumor ensures high efficiency and low toxicity of ADEPT therapy (based on [11])

- pentavalent [89,90];
- decavalent [76];
- fused to an enzyme [12];
- fused to another protein to increase the size and half-life, such as an Fc fragment [10].

## 6. Applications of VHH fragments

The unique properties of VHHs make them useful in many applications, such as therapy, diagnostics and laboratory praxis. Some of the applications are described below and more are reviewed in [54,61,85].

### 6.1. VHHs in therapy

#### *6.1.1. Anti-cancer therapy*

Since it was demonstrated that VHHs can bind antigens on the surface of tumor cells and their immunogenicity is low [12], VHHs are under intensive scrutiny as potential therapeutic tools, and among the most studied are VHHs with potential anti-cancer activity. To date, VHHs recognizing antigens that are expressed by cells of many types of cancer have been obtained, and many of them reveal prospective therapeutic values.

A high-affinity VHH recognizing carcinoembryonic antigen (CEA), a protein overexpressed in many types of tumors, was used as a vehicle in the technique called antibody-dependent enzyme pro-drug therapy (ADEPT). The anti-CEA VHH was used as a transporter fused to β-lactamase, an enzyme that can cleave a pro-drug of low toxicity into its toxic form (Fig. 8). The method was proved effective using a mouse model of adenocarcinoma: it was found that the tumor mass had decreased significantly, while the toxicity of the drug was relatively low [11]. Another VHH with therapeutic potential is endoglin, a receptor involved in angiogenesis, which is up-regulated in cancer tissues. It was shown that the VHH recognizes the antigen with high affinity and can act as an anti-angiogenic agent by binding endoglin and inhibiting angiogenesis in the tumor [3].

A VHH recognizing Fcγ receptor III was shown to activate natural killer (NK) cells and induce production of interferon γ. The mono- and bispecific hcAb fragments activated FcγRIII and recruited NK cells to the target site. Such a VHH, if fused to another VHH that recognizes a protein present on the surface of the tumor, may be used in anti-cancer therapy [5]. It was also shown that VHH recognizing integrin α3β1 (VLA3), an adhesion protein involved in cancer metastasis, can decrease the metastatic potential of malignant tumors. The same VHH can block cell-mediated adhesion by modulation of VLA3 functions, so that VHH can be used to decrease tumor metastasis [31].

#### *6.1.2. Hematological disorders*

VHHs can also be used in other therapeutic applications. For example, advanced studies on therapeutic use of VHHs are focused on von Willebrand disease. Von Willebrand factor (VWF) is involved in the formation of blood clots by interactions with clotting factor VIII and platelets. There are two disorders involving VWF that may be treated with VHHs: vWFD type 2B that is caused by a decreased level of VWF and leads to elongated bleeding time, and thrombotic thrombocytopenic purpura, which is caused by the lack of an enzyme that cleaves ultra-large VWF multimers which bind platelets. Such aggregates may accumulate in vessels, causing obstruction of blood flow. Hulestin et al. [40] obtained a VHH that binds to the active form of VWF and may be used as a diagnostic tool to detect VWF in serum, since an increased level of active VWF is a key factor in both kinds of disease. Two VHHs recognizing VWF are currently undergoing clinical trials [69]. A VHH that prevents platelet aggregation binds to ultra-large VWF multimers and may be used to prevent spontaneous clotting in acute coronary syndrome and percutaneous coronary intervention [78,80]. The other VHH improves the time course of normalization of the platelet level in blood and prevents capture of platelets by VWF, so it may lead to improved treatment of von Willebrand disease type 2B in addition to the standard plasma-exchange therapy. Currently (March 2012), both drugs are in phase II clinical trials.

*6.1.3. Autoimmune diseases*

It was shown that VHHs derived from an animal immunized with TNFα can be used to inhibit functions of TNFα in a murine model. Such VHHs may be a cheaper alternative to the currently available anti-TNFα drugs used in rheumatoid arthritis therapy [10]. It is noteworthy that anti-TNFα VHH in its bivalent form (fused to a human Fc fragment) is now in the second phase of clinical trials. It was also shown that bacteria of a *Lactococcus lactis* strain can be used as a carrier for VHHs recognizing TNFα: antibody fragments were secreted by bacteria directly in the colon, acting as therapeutic agents for patients suffering from chronic colon inflammation [81]. The major advantage of such a strategy is that it may allow one to avoid proteolysis of orally administered VHHs [34,35].

*6.1.4. Toxins*

Another application of VHHs is neutralization of toxins. It was shown that IgGs isolated from the serum of an immunized dromedary can neutralize toxins of the scorpion *Androctonus australis* [52]. VHHs recognizing the most important toxin components, AahII [1] and AahI', have also been obtained [1,37]. Furthermore, it was shown that the bivalent VHHs consisting of two identical anti-AahI' VHH domains linked together or bispecific VHHs containing anti-AahI' and anti--AahII VHHs can neutralize scorpion toxins. The additional advantage of such constructs is higher molecular mass that increases their blood circulation time. Such bivalent or bispecific antibody produced in bacteria can be a reliable alternative to currently used horse-derived Fab'2 fragments [38]. A VHH that inhibits enzymatic activity of botulinum neurotoxin has also been described, and it was suggested that it may be used to treat patients poisoned by botulinum toxin [9,23].

*6.1.5. Infectious diseases*

VHHs are also being tested in therapy of disease caused by protozoa, such as trypanosomiasis (e.g. sleeping sickness) caused by parasites from the *Trypanosoma genus*. The outer surface of the parasite is covered with hypervariable proteins, so obtaining antibodies that recognize these proteins is difficult. The VHHs, however, due to their small size, can access more conserved epitopes that are located deeper under the surface of the parasite. It was shown that a VHH linked to β-lactamase, which can convert a pro-drug to an active drug, might be used in the therapy of sleeping sickness [75]. A therapy based on a VHH fused to the trypanolytic factor present in human serum was also proposed [4]. Finally, it was shown that a nanobody may be *per se* trypanolytic when interacting with the parasite, despite missing the Fc fragment (which mediates ADCC function) [74]. Another example of a VHH that can inhibit invasion of protozoa is a VHH recognizing the Duffy antigen receptor for chemokines (DARC) from human erythrocytes. It was shown that it can inhibit binding of *Plasmodium vivax* to red blood cells [71].

VHHs have been shown to inhibit bacterial and viral infections. For example, a VHH recognizing β-lactamase may inhibit enzyme function and may increase bacterial sensitivity to β-lactam antibiotics when administered together with an antibiotic [8]. VHHs recognizing proteins involved in HIV infections were shown to block HIV infection and thus such VHHs may be considered as prospective drugs. These proteins are virus envelope protein gp120, which is involved in recognition of CD4 antigen [26,45], and human CXCR4, which is a co-receptor for virus entry into CD4+ T-cells [44,45].

VHHs recognizing other viruses have also been obtained. For example, a VHH against capsid protein of hepatitis B virion, which is involved in virus secretion, was shown to bind to the virion protein *in vitro* and *in vivo* and to inhibit release of the virus [68]. It was also shown that a VHH recognizing rotavirus can provide cheap and efficient protection to pigs against virus infections if secreted by or anchored to bacteria belonging to the *Lactobacilli strains* and added to chow fed to animals [59]. Recently, a VHH that recognizes native M2 channel protein from influenza A virion was shown to inhibit replication of influenza A viruses *in vitro*, block proton influx through M2 channels and to protect mice from lethal influenza virus challenge. Such broad-specificity VHH may provide potential protection against variants and subtypes of influenza A viruses [84]. A VHH that binds to H5 hemagglutinin was shown to reduce viral replication of H5N1 influenza virus in mice and significantly delay time to death [41].

VHHs recognizing the tail of infectious phages were also shown to protect *Lactococcus bacteria*, strains of which are important in the dairy industry, against infection by phages [15,49].

Finally, an unusual application of VHHs is anti-dandruff shampoo. Dolk's group showed that it is possible to obtain VHHs that are active even in a high concentration of surfactants. They obtained a VHH recognizing a surface protein of *Malassezia furfur*, a microorganism implicated in dandruff formation, and suggested that such an antibody may reduce the number of *M. furfur* and serve as a carrier of a therapeutic agent if used as a shampoo ingredient [22].

**6.2. Applications of VHHs in diagnostics and biotechnology**

*6.2.1. Imaging*

Due to their small size and high affinity, VHHs are being increasingly used in applications such as biotechnology and diagnostics. In particular, VHHs have all the properties of a perfect imaging tool because they can easily penetrate tissues, accessing also cryptic antigens (e.g. located behind the blood-brain barrier), and bind antigens with high affinity. Several VHHs showing such activity have been described so far; e.g. a VHH recognizing epidermal growth factor receptor (EGFR), an antigen overexpressed on the surface of many tumors, was shown to bind EGFR in biopsy samples and *in vivo* [6,29,39]. The main disadvantage of VHHs as imaging tools is their relatively high renal uptake. The retention of VHHs in kidneys was shown to be dependent on megalin, a receptor involved in re-absorption of proteins, but avenues are being explored to decrease interactions with megalin-mediated VHH renal intake [28].

*6.2.2. Diagnostics*

VHHs have been shown to pass the blood-brain barrier, most probably by transcytosis [2]; however, due to their

small size they are rapidly cleared from the blood. Possible ways to overcome this problem may be preparing multivalent (bivalent, pentavalent) VHHs, or fusing VHHs to human Fc fragments [42]. The latter seems to be a better option, since such protein has a longer half-life and better tumor penetration ability. A VHH that can pass through the blood-brain barrier and can recognize oligomeric forms of amyloid β has been described; it was suggested that it can be used in diagnosis of Alzheimer's disease [48].

VHHs can also be used for intracellular imaging: a VHH fused to green fluorescent protein (GFP), and called a chromobody, can serve as an imaging tool if expressed in the intracellular compartment in cell lines [53,65].

### *6.2.3. Protein purification*

An important application of VHHs may be purification of proteins by affinity chromatography. In fact, all procedures using antibodies or their recombinant derivatives may be adapted to the use of VHHs, with all the advantages of VHHs such as easy expression and high thermostability being preserved. Thus, VHHs can be an efficient tool for purification of proteins: a VHH recognizing GFP could serve as a tool for purification of GFP-fused proteins [64]. Another example is nitric oxide reductase, which was purified from a cell lysate membrane fraction using specific His-tagged VHHs and nickel affinity chromatography [7]. A different approach was used for purification of the Duffy antigen receptor for chemokines (DARC) from DARC-expressing K562 cells. The anti-DARC VHHs were immobilized on the column using amine coupling chemistry and cell lysate was applied. A peptide whose sequence is the same as the sequence recognized by VHH was used to elute the target protein, and the result was highly purified protein [71].

### *6.2.4. Enzymology*

Recently, it was shown that the VHH library obtained after immunization with an enzyme can be used to obtain not only the anti-enzyme VHHs that can inhibit the enzyme, but also anti-idiotypic VHHs with catalytic activity. Thus, camel immunized with alliinase (*S*-alkyl-L-cysteine *S*-oxide alkyl-sulfenate-lyase) produced VHHs with alliinase activity which can convert the prodrug alliin into allicin [50]. It was also shown that such VHHs can significantly suppress growth of B16 tumor cells in the presence of alliin *in vitro* [50].

## 7. Summary

VHHs, which are the smallest fully active antibody fragments, can be a "coming of age" in medicine and biotechnology. Their unique features – small size, high affinity, ability to bind epitopes inaccessible for regular antibodies or their fragments, amazing stability, and feasibility of expression – suggest that they may be considered as a new magic bullet of medicine. In addition, they may be employed in biotechnology, therapy, imaging and laboratory practice.

## References


[1] Abderrazek R.B., Hmila I., Vincke C., Benlasfar Z., Pellis M., Dabbek H., Saerens D., El Ayeb M., Muyldermans S., Bouhaouala-Zahar B.: Identification of potent nanobodies to neutralize the most poisonous polypeptide from scorpion venom. Biochem. J., 2009; 424: 263–272

[2] Abulrob A., Sprong H., Van Bergen en Henegouwen P., Stanimirovic D.: The blood-brain barrier transmigrating single domain antibody: mechanisms of transport and antigenic epitopes in human brain endothelial cells. J. Neurochem., 2005; 95: 1201–1214

[3] Ahmadvand D., Rasaee M.J., Rahbarizadeh F., Kontermann R.E., Sheikholislami F.: Cell selection and characterization of a novel human endothelial cell specific nanobody. Mol. Immunol., 2009; 46: 1814–1823

[4] Baral T.N., Magez S., Stijlemans B., Conrath K., Vanhollebeke B., Pays E., Muyldermans S., De Baetselier P.: Experimental therapy of African trypanosomiasis with a nanobody-conjugated human trypanolytic factor. Nat. Med., 2006; 12: 580–584

[5] Behar G., Sibéril S., Groulet A., Chames P., Pugniere M., Boix C., Sautes-Fridman C., Teillaud J.L., Baty D.: Isolation and characterization of anti-FcγRIII (CD16) llama single-domain antibodies that activate natural killer cells. Protein Eng. Des. Sel., 2008; 21: 1–10

[6] Chopra A.: [99mTc]Epidermal growth factor receptor-specific nanobody. Molecular Imaging and Contrast Agent Database, 2008

[7] Conrath K., Pereira A.S., Martins. C.E., Timóteo C.G., Tavares P., Spinelli S., Kinne J., Flaudrops C., Cambillau C., Muyldermans S., Moura I., Moura J.J., Tegoni M., Desmyter A.: Camelid nanobodies raised against an integral membrane enzyme, nitric oxide reductase. Protein Sci., 2009; 18: 619–628

[8] Conrath K.E., Lauwereys M., Galleni M., Matagne A., Frere J.M., Kinne J., Wyns L., Muyldermans S.: β-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae. Antimicrob. Agents Chemother., 2001; 45: 2807–2812

[9] Conway J.O., Sherwood L.J., Collazo M.T., Garza J.A., Hayhurst A.: Llama single domain antibodies specific for the 7 botulinum neurotoxin serotypes as heptaplex immunoreagents. PLoS One, 2010; 5: e8818

[10] 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.: Formatted anti-tumor necrosis factor α VHH proteins derived from camelids show superior potency and targeting to inflamed joints in a murine model of collagen-induced arthritis. Arthritis Rheum., 2006; 54: 1856–1866

[11] Cortez-Retamozo V., Backmann N., Senter P.D., Wernery U., De Baetselier P., Muyldermans S., Revets H.: Efficient cancer therapy with a nanobody-based conjugate. Cancer Res., 2004; 64: 2853–2857

[12] Cortez-Retamozo V., Lauwereys M., Hassanzadeh Gh G., Gobert M., Conrath K., Muyldermans S., De Baetselier P., Revets H.: Efficient tumor targeting by single-domain antibody fragments of camels. Int. J. Cancer, 2002; 98: 456–462

[13] Czerwiński M., Krop-Wątorek A.: Heavy-chain antibodies of the Camelidae and their possible applications. Postępy Hig. Med. Dośw., 2005; 59: 193–202

[14] De Genst E., Saerens D., Muyldermans S., Conrath K.: Antibody repertoire development in camelids. Dev. Comp. Immunol., 2006; 30: 187–198

[15] De Haard H.J., Bezemer S., Ledeboer A.M., Müller W.H., Boender P.J., Moineau S., Coppelmans M.C., Verkleij A.J., Frenken L.G., Verrips C.T.: Llama antibodies against a lactococcal protein located at the tip of the phage tail prevent phage infection. J. Bacteriol., 2005; 187: 4531–4541

[16] Deschacht N., De Groeve K., Vincke C., Raes G., De Baetselier P., Muyldermans S.: A novel promiscuous class of camelid single-domain antibody contributes to the antigen-binding repertoire. J. Immunol., 2010; 184: 5696–5704

[17] Desmyter A., Decanniere K., Muyldermans S., Wyns L.: Antigen specificity and high affinity binding provided by one single loop of a camel single-domain antibody. J. Biol. Chem., 2001; 276: 26285–26290

[18] Desmyter A., Spinelli S., Payan F., Lauwereys M., Wyns L., Muyldermans S., Cambillau C.: Three camelid VHH domains in complex with porcine pancreatic α-amylase. Inhibition and versatility of binding topology. J. Biol. Chem., 2002; 277: 23645–23650

[19] Desmyter A., Transue T.R., Ghahroudi M.A., Thi M.H., Poortmans F., Hamers R., Muyldermans S., Wyns L.: Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme. Nat. Struct. Biol., 1996; 3: 803–811

[20] Diaz M., Greenberg A.S., Flajnik M.F.: Somatic hypermutation of the new antigen receptor gene (NAR) in the nurse shark does not generate the repertoire: possible role in antigen-driven reactions in the absence of germinal centers. Proc. Natl. Acad. Sci. USA, 1998; 95: 14343–14348

[21] Diaz M., Stanfield R.L., Greenberg A.S., Flajnik M.F.: Structural analysis, selection, and ontogeny of the shark new antigen receptor (IgNAR): identification of a new locus preferentially expressed in early development. Immunogenetics, 2002; 54: 501–512

[22] Dolk E., van der Vaart M., Lutje Hulsik D., Vriend G., de Haard H., Spinelli S., Cambillau C., Frenken L., Verrips T.: Isolation of llama antibody fragments for prevention of dandruff by phage display in shampoo. Appl. Environ. Microbiol., 2005; 71: 442–450

[23] Dong J., Thompson A.A., Fan Y., Lou J., Conrad F., Ho M., Pires-Alves M., Wilson B.A., Stevens R.C., Marks J.D.: A single-domain llama antibody potently inhibits the enzymatic activity of botulinum neurotoxin by binding to the non-catalytic α-exosite binding region. J. Mol. Biol., 2010; 397: 1106–1118

[24] Dudek J., Benedix J., Cappel S., Greiner M., Jalal C., Müller L., Zimmermann R.: Functions and pathologies of BiP and its interaction partners. Cell. Mol. Life Sci., 2009; 66: 1556–1569

[25] Flajnik M.F., Deschacht N., Muyldermans S.: A case of convergence: why did a simple alternative to canonical antibodies arise in sharks and camels? PLoS Biol., 2011; 9: e1001120

[26] Forsman A., Beirnaert E., Aasa-Chapman M.M., Hoorelbeke B., Hijazi K., Koh W., Tack V., Szynol A., Kelly C., McKnight A., Verrips T., de Haard H., Weiss R.A.: Llama antibody fragments with cross-subtype human immunodeficiency virus type 1 (HIV-1)-neutralizing properties and high affinity for HIV-1 gp120. J. Virol., 2008; 82: 12069–12081

[27] Frenken L.G., van der Linden R.H., Hermans P.W., Bos J.W., Ruuls R.C., de Geus B., Verrips C.T.: Isolation of antigen specific llama VHH antibody fragments and their high level secretion by *Saccharomyces cerevisiae*. J. Biotechnol., 2000; 78: 11–21

[28] Gainkam L.O., Caveliers V., Devoogdt N., Vanhove C., Xavier C., Boerman O., Muyldermans S., Bossuyt A., Lahoutte T.: Localization, mechanism and reduction of renal retention of technetium-99m labeled epidermal growth factor receptor-specific nanobody in mice. Contrast Media Mol. Imaging, 2011; 6: 85–92

[29] Gainkam L.O., Huang L., Caveliers V., Keyaerts M., Hernot S., Vaneycken I., Vanhove C., Revets H., De Baetselier P., Lahoutte T.: Comparison of the biodistribution and tumor targeting of two 99mTc--labeled anti-EGFR nanobodies in mice, using pinhole SPECT/micro--CT. J. Nucl. Med., 2008; 49: 788–795

[30] Greenberg A.S., Avila D., Hughes M., Hughes A., McKinney E.C., Flajnik M.F.: A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. Nature, 1995; 374: 168–173

[31] Groot A.J., El Khattabi M., Sachs N., van der Groep P., van der Wall E., van Diest P.J., Sonnenberg A., Verrips C.T., Vooijs M.: Reverse proteomic antibody screening identifies anti adhesive VHH targeting VLA-3. Mol. Immunol., 2009; 46: 2022–2028

[32] Habib I., Smolarek D., Hattab C., Gangnard S., Cochet S., Nunes de Silva S., Le Van Kim C., Colin Y., Czerwinski M., Bertrand O.: Methods for efficient, straightforward expression and purification of VHH, recombinant derivatives of heavy chain only camelid antibodies. 19th Biennial Meeting of the International Society for Molecular Recognition Tavira, Portugal, 16–19 June, 2011

[33] Hamers-Casterman C., Atarhouch T., Muyldermans S., Robinson G., Hamers C., Songa E.B., Bendahman N., Hamers R.: Naturally occurring antibodies devoid of light chains. Nature, 1993; 363: 446–448

[34] Harmsen M.M., De Haard H.J.: Properties, production, and applications of camelid single-domain antibody fragments. Appl. Microbiol. Biotechnol., 2007; 77: 13–22

[35] Harmsen M.M., van Solt C.B., van Zijderveld-van Bemmel A.M., Niewold T.A., van Zijderveld F.G.: Selection and optimization of proteolytically stable llama single-domain antibody fragments for oral immunotherapy. Appl. Microbiol. Biotechnol., 2006; 72: 544–551

[36] Hendershot L., Bole D., Kearney J.F.: The role of immunoglobulin heavy chain binding protein in immunoglobulin transport. Immunology Today, 1987; 8: 111–114

[37] Hmila I., Abdallah R.B., Saerens D., Benlasfar Z., Conrath K., Ayeb M.E., Muyldermans S., Bouhaouala-Zahar B.: VHH, bivalent domains and chimeric Heavy chain-only antibodies with high neutralizing efficacy for scorpion toxin AahI'. Mol. Immunol., 2008; 45: 3847–3856

[38] Hmila I., Saerens D., Ben Abderrazek R., Vincke C., Abidi N., Benlasfar Z., Govaert J., El Ayeb M., Bouhaouala-Zahar B., Muyldermans S.: A bispecific nanobody to provide full protection against lethal scorpion envenoming. FASEB J., 2010; 24: 3479–3489

[39] Huang L., Gainkam L.O., Caveliers V., Vanhove C., Keyaerts M., De Baetselier P., Bossuyt A., Revets H., Lahoutte T.: SPECT imaging with 99mTc-labeled EGFR-specific nanobody for *in vivo* monitoring of EGFR expression. Mol. Imaging Biol., 2008; 10: 167–175

[40] Hulstein J.J., de Groot P.G., Silence K., Veyradier A., Fijnheer R., Lenting P.J.: A novel nanobody that detects the gain-of-function phenotype of von Willebrand factor in ADAMTS13 deficiency and von Willebrand disease type 2B. Blood, 2005; 106: 3035–3042

[41] Ibañez L.I., De Filette M., Hultberg A., Verrips T., Temperton N., Weiss R.A., Vandevelde W., Schepens B., Vanlandschoot P., Saelens X.: Nanobodies with *in vitro* neutralizing activity protect mice against H5N1 influenza virus infection. J. Infect. Dis., 2011; 203: 1063–1072

[42] Iqbal U., Trojahn U., Albaghdadi H., Zhang J., O'Connor-McCourt M., Stanimirovic D., Tomanek B., Sutherland G., Abulrob A.: Kinetic analysis of novel mono- and multivalent VHH-fragments and their application for molecular imaging of brain tumours. Br. J. Pharmacol., 2010; 160: 1016–1028

[43] Ismaili A., Jalali-Javaran M., Rasaee M.J., Rahbarizadeh F., Forouzandeh-Moghadam M., Memari H.R.: Production and characterization of anti-(mucin MUC1) single-domain antibody in tobacco (*Nicotiana tabacum cultivar Xanthi*). Biotechnol. Appl. Biochem., 2007; 47: 11–19

[44] Jähnichen S., Blanchetot C., Maussang D., Gonzalez-Pajuelo M., Chow K.Y., Bosch L., De Vrieze S., Serruys B., Ulrichts H., Vandevelde W., Saunders M., De Haard H.J., Schols D., Leurs R., Vanlandschoot P., Verrips T., Smit M.J.: CXCR4 nanobodies (VHH-based single variable domains) potently inhibit chemotaxis and HIV-1 replication and mobilize stem cells. Proc. Natl. Acad. Sci. USA, 2010; 107: 20565–20570

[45] Koh W.W., Steffensen S., Gonzalez-Pajuelo M., Hoorelbeke B., Gorlani A., Szynol A., Forsman A., Aasa-Chapman M.M., de Haard H., Verrips T., Weiss R.A.: Generation of a family-specific phage library of llama single chain antibody fragments that neutralize HIV-1. J. Biol. Chem., 2010; 285: 19116–19124

[46] Köhler G., Milstein C.: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 1975; 256: 495–497

[47] Ladenson R.C., Crimmins D.L., Landt Y., Ladenson J.H.: Isolation and characterization of a thermally stable recombinant anti-caffeine heavy-chain antibody fragment. Anal. Chem., 2006; 78: 4501–4508

[48] Lafaye P., Achour I., England P., Duyckaerts C., Rougeon F.: Single-domain antibodies recognize selectively small oligomeric forms of amyloid beta, prevent Abeta-induced neurotoxicity and inhibit fibril formation. Mol. Immunol., 2009; 46: 695–704

[49] Ledeboer A.M., Bezemer S., de Hiaard J.J., Schaffers I.M., Verrips C.T., van Vliet C., Düsterhöft E.M., Zoon P., Moineau S., Frenken L.G.: Preventing phage lysis of *Lactococcus lactis* in cheese production using a neutralizing heavy-chain antibody fragment from llama. J. Dairy Sci., 2002; 85: 1376–1382

[50] Li J.W., Xia L., Su Y., Liu H., Xia X., Lu Q., Yang C., Reheman K.: Molecular imprint of enzyme active site by camel nanobodies: rapid and efficient approach to produce abzymes with alliinase activity. J. Biol. Chem., 2012; 287: 13713–13721

[51] Maattanen P., Gehring K., Bergeron J.J., Thomas D.Y.: Protein quality control in the ER: the recognition of misfolded proteins. Semin. Cell Dev. Biol., 2010; 21: 500–511

[52] Meddeb-Mouelhi F., Bouhaouala-Zahar B., Benlasfar Z., Hammadi M., Mejri T., Moslah M., Karoui H., Khorchani T., El Ayeb M.: Immunized camel sera and derived immunoglobulin subclasses neutralizing *Androctonus australis* hector scorpion toxins. Toxicon, 2003; 42: 785–791

[53] Moutel S., Perez F.: Intrabodies, potent tools to unravel the function and dynamics of intracellular proteins. Med. Sci., 2009; 25: 1173–1176

[54] Muyldermans S.: Single domain camel antibodies: current status. J. Biotechnol., 2001; 74: 277–302

[55] Muyldermans S., Atarhouch T., Saldanha J., Barbosa J.A., Hamers R.: Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. Protein Eng., 1994; 7: 1129–1135

[56] Nguyen V.K., Desmyter A., Muyldermans S.: Functional heavy-chain antibodies in Camelidae. Adv. Immunol., 2001; 79: 261–296

[57] Nguyen V.K., Hamers R., Wyns L., Muyldermans S.: Loss of splice consensus signal is responsible for the removal of the entire C(H)1 domain of the functional camel IGG2A heavy-chain antibodies. Mol. Immunol., 1999; 36: 515–524

[58] Nguyen V.K., Hamers R., Wyns L., Muyldermans S.: Camel heavy--chain antibodies: diverse germline V(H)H and specific mechanisms enlarge the antigen-binding repertoire. EMBO J., 2000; 19: 921–930

[59] Pant N., Hultberg A., Zhao Y., Svensson L., Pan-Hammarstrom Q., Johansen K., Pouwels P.H., Ruggeri F.M., Hermans P., Frenken L., Boren T., Marcotte H., Hammarstrom L.: Lactobacilli expressing variable domain of llama heavy-chain antibody fragments (lactobodies) confer protection against rotavirus-induced diarrhea. J. Infect. Dis., 2006; 194: 1580–1588

[60] Perk K., Frei Y.F., Herz A.: Osmotic fragility of red blood cells of young and mature domestic and laboratory animals. Am. J. Vet. Res., 1964; 25: 1241–1248

[61] Rahbarizadeh F., Ahmadvand D., Sharifzadeh Z.: Nanobody; an old concept and new vehicle for immunotargeting. Immunol. Invest., 2011; 40: 299–338

[62] Rahbarizadeh F., Rasaee M.J., Forouzandeh M., Allameh A.A.: Over expression of anti-MUC1 single-domain antibody fragments in the yeast Pichia pastoris. Mol. Immunol., 2006; 43: 426–435

[63] Rajabi-Memari H., Jalali-Javaran M., Rasaee M.J., Rahbarizadeh F., Forouzandeh-Moghadam M., Esmaili A.: Expression and characterization of a recombinant single-domain monoclonal antibody against MUC1 mucin in tobacco plants. Hybridoma, 2006; 25: 209–215

[64] Rothbauer U., Zolghadr K., Muyldermans S., Schepers A., Cardoso M.C., Leonhardt H.: A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. Mol. Cell. Proteomics, 2008; 7: 282–289

[65] Rothbauer U., Zolghadr K., Tillib S., Nowak D., Schermelleh L., Gahl A., Backmann N., Conrath K., Muyldermans S., Cardoso M.C., Leonhardt H.: Targeting and tracing antigens in live cells with fluorescent nanobodies. Nat. Methods, 2006; 3: 887–889

[66] Roux K.H., Greenberg A.S., Greene L., Strelets L., Avila D., McKinney E.C., Flajnik M.F.: Structural analysis of the nurse shark (new) antigen receptor (NAR): molecular convergence of NAR and unusual mammalian immunoglobulins. Proc. Natl. Acad. Sci. USA, 1998; 95: 11804–11809

[67] Saerens D., Pellis M., Loris R., Pardon E., Dumoulin M., Matagne A., Wyns L., Muyldermans S., Conrath K.: Identification of a universal VHH framework to graft non-canonical antigen-binding loops of camel single-domain antibodies. J. Mol. Biol., 2005; 352: 597–607

[68] Serruys B., Van Houtte F., Farhoudi-Moghadam A., Leroux-Roels G., Vanlandschoot P.: Production, characterization and *in vitro* testing of HBcAg-specific VHH intrabodies. J. Gen. Virol., 2010; 91: 643–652

[69] Siller-Matula J.M., Krumphuber J., Jilma B.: Pharmacokinetic, pharmacodynamic and clinical profile of novel antiplatelet drugs targeting vascular diseases. Br. J. Pharmacol., 2010; 159: 502–517

[70] Smith M.H., Ploegh H.L., Weissman J.S.: Road to ruin: targeting proteins for degradation in the endoplasmic reticulum. Science, 2011; 334: 1086–1090

[71] Smolarek D., Hattab C., Hassanzadeh-Ghassabeh G., Cochet S., Gutiérrez C., de Brevern A.G., Udomsangpetch R., Picot J., Grodecka M., Wasniowska K., Muyldermans S., Colin Y., Le Van Kim C., Czerwinski M., Bertrand O.: A recombinant dromedary antibody fragment (VHH or nanobody) directed against human Duffy antigen receptor for chemokines. Cell. Mol. Life Sci., 2010; 67: 3371–3387

[72] Spinelli S., Frenken L.G., Hermans P., Verrips T., Brown K., Tegoni M., Cambillau C.: Camelid heavy-chain variable domains provide efficient combining sites to haptens. Biochemistry, 2000; 39: 1217–1222

[73] Spinelli S., Tegoni M., Frenken L., van Vliet C., Cambillau C.: Lateral recognition of a dye hapten by a llama VHH domain. J. Mol. Biol., 2001, 311: 123–129

[74] Stijlemans B., Caljon G., Natesan S.K., Saerens D., Conrath K., Pérez-Morga D., Skepper J.N., Nikolaou A., Brys L., Pays E., Magez S., Field M.C., De Baetselier P., Muyldermans S.: High affinity nanobodies against the Trypanosome brucei VSG are potent trypanolytic agents that block endocytosis. PLoS Pathog., 2011; 7: e1002072

[75] Stijlemans B., Conrath K., Cortez-Retamozo V., Van Xong H., Wyns L., Senter P., Revets H., De Baetselier P., Muyldermans S., Magez S.: Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies. African trypanosomes as paradigm. J.Biol. Chem., 2004; 279: 1256–1261

[76] Stone E., Hirama T., Chen W., Soltyk A.L., Brunton J., MacKenzie C.R., Zhang J.: A novel pentamer versus pentamer approach to generating neutralizers of verotoxin 1. Mol. Immunol., 2007, 44: 2487–2491

[77] Thomassen Y.E., Verkleij A.J., Boonstra J., Verrips C.T.: Specific production rate of VHH antibody fragments by *Saccharomyces cerevisiae* is correlated with growth rate, independent of nutrient limitation. J. Biotechnol., 2005; 118: 270–277

[78] Ulrichts H., Silence K., Schoolmeester A., de Jaegere P., Rossenu S., Roodt J., Priem S., Lauwereys M., Casteels P., Van Bockstaele F., Verschueren K., Stanssens P., Baumeister J., Holz J.B.: Antithrombotic drug candidate ALX-0081 shows superior preclinical efficacy and safety compared with currently marketed antiplatelet drugs. Blood, 2011; 118: 757–765

[79] Ungar-Waroni H., Elias E., Gluckman A., Trainin Z.: Dromedary IgG: purification, characterization and quantitation in sera of dams and newborns. Israel J. Vet. Med., 1987; 43: 198–203

[80] van Loon J.E., de Jaegere P.P., Ulrichts H., van Vliet H.H., de Maat M.P., de Groot P.G., Simoons M.L., Leebeek F.W.: The *in vitro* effect of the new antithrombotic drug candidate ALX-0081 on blood samples of patients undergoing percutaneous coronary intervention. Thromb. Haemost., 2011; 106: 165–171

[81] Vandenbroucke K., de Haard H., Beirnaert E., Dreier T., Lauwereys M., Huyck L., Van Huysse J., Demetter P., Steidler L., Remaut E., Cuvelier C., Rottiers P.: Orally administered L. lactis secreting an anti-TNF Nanobody demonstrate efficacy in chronic colitis. Mucosal Immunol., 2010; 3: 49–56

[82] Vu K.B., Ghahroudi M.A., Wyns L., Muyldermans S.: Comparison of llama VH sequences from conventional and heavy chain antibodies. Mol. Immunol., 1997; 34: 1121–1131

[83] Wahner-Roedler D.L., Kyle R.A.: Heavy chain diseases. Best Pract. Res. Clin. Haematol., 2005; 18: 729–746

[84] Wei G., Meng W., Guo H., Pan W., Liu J., Peng T., Chen L., Chen C.Y.: Potent neutralization of influenza A virus by a single-domain antibody blocking M2 ion channel protein. PLoS One, 2011; 6: e28309

[85] Wesolowski J., Alzogaray V., Reyelt J., Unger M., Juarez K., Urrutia M., Cauerhff A., Danquah W., Rissiek B., Scheuplein F., Schwarz N., Adriouch S., Boyer O., Seman M., Licea A., Serreze D.V., Goldbaum F.A., Haag F., Koch-Nolte F.: Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. Med. Microbiol. Immunol., 2009; 198: 157–174

[86] Woolven B.P., Frenken L.G., van der Logt P., Nicholls P.J.: The structure of the llama heavy chain constant genes reveals a mechanism for heavy-chain antibody formation. Immunogenetics, 1999; 50: 98–101

[87] Wörn A., Plückthun A.: Different equilibrium stability behavior of ScFv fragments: identification, classification, and improvement by protein engineering. Biochemistry, 1999; 38: 8739–8750

[88] Wu T.T., Johnson G., Kabat E.A.: Length distribution of CDRH3 in antibodies. Proteins, 1993; 16: 1–7

[89] Zhang J., Li Q., Nguyen T.D., Tremblay T.L., Stone E., To R., Kelly J., MacKenzie R.C.: A pentavalent single-domain antibody approach to tumor antigen discovery and the development of novel proteomics reagents. J. Mol. Biol., 2004; 341: 161–169

[90] Zhang J., Tanha J., Hirama T., Khieu N.H., To R., Tong-Sevinc H., Stone E., Brisson J.R., MacKenzie C.R.: Pentamerization of single--domain antibodies from phage libraries: a novel strategy for the rapid generation of high-avidity antibody reagents. J. Mol. Biol., 2004; 335: 49–56

---

The authors have no potential conflicts of interest to declare.