Nano antibodies (Single-domain antibodies) in Arabian camels (Single hump) milk, diagnostic, therapeutic and medical functions (Review)

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Abstract: Nano-Antibodies or "nAb" are single-domain VHH antibodies derived from camelidae (camels, llamas, alpacas, etc.). Compared with conventional monoclonal and polyclonal antibodies, nAb antibodies possess many superior properties, including small size (~15kd), high affinity (frequently nanomolar range), high specificity, and unsurpassed stability. Each nAb is available in the following formats: stable purified protein, fluorescent dye conjugates, and conjugates to various matrices (including agarose resin and magnetic beads). 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.
[Hussien Abd El-Fattah Osman, Mona S. Zaki, Sami I Shalaby, Olfat M. Fawzi. **Nano antibodies (Single-domain antibodies) in Arabian camels (Single hump) milk, diagnostic, therapeutic and medical functions (Review).** *Researcher* 2019;11(7):51-65]. ISSN 1553-9865 (print); ISSN 2163-8950 (online). http://www.sciencepub.net/researcher. 5. doi:10.7537/marsrsj110719.05.

Key words: camels- milk**-** conventional antibodies -nano-antibodies- single-domain VHH - unique features.

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 [74]. Monoclonal antibodies usually are monospecific (they recognize only one type of antigen), and once cloned, they can be produced forever. 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 [35]. 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. In addition, scFv usually have lower affinity than Fab fragments or antibodies and show a tendency to aggregate [114].

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 [36]. Variable regions of heavy chain antibodies called VHHs or nanobodies TM 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 [41, 82].

A single-domain antibody (sdAb, called Nanobody by Ablynx, the developer[1]) is an antibody fragment consisting of a single monomeric variable antibody domain. Like a whole antibody, it is able to bind selectively to a specific antigen. With a molecular weight of only 12–15 kDa, single-domain antibodies are much smaller than common antibodies (150–160 kDa) which are composed of two heavy protein chains and two light chains, and even smaller than Fab fragments (~50 kDa, one light chain and half a heavy chain) and single-chain variable fragments (~25 kDa, two variable domains, one from a light and one from a heavy chain).[2]

The first single-domain antibodies were engineered from heavy-chain antibodies found in camelids; these are called **V_HH fragments**. Cartilaginous fishes also have heavy-chain antibodies (IgNAR, 'immunoglobulin new antigen receptor'), from which single-domain antibodies called **V_{NAR} fragments** can be obtained. An alternative approach is

to split the dimeric variable domains from common immunoglobulin G (IgG) from humans or mice into monomers [42]. Although most research into single-domain antibodies is currently based on heavy chain variable domains, nanobodies derived from light chains have also been shown to bind specifically to target epitopes (antigen) [3].

Single-domain camelids antibodies have been shown to be just as specific as a regular antibody and in some cases they are more robust. As well, they are easily isolated using the same phage panning procedure used for traditional antibodies, allowing them to cultured in vitro in large concentrations. The smaller size and single domain make these antibodies easier to transform into bacterial cells for bulk production, making them ideal for research purposes [4].

Single-domain antibodies are being researched for multiple pharmaceutical applications and have potential for use in the treatment of acute coronary syndrome, cancer and Alzheimer's disease [5, 6]. Thus present review aimed to concentrate or focus the light on the nanobodies produced naturally in camels milk production or preparation in vivo and vitro in addition of its uses in diagnosis, treatment of different human being and animal diseases and biotechnology.

Camels and their milk:

Human knew camel family since three thousand years BC; preferred as a means to him to travel and carry weight, diet and as a source of his drink of milk, ate the meat, has spread beauty in all continents of the world which is of two types: single-hump Arab camels and spread in the Arab desert island Africa, including the spread of the Asian countries and others [17]. The camels of Alsnaman are found in the Himalayan region, India and some other country. And camel milk is the staple food of the Bedouins in the desert and they consider the best whole milk And they prefer fresh in most cases [8,19]. And it varies the intensity of the taste of the milk to lukewarm sweetness and salty, and the taste depends on the type of food provided to Apple [47]. Scientists have been interested in research on infant formula camels in the last quarter of the twentieth century were conducted research on hundreds of types of camels, the amount generated by the dairy a day and period Adrarha milk after birth, and the components of camel milk. After extensive studies, scientists concluded that camel milk is an essential element in improving the human food quality and quantity [44,13].

Camel milk components:

Graded camel milk in its components based on the stage of lactation and age of the camel and the number of children and the amount and type of food you feed it, as well as on the amount of water available for drinking [12]. The camels High alkaline milk but soon becomes acidic if you leave a period of time if the range PH of 6.5 to 6.7% and turns of the acidic quickly where increases lactic acid of 0.03 after two hours to 14% after 6 hours, and the ranges of water in camel's milk from 84 to 90 % of its components range from fat in the milk an average of about 5.4, and the protein is about 3%, and the proportion of lactose about 3.4 and minerals about 0.7%, such as iron, calcium, phosphorus, manganese, potassium, magnesium, there are significant differences in these compounds between different types of camels and depend on the food and drink provided her [45]. And the proportion of fat to solids in camel milk lower than in milk buffalo with an in camel milk 31.6%, while in Buffalo 40.9% and the fat camel milk exist in the form of minute concentric grains with protein so it is difficult to separate them in the camel milk usual ways in other dairy, fatty acids in short camel milk chain which is less than in other dairy, and camel milk contains the greatest concentration of fatty acids volatile especially linoleic multiple non Almicheah fatty and amino acid, which are vital in the human diet, especially heart patients. The percentage of low Alklestarol in camel milk compared to cow's milk by about 40% [49]. And the percentage of casein protein in the total protein camel milk to 70%, making camel milk is easy to digest. Camel milk contains vitamin C at a rate of three times its presence in cow's milk and increase if camels fed on herbs and food rich in this vitamin. The vitamin B 1.2 is present in sufficient quantity at the top of the sheep's milk yogurt and there is a vitamin A (A) and beta carotene rates sufficient [25].

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 [107]. However, the detailed characterization and demonstration of potential usefulness of heavy chain antibodies stemmed from the work of the Hamers group in Brussels [53,61]. 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 [61]. 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. Contrasting with regular antibody, there is no CH1 domain [83] 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 [84]. 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. proteins is called heavy chain binding protein (BiP) [52,79]. A nascent heavy chain 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 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 [98]. BiP binds specifically to the CH1 segment of the heavy chain [64] 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. 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 [84,113].

Uses camel milk:

The Arabs have benefited from the old camel milk in the treatment of many illnesses and Kaljdra wounds and dental diseases and diseases of the digestive system and resistance to toxins.

They said that the best milk camels as a treatment milk after birth forty days and his superiors what intensified the egg-white and merry stench Olz taste was the sweetness easy and Dsamh moderate and mild texture in Raqqa and Aleppo from camel correct moderate meat commendable pasture and Almcherb. oicol Arabs to milk camels medicine. And milk camels Mahmoud generates a good blood and moisturizes the body dry and benefit from obsessive-compulsive, distress and disease melancholy, and if you drink with honey pure sores esoteric of the Blends rotten, and drink milk with sugar improves the very color and liquidate the skin and is good for chest diseases, especially lung and good for people with tuberculosis [58,59]. The vaccine has been received milk evacuation and Tliyna and pass more and Faha to hit and good for ascites. Razi said in camel milk (milk vaccine cure aches liver and corruption mood " said Ibn Sina in the book of the law " that camel milk medication beneficial because it involves evacuation gently and its property, and that this severe milk benefit if that man lived it instead water and food cured it, Try it has pushed people to the land of the Arabs. It is recommended that the patient takes the camel milk to take him for treatment Balgdah, something it does not include, and must complete rest after drinking it. The warm fresh camel milk the best thing to clean the digestive system and is considered the best laxatives. The spread between the Bedouins that any disease at home can be treated with camel milk. Milk is not only a donor of force, but also for health. Scientific research has demonstrated the unique advantages of the modern camel milk [85,86].

Immunological characteristics and medical uses of camel's milk:

Numerous studies carried out by **Ajami** explained (**1994 and 2000**) and **Ajami *et al.* (1992, 1996 and 1998**) that camel milk is characterized by features immune unique, as it contains very high concentrations of some inhibitory compounds to do some pathogenic bacteria and some viruses. In India uses camel milk as a treatment for ascites, jaundice and trouble spleen, tuberculosis, asthma, anemia, hemorrhoids (**Rao *et al.*, 1970**) in the treatment of hepatitis, chronic disease and improve liver function has liver function improved in patients with hepatitis after being treated milk camels **Sharmanov *et al.*, 1978** And give the milk to the elderly, youth and children which is important in bone formation. As proven that camel milk lowers blood levels of glucose and thus can have a role in the treatment of diabetes [1, 2]. It is amazing that he has found in camel milk high levels of insulin and insulin-like proteins, and if you drink milk, these compounds are carried out through the stomach into the blood of non-shatter, while the acid destroys normal intestinal insulin [3]. This has given hope for the manufacture of human insulin intake by mouth, and is working today on the drug companies manufactured and marketed in the near future. It has been found in a recent study [3] that patients with Type I diabetes have benefited from Cuba when he ate camel milk have decreased the level

of sugar in the blood and reduced the amount of insulin prescribed to them.

What is nAb?

Nano-Antibodies or "nAb" are single-domain VHH antibodies derived from camelidae (camels, llamas, alpacas, etc.). Compared with conventional monoclonal and polyclonal antibodies, nAb antibodies possess many superior properties, including small size (~15kd), high affinity (frequently nanomolar range), high specificity, and unsurpassed stability. Each nAb is available in the following formats: stable purified protein, fluorescent dye conjugates, and conjugates to various matrices (including agarose resin and magnetic beads) [25].

During development of each nAb, researchers apply unique screening processes and protein engineering expertise to generate single-domain antibodies that are improved over natural camelid antibody fragments [94]. As part of this engineering process, all points of linkage between the nAb and the optimized affinity resins are strategically confined to non-binding surfaces of the antibody, increasing specific affinity and reducing the potential for non-specific interactions. Beyond the flagship GFP-nAb™ and mNeon Green-nAb™ products, it will soon be providing nAb antibodies in multiple formats against stem cell markers, cancer markers, functional repressors, additional fluorescent protein families, and many common epitope tags [30].

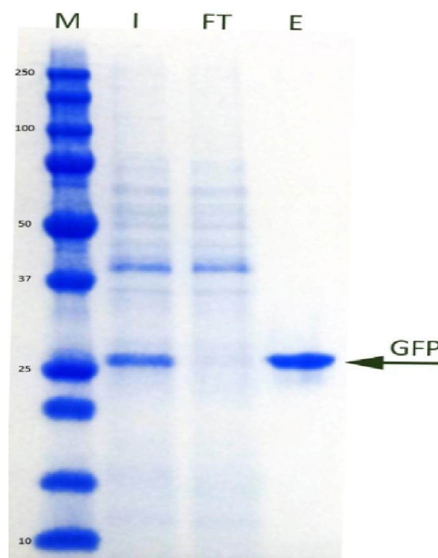
Why Should You Choose nAb?

nAb antibodies are robust tools for a number of biological assays and applications due to their high affinity and binding specificity, allowing for clean pull down of your target protein/fusion with essentially zero background. Some of the most common applications of nAb include:

- ChIP-CHIP assays
- Identifying Interacting Proteins
- RIP Assays (RNA Immunoprecipitation)
- CLIP Assays (in vivo Cross Linking and Immunoprecipitation)3

History of nAb Development

Allele's involvement in the field of VHH antibodies goes back a number of years to 2008, when the first trial began to examine and appreciate the unmatched potential of nano-antibodies [12]. In 2009, GFP-trap was introduced along with other VHH products to the US market through our distribution deal with Chromotek [17]. Moving forward to 2011, with NIH funding to carry out a research and development project exploring alternative VHH antibody development and isolation technology. Shortly after, introduction camelid antibody service, harnessing the latest technology and techniques to generate unique single domain antibodies for customers. In 2012, a new, two-story facility in San Diego with a dedicated antibody screening area, drastically increasing internal research and custom service capacity.



Complete Pulldown using the GFP-nAb™ Spin Kit.

EGFP-expressing Sf9 (insect) cell lysate contained a total of 16µg of EGFP in total volume of 500µl, determined spectrophotometrically. Following the GFP-nAb™ Spin Kit binding and wash protocols, the protein was eluted in 2 x 50 µl elution buffer (0.2M glycine pH 2.5), pooled, and neutralized with 10µl of 1M Tris base. Equal volumes of lysate input (I), flow-through (FT) after binding to GFP-nAb™ agarose resin, and elution (E) fractions were analyzed by SDS-PAGE followed by Coomassie staining. In this experiment, EGFP was quantitatively removed from the lysate.

Properties a single-domain antibody:

A single-domain antibody is a peptide chain of about 110 amino acids long, comprising one variable domain (V_H) of a heavy-chain antibody, or of a common IgG. These peptides have similar affinity to antigens as whole antibodies, but are more heat-resistant and stable towards detergents and high concentrations of urea. Those derived from camelid and fish antibodies are less lipophilic and more soluble in water, owing to their complementarity determining region 3 (CDR3), which forms an extended loop (coloured orange in the ribbon diagram above) covering the lipophilic site that normally binds to a light chain.[7][8] In contrast to common antibodies, two out of six single-domain antibodies survived a temperature of 90 °C (194 °F) without losing their ability to bind antigens in a 1999 study.[9] Stability towards gastric acid and proteases depends on the amino acid sequence. Some species have been shown

to be active in the intestine after oral application,[10][11] but their low absorption from the gut impedes the development of systemically active orally administered single-domain antibodies.

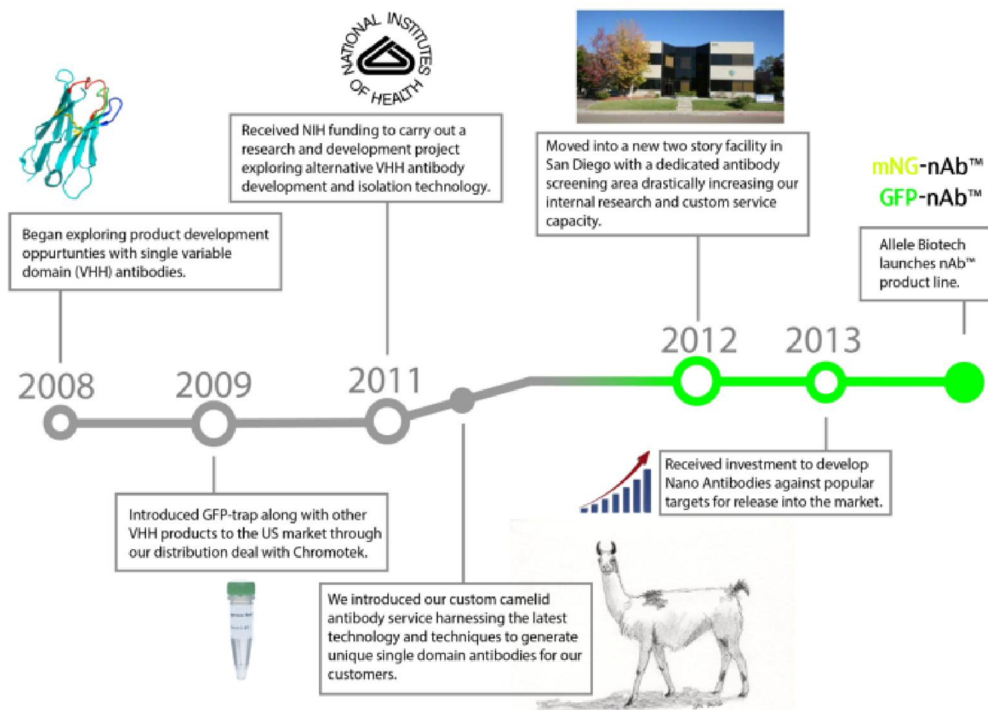


Fig. 1: Showing Stages of development and manufacturing nanobodies.

The comparatively low molecular mass leads to a better permeability in tissues, and to a short plasma half-life since they are eliminated renally.[2] Unlike whole antibodies, do not show complement system triggered cytotoxicity because they lack an Fc region. Camelid and fish derived sdAbs are able to bind to hidden antigens that are not accessible to whole antibodies, for example to the active sites of enzymes. This property has been shown to result from their extended CDR3 loop, which is able to penetrate such sites.[8][12]

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, 48].

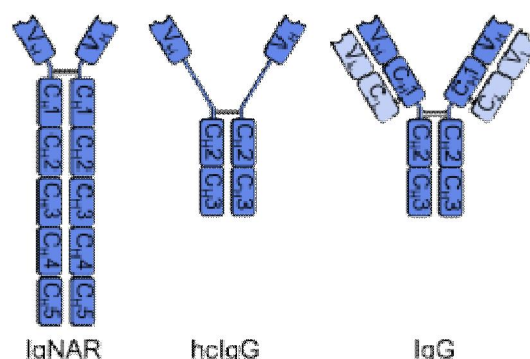
• Since VHHs consist of only one domain, they are easy to clone and can be expressed with high yields using different expression systems [62].

• 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 [45], but also buried deep in the clefts [32]. VHHs have been shown to recognize a wide range of epitopes, ranging from small haptens [19,100,101] to binding sites of enzymes [19, 75].

• 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 [30,76].

• VHHs show high solubility and stability even in denaturing conditions or high temperatures [19,102].

Production of nanobodies:



From heavy-chain antibodies A shark (left) and a camelid (middle) heavy-chain antibody in to a common antibody (right). Heavy chains are shown in a darker shade, light chains in a lighter shade. V_H and V_L are the variable domains.

A single-domain antibody can be obtained by immunization of dromedaries, camels, llamas, alpacas or sharks with the desired antigen and subsequent isolation of the mRNA coding for heavy-chain antibodies. By reverse transcription and polymerase chain reaction, a gene library of single-domain antibodies containing several million clones is produced. Screening techniques like phage display and ribosome display help to identify the clones binding the antigen [12].

A different method uses gene libraries from animals that have not been immunized beforehand. Such naïve libraries usually contain only antibodies with low affinity to the desired antigen, making it necessary. to apply affinity maturation by random mutagenesis as an additional step [14].

When the most potent clones have been identified, their DNA sequence is optimized, for example to improve their stability towards enzymes. Another goal is humanization to prevent immunological reactions of the human organism against the antibody. Humanization is unproblematic because of the homology between camelid V_HH and human V_H fragments.[14] The final step is the translation of the optimized single-domain antibody in *E. coli*, *Saccharomyces cerevisiae* or other suitable organisms.

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 [82]. 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. 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 in-jections. 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 [82, 95,106].

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-im-mune 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, 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 [41,112, 109].

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 [55,60]. 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 [105]. The yields of protein can be even higher if *Pichia pastoris* yeast is selected for protein expression [90]. Production of VHHs is also possible in tobacco [71, 91,116].

VHHs can be fused to various partners, either in order to increase their size or to confer new properties or effect or functions. Thus the VHHs can be:

• bivalent

– fused by a linker [63].

– fused to the IgG Fc domain, which can in addition confer antibody-dependent cell cytotoxicity [65].

• bispecific [38,70].

• pentavalent [115,90].

• decavalent [104].

• fused to an enzyme [40].

• fused to another protein to increase the size and half-life, such as an Fc fragment [38].

From conventional antibodies:

Alternatively, single-domain antibodies can be made from common murine or human IgG with four chains.[15] The process is similar, comprising gene libraries from immunized or naïve donors and display techniques for identification of the most specific antigens. A problem with this approach is that the binding region of common IgG consists of two domains (V_H and V_L), which tend to dimerize or aggregate because of their lipophilicity. Monomerization is usually accomplished by replacing lipophilic by hydrophilic amino acids, but often results in a loss of affinity to the antigen.[16] If affinity can be retained, the single-domain antibodies can likewise be produced in *E. coli*, *S. cerevisiae* or other organisms.

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 [82,89, 112].

A. VHHs in therapy:

1. Anti-cancer therapy:

Since it was demonstrated that VHHs can bind antigens on the surface of tumor cells and their immunogenicity is low [40], 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 pro-spective 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 b-lactamase, an enzyme that can cleave a pro-drug of low toxicity into its toxic form. 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 [39]. 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 Fcg receptor III was shown to activate natural killer (NK) cells and induce production of in-terferon g. The mono- and bispecific hcAb fragments activated FcgRIII 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 [33]. It was also shown that VHH recognizing integrin a3b1 (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 [60].

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.* [68] 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 [97]. 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 [107,108]. 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.

3. Autoimmune diseases:

It was shown that VHHs derived from an animal immunized with TNFa can be used to inhibit functions of TNFa in a murine model. Such VHHs may be a cheaper alternative to the currently available anti-TNFa drugs used in rheumatoid arthritis therapy [38]. It is noteworthy that anti-TNFa 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 TNFa: 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 [62,63].

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* [80]. VHHs recognizing the most important toxin components, AahII [29] and AahI', have also been obtained [29,65]. 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 [66]. 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 [37,51].

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 *Trypanosome 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 b-lactamase, which can convert a pro-drug to an active drug, might be used in the therapy of sleeping sickness [103]. A therapy based on a VHH fused to the trypanolytic factor present in human serum was also proposed [32]. 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) [104]. 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 b-lactamase may inhibit enzyme function and may increase bacterial sensitivity to b-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 [54, 73], and human CXCR4, which is a co-receptor for virus entry into CD4+ T-cells [72, 73].

VHHs recognizing other viruses have also been obtained. For example, a VHH against capsid protein of hepatitis B virus, 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 [96]. 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 [87]. 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 [111]. 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 [69].

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 [15,77].

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 [50].

B. Applications of VHHs in diagnostics and biotechnology:

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* [34,57,67]. 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 [56].

2. Diagnostics:

VHHs have been shown to pass the blood-brain barrier, most probably by transcytosis [30]; 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 [70]. 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 b has been described; it was suggested that it can be used in diagnosis of Alzheimer's disease [75].

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 [81,93].

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 [92]. 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 [99].

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* [78].

Future approach:

Now is working about 800 world of biotechnology scientists who specialize in human health Plant Biotechnology and Systems Research, and the togetherness of several universities on nano research antibodies for the implementation of the future project in the treatment of stubborn diseases.

Abbreviations:

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

Conclusion:

The latest study published by the American Science magazine in its edition in August 2005 he found that the camel family, especially the one-humped Arabian camel is distinct from other the rest of the mammals in that they have in their blood and tissues small antibodies are composed of short chains of amino acids and form the image of a character V and called scientists antibodies missing or nanoparticles Nano Antibodies or Acronym Nanobodies There is no such antibodies only in the Arab camels, increase the presence of other antibodies in human and other mammals which also, and that the shape of the letter Y, and that the size of these antibodies is ten the size of regular antibiotics and more slender than chemical and able to lock onto its targets and destroy the ability of the same regular antibodies, and pass easily through cell membranes and reach each body's cells. The advantage of these objects as being more stable nanoparticles to resist temperature change and extreme pH change, and reserves the Pfaalatha as it passed through the stomach and intestines, unlike regular antibodies that damage the thermal changes and digestive enzymes. Thus enhancing the prospects for the emergence of drug pills contain nano-objects for the treatment of inflammatory bowel disease and colon cancer and rheumatoid arthritis and possibly Alzheimer's patients also. Scientific research has focused on these antibodies about 2001, since in the treatment of tumors in experimental animals and human and proven to be effective in eradicating tumors where stick with high efficiency wall of a cancer cell and destroy it has succeeded some interested research for Biotechnology in Britain and America in the production of drug companies body component tablets of anti-camel-like Palmugodh valuable for the treatment of cancer and chronic diseases and many bacterial and viral infections. And developed Ablynx company these objects nanoparticles to check sixteen therapeutically target the most important diseases that affect human cover, the first of the cancer, followed by some inflammatory diseases, diseases of the heart and blood vessels.

References:

1. Gibbs, W. Wayt (August 2005). "Nanobodies". Scientific American Magazine.
2. Harmsen MM, De Haard HJ (November 2007). "Properties, production, and applications of camelid single-domain antibody fragments". Appl. Microbiol. Biotechnol. 77 (1): 13–22.
3. Möller, A.; Pion, E; Narayan, V; Ball, KL (September 2010). "Intracellular activation of

interferon regulatory factor-1 by nanobodies to the multi-functional (Mf1) domain". The Journal of Biological Chemistry (J Biol Chem) 285 (49): 38348–38361.
4. Ghannam, A., Kumari, S., Muyldermans, S., & Abbady, A. Q. (2015). Camelid nanobodies with high affinity for broad bean mottle virus: a possible promising tool to immunomodulate plant resistance against viruses. Plant Molecular Biology, 1-15.
5. "Nanobodies herald a new era in cancer therapy". The Medical News. 12 May 2004.
6. "Pipeline". Ablynx. Retrieved 20 January 2010.
7. Dolk, E.; Van Der Vaart, M.; Lutje Hulsik, D.; Vriend, G.; De Haard, H.; Spinelli, S.; Cambillau, C.; Frenken, L.; Verrips, T. (2005). "Isolation of Llama Antibody Fragments for Prevention of Dandruff by Phage Display in Shampoo". Applied and Environmental Microbiology 71 (1): 442–450.
8. Stanfield, R.; Dooley, H.; Flajnik, M.; Wilson, I. (2004). "Crystal structure of a shark single-domain antibody V region in complex with lysozyme". Science 305 (5691): 1770–1773.
9. Van Der Linden, R.; Frenken, L.; De Geus, B.; Harmsen, M.; Ruuls, R.; Stok, W.; De Ron, L.; Wilson, S.; Davis, P.; Verrips, C. T. (1999). "Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies". Biochimica et Biophysica Acta 1431 (1): 37–46.
10. Harmsen, M.; Vansolt, C.; Hoogendoorn, A.; Vanzijderveld, F.; Niewold, T.; Vandermeulen, J. (2005). "Escherichia coli F4 fimbriae specific llama single-domain antibody fragments effectively inhibit bacterial adhesion in vitro but poorly protect against diarrhoea". Veterinary Microbiology 111 (1–2): 89–98.
11. Harmsen, M. M.; Van Solt, C. B.; Van Zijderveld-Van Bemmel, A. M.; Niewold, T. A.; Van Zijderveld, F. G. (2006). "Selection and optimization of proteolytically stable llama single-domain antibody fragments for oral immunotherapy". Applied Microbiology and Biotechnology 72 (3): 544–551.
12. Desmyter, A.; Transue, T. R.; Ghahroudi, M. A.; Thi, M. H.; Poortmans, F.; Hamers, R.; Muyldermans, S.; Wyns, L. (1996). "Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme". Nature Structural Biology 3 (9): 803–811.
13. 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 Letters 414 (3): 521–526.
14. Saerens, D.; Ghassabeh, G.; Muyldermans, S. (2008). "Single-domain antibodies as building blocks for novel therapeutics". Current Opinion in Pharmacology 8 (5): 600–608.
15. Holt, L. J.; Herring, C.; Jespers, L. S.; Woolven, B. P.; Tomlinson, I. M. (2003). "Domain antibodies: proteins for therapy". Trends in Biotechnology 21 (11): 484–490.
16. Borrebaeck, C. A. K.; Ohlin, M. (2002). "Antibody evolution beyond Nature". Nature Biotechnology 20 (12): 1189–90.
17. Muyldermans, S. (2013). "Nanobodies: Natural Single-Domain Antibodies". Annual Review of Biochemistry 82: 775–797.
18. Rothbauer, U.; Zolghadr, K.; Tillib, S.; Nowak, D.; Schermelleh, L.; Gahl, A.; Backmann, N.; Conrath, K.; Muyldermans, S.; Cardoso, M. C.; Leonhardt, H. (2006). "Targeting and tracing antigens in live cells with fluorescent nanobodies". Nature Methods 3 (11): 887–889.
19. Rothbauer, U.; Zolghadr, K.; Muyldermans, S.; Schepers, A.; Cardoso, M. C.; Leonhardt, H. (2007). "A Versatile Nanotrap for Biochemical and Functional Studies with Fluorescent Fusion Proteins". Molecular & Cellular Proteomics 7 (2): 282–289.
20. Ries, J.; Kaplan, C.; Platonova, E.; Eghlidi, H.; Ewers, H. (2012). "A simple, versatile method for GFP-based super-resolution microscopy via nanobodies". Nature Methods 9 (6): 582–584.
21. Koide, S. (2009). "Engineering of recombinant crystallization chaperones". Current Opinion in Structural Biology 19 (4): 449–457.
22. Saerens, D.; Frederix, F.; Reekmans, G.; Conrath, K.; Jans, K.; Brys, L.; Huang, L.; Bosmans, E. N.; Maes, G.; Borghs, G.; Muyldermans, S. (2005). "Engineering Camel Single-Domain Antibodies and Immobilization Chemistry for Human Prostate-Specific Antigen Sensing". Analytical Chemistry 77 (23): 7547–7555.
23. Ibanez, L. I.; De Filette, M.; Hultberg, A.; Verrips, T.; Temperton, N.; Weiss, R. A.; Vandevelde, W.; Schepens, B.; Vanlandschoot, P.; Saelens, X. (2011). "Nanobodies with in Vitro Neutralizing Activity Protect Mice Against H5N1 Influenza Virus Infection". Journal of Infectious Diseases 203 (8): 1063–1072.
24. Hussack, G.; Arbabi-Ghahroudi, M.; Van Faassen, H.; Songer, J. G.; Ng, K. K. - S.; MacKenzie, R.; Tanha, J. (2011). "Neutralization of Clostridium difficile Toxin a with Single-domain Antibodies Targeting the Cell Receptor Binding Domain". Journal of Biological Chemistry 286 (11): 8961–8976.

25. Yagil Reuven (2013) Comparative alternative Medicinal (CAM) Properties in Camel Milk for Treatment of Epidemic Diseases. Journal of Agricultural Science and Technology A 3; 575-580.
26. Van De Broek, B.; Devoogdt, N.; d'Hollander, A.; Gijs, H. L.; Jans, K.; Lagae, L.; Muyldermans, S.; Maes, G.; Borghs, G. (2011). "Specific Cell Targeting with Nanobody Conjugated Branched Gold Nanoparticles for Photothermal Therapy". ACS Nano 5 (6): 4319–4328.
27. "Ablynx Announces Interim Results of First Nanobody Phase I Study of, ALX-0081 (ANTI-VWF)". Bio-Medicine.org. 2 July 2007.
28. Clinical trial number NCT01020383 for "Comparative Study of ALX-0081 Versus GPIIb/IIIa Inhibitor in High Risk Percutaneous Coronary Intervention (PCI) Patients" at Clinical Trials.gov.
29. 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.
30. 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.
31. 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.
32. 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.
33. 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-FcgRIII (CD16) llama single-domain antibodies that activate natural killer cells. Protein Eng. Des. Sel., 2008; 21: 1–10.
34. Chopra A. 2008 Epidermal growth factor receptor-specific nanobody. Molecular Imaging and Contrast Agent Database.
35. 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.
36. Conrath K.E., Lauwereys M., Galleni M., Matagne A., Frere J.M., Kinne J., Wyns L., Muyldermans S.: b-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae. Antimicrob. Agents Chemother., 2001; 45: 2807–2812.
37. 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.
38. 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 a 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.
39. 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.
40. 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.
41. Czerwiński M., Krop-Wątorek A.: Heavy-chain antibodies of the Camelidae and their possible applications. Postępy Hig. Med. Dośw., 2005; 59: 193–202.
42. De Genst E., Saerens D., Muyldermans S., Conrath K.: Antibody repertoire development in camelids. Dev. Comp. Immunol., 2006; 30: 187–198.
43. 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.
44. 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.
45. 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.

46. Desmyter A., Spinelli S., Payan F., Lauwereys M., Wyns L., Muyldermans S., Cambillau C.: Three camelid VHH domains in complex with porcine pancreatic a-amylase. Inhibition and versatility of binding topology. J. Biol. Chem., 2002; 277: 23645–23650.
47. 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.
48. 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.
49. 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.
50. 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.
51. 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 a-exosite binding region. J. Mol. Biol., 2010; 397: 1106–1118.
52. 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.
53. 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.
54. 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.
55. 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.
56. 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.
57. 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.
58. Greenberg A.S., Avila D., Hughes M., Hughes A., McKinney E.C., Flajnik M.F.: A new antigen receptor gene family that undergoes re-arrangement and extensive somatic diversification in sharks. Nature, 1995; 374: 168–173.
59. 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.
60. 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.
61. 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.
62. Harmsen M.M., De Haard H.J.: Properties, production, and applications of camelid single-domain antibody fragments. Appl. Microbiol. Biotechnol., 2007; 77: 13–22.
63. Harmsen M.M., van Solt C.B., van Zijderveld-van Bemmel A.M., Niewold T.A., van Zijderveld F.G.: Selection and optimization of pro-

teolytically stable llama single-domain antibody fragments for oral immunotherapy. Appl. Microbiol. Biotechnol., 2006; 72: 544–551.

64. Hendershot L., Bole D., Kearney J.F.: The role of immunoglobulin heavy chain binding protein in immunoglobulin transport. Immunology Today, 1987; 8: 111–114.
65. 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 Aahl'. Mol. Immunol., 2008; 45: 3847–3856.
66. 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.
67. 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.
68. 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.
69. 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.
70. 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.
71. 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.
72. 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.
73. 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.
74. Köhler G., Milstein C.: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 1975; 256: 495–497.
75. 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.
76. 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.
77. 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.
78. 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.
79. 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.
80. 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.
81. Moutel S., Perez F.: Intrabodies, potent tools to unravel the function and dynamics of intracellular proteins. Med. Sci., 2009; 25: 1173–1176.
82. Muyldermans S.: Single domain camel antibodies: current status. J. Biotechnol., 2001; 74: 277–302.

83. 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.
84. Nguyen V.K., Desmyter A., Muyldermans S.: Functional heavy-chain antibodies in Camelidae. Adv. Immunol., 2001; 79: 261–296.
85. 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.
86. 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.
87. 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.
88. 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.
89. Rahbarizadeh F., Ahmadvand D., Sharifzadeh Z.: Nanobody; an old concept and new vehicle for immunotargeting. Immunol. Invest., 2011; 40: 299–338.
90. 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.
91. 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.
92. 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.
93. 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.
94. 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.
95. 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.
96. 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.
97. 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.
98. 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.
99. 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.
100. 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.
101. 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.
102. 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

103. 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.
104. 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.
105. 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.
106. 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.
107. 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.
108. 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.
109. 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.
110. 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.
111. Wahner-Roedler D.L., Kyle R.A.: Heavy chain diseases. Best Pract. Res. Clin. Haematol., 2005; 18: 729–746.
112. 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.
113. 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.
114. 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.
115. Wörn A., Plückthun A.: Different equilibrium stability behavior of ScFv fragments: identification, classification, and improvement by protein engineering. Biochemistry, 1999; 38: 8739–8750.
116. Wu T.T., Johnson G., Kabat E.A.: Length distribution of CDRH3 in antibodies. Proteins, 1993; 16: 1–7.
117. 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.
118. 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.

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