



## Nanobody as a New Generation of Functional Proteins

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### Abstract

Nanobody (Nb) or VHH is the smallest binding domain of camelid heavy chain antibody (HcAb). Light chains of HcAb naturally removed and because of some evolutionary changes, Nbs have unique properties rather than conventional antibodies. The size of Nb is about one-tenth (0.1) of whole antibodies and this size improved some problems of four chains antibodies such as high yield of expression in prokaryotic systems and penetration to tissues. Some other characteristics of Nb like close homology to human VH, high stability in the extended range of pH and temperature, and the capability to the identification of unusual epitope are very attractive for research and development of new Nb candidates for diagnosis, research, and therapeutic applications. Discovery of Nb almost coincided with advancement in phage display technology that was used along with Hybridoma technology in monoclonal antibody development. Currently, many of research groups focused on high-quality Nbs development against different targets especially in cancers and fortunately there are many of Nbs in clinical trial stages for use in extended ranges of diseases such as cancers, autoimmune, inflammatory diseases, and infectious diseases. Recently two of them were approved for clinical use. Big companies like Ablynx and Merck have been invested in this field and in future, further drugs base Nbs were approved in different areas of health science. In this review, we focused on production, features, and clinical application of Nbs and will be noted to Nbs in clinical trials.

**Keywords:** Monoclonal antibodies, Phage display, Camel heavy chain antibody, Nanobody/ (VHH), Cancer

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### 1. Introduction

Antibodies are glycoprotein compounds that were produced by B lymphocytes in response to antigens. First monoclonal antibodies were produced after invention of Hybridoma technology in 1975 by Milstein and Köhler [1]. Currently, there are about 40 monoclonal

antibodies in the pharmaceutical market and more than 350 monoclonal antibodies are in various stages of clinical trials. The first product in this field was the Muromonab-murrian anti-CD3 monoclonal antibody (Orthoclone or OKT3), designed against CD3 receptor of T lymphocyte cell [2]. These groups of monoclonal antibodies because of the murine nature have some disadvantages such as activation of human immune responses against murrian backbone and so did not earn much success in the pharmaceutical market. Therefore, great efforts were made for changing the murine scaffold. Consequently, the chimeric and humanized antibody was developed via grafting of mouse antigen-binding regions and CDRs respectively to human antibody scaffolds. In addition to these mAb, fully human mAb was produced [3]. Currently, some of mAbs such as Adalimumab, Rituximab, Bevacizumab, Trastuzumab, Cetuximab, and Infliximab which are respectively against rheumatoid arthritis, non-Hodgkin's lymphoma, colon cancer, breast cancer, colon cancer, and rheumatoid arthritis are a part of top selling biopharmaceutical mAbs in the world market [4]. Despite the importance and high advantages, these magic molecules have some drawbacks in industry, including large size about 150 kDa, the complexity of the structure, mispairing of light, heavy chains in expression, high cost of production in eukaryotic systems, and also high doses compared to the other therapeutic proteins [5].

Therefore, the scientist try to overcome these critical problems by the antibody engineering and production of smaller antibody fragments, such as antigen binding fragment (fab) and single chain variable fragment (scFv). Antibody fragments have certain advantages rather than the whole antibody including high expression level in microbial systems, high penetration to the solid tissue, low immunogenicity, high expression level in microbial systems, high penetration to the solid tissue, and low immunogenicity [6, 7].

In 1993 heavy chain antibodies were discovered in camelid serum by Hamers Casterman [8]. The other similar structures were identified in shark and ratfish. These kinds of antibodies have two heavy chains and light chains naturally were missed. The antigen binding domain of it called Nb or VHH (Vh of heavy chain antibody) or single domain antibody (sdAb) with a molecular weight of about one-tenth of the monoclonal antibody [9].

Due to the unique and natural characteristics of nanobodies, these molecules have a high potential for various therapeutic and diagnostic applications and many research groups and some big companies invested for identification and development Nbs candidate for treatment and diagnostics purposes. In this review, various aspects of the use and production of Nb have been considered. It should be noted that a lot of nanobodies are in various stages of clinical trials by big companies such as Ablynx [10].

### 1.1. Nanobody Features

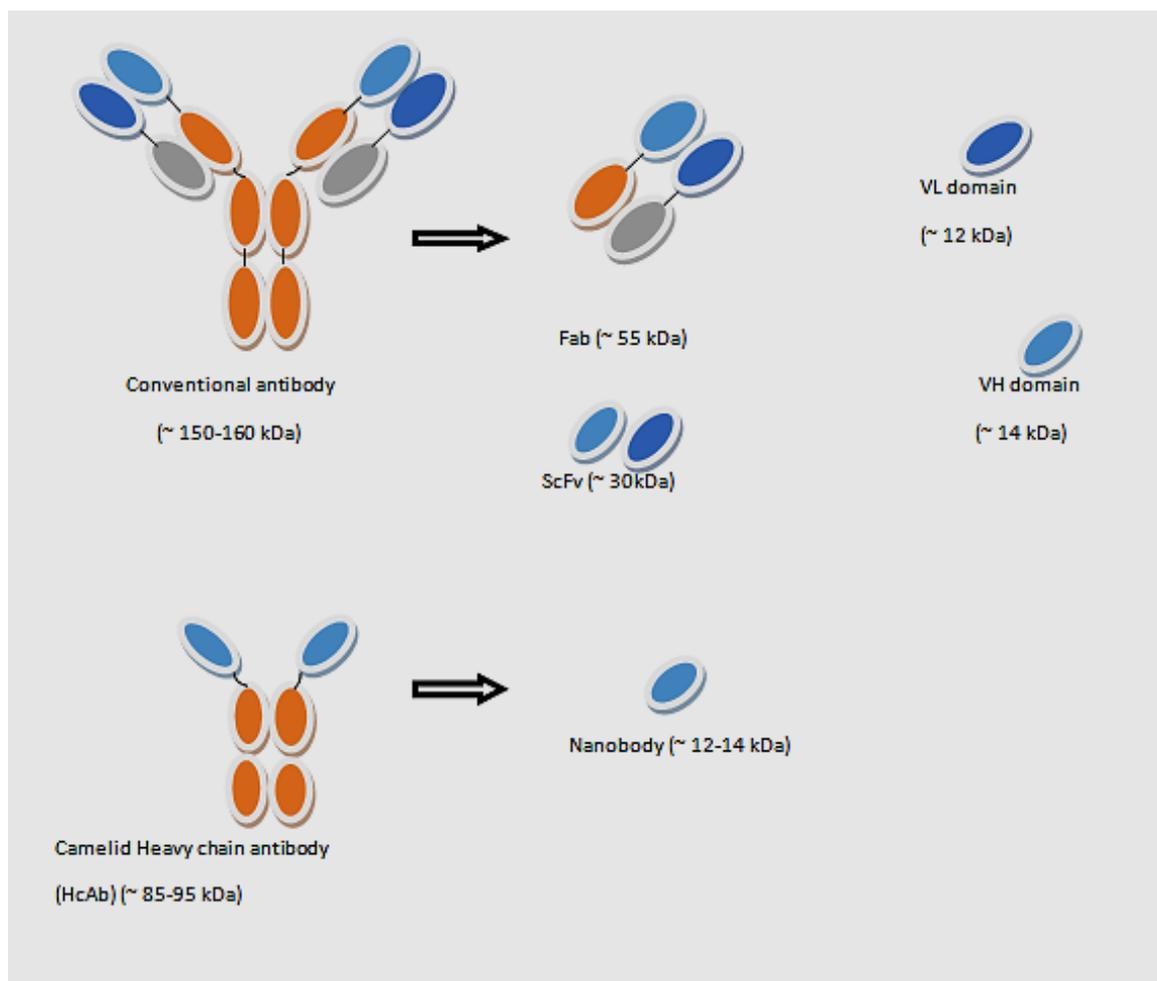
As noted in the serum of the camelid family (dromedaries and llamas) in addition to classic antibodies, there is a naturally certain type of antibodies with a unique structure, they have two heavy chains (VH) and do not have the light chain (Figure1). Moreover, they lack the first constant domain (CH1) and are called heavy chain antibody (HcAb) [11]. The molecular weight of HcAb is approximately 95-96 kDa. Antigen binding domains of HcAb are called VHH or Nb which have a molecular weight of 12-15 kDa [12]. Camel VHH indicates a high degree of homology with human VH sequences (gene family of VH3). Crystal structure of Nb as same as human VH indicates that their frameworks contain 2  $\alpha$ -sheeted structures. In contrary to murrain mAb this similarity leads to a low immune response in humans [13].

Similar to VH of conventional antibody, three hypervariable region or complementary determining region (CDR1-3) in VHH was surrounded by four frameworks (FR1-4). FR regions contain conserved residues sequences [14]. CDRs are very diverse and responsible for antigen binding (particularly CDR3), and in Nb, some evolutionary changes created unique properties which compensated lack of light chain. FR2 region contains important residues, called Nb hallmarks. These hallmarks, unlike the corresponding amino acids in VH that are hydrophobic, naturally are hydrophilic and this feature enhances the solubility, stability, and eliminates the need to light chain in Nbs [15]. Hydrophobic residues

in FR2 region of VH includes V37, G 44, L 45, and W47 that, in VHH have respectively converted to the hydrophilic amino acids, including F37, E44, R45, and G 47. Many of studies have demonstrated that CDR1 and CDR3 in camelid antibodies have been longer than VH and are linked to each other by disulfide bonds [16]. The average length of CDR3 in VH is 13 amino acids, while VHH has 18 amino acids lengths. In fact, CDR3 of VHH in comparison to VH has a convex and flexible structure and can easily penetrate into the epitopes grooves or even hidden epitope of unusual antigens which normally are not accessible in other antibodies [17, 18].

The half-life of VHH is about 2-18 hours. This half is not suitable for some applications such as tumor targeting and must be increased by some strategies such as binding of Nb to albumin (10-20 days) or anti-albumin Nb fusion protein, PEGylation, N- glycosylation, pentamerization (up to 14 days) and also by combining the Fc region of Igs with Nb (4-15 days) [19].

In addition to the above, the small size of Nb provides many advantages including a high level of production in prokaryotic systems, genetic manipulation capabilities, high tissue penetration, and faster clearance from the liver and kidney system that are beneficial in the application of Nb in imaging [20]. Solubility and stability of Nb in presence of proteases, a wide range of pH that is a high and low tendency to aggregation bring up Nbs applicable in different route of administration including intravenous injection, oral and



**Figure 1.** Schematic figures of conventional antibody and antibody derived fragments and camelid heavy chain only antibodies (HcAb).

sprays that are other advantages of nanobodies [21]. Other studies have demonstrated that nanobodies are more resistant to heat and detergents [22].

## 2. Materials and Methods

### 2.1. Production Method

Phage display technology is powerful tools for isolation of antibody fragment such as Nb from a library. This technology is based on a direct linkage between phenotype and genotype, which leads to displaying of small proteins or peptides on the surface of

filamentous phage which encoding genes are in the genome of phages [23].

In isolation of Nb, camel peripheral blood lymphocytes are isolated and total mRNA is extracted. Quality of mRNA ensures the isolation of suitable Nb. In addition, cDNA library is made using the reverse transcriptase reaction. In the general procedure, using two steps PCR reaction coding regions of Nb were specifically amplified. The primers of the first step of PCR are designed for hinge regain and the leader sequence. In the second step, the primers were designed for FR1 and FR4 regions. In the following, sequences of

nanobodies were cloned in the phagemid vector and transformed in the suitable host. Because of the high diversity of antibody genes in the body, the size of a library could be considered as reflection of diversity of antibody genes *in vivo*. The number of individual clones in library has the most important role in isolation of high-quality Nbs. So, the immune library should had been  $10^7$ - $10^9$  individual clones and a non-immune library should had been up to  $10^{11}$  individual clones. Phage library was constructed by library helper phage infection. Biopanning based on affinity selection on immobilized antigen or cell and even *in vivo* were used to high-quality Nb. After Nb selection, the efficiency and functional activity should be determined with functional assay methods.

The important note is that identification of high quality Nb via the phage display method is dependent on preparing the high quality RNA and cDNA.

Unique Nb features such as access to hidden epitopes and high binding capacity lead to Nb candidate as powerful diagnostic and therapeutic candidates. In many studies, Nbs were used in the therapeutic areas in the field of inhibition of cancer cells proliferation, inflammation, autoimmune, and infectious diseases. Currently, many nanobodies in the field of treatment of these diseases have reached the clinical trial stages. In the field of diagnostics especially for tumors, nanobodies have more advantages compared to conventional antibodies. Small size compared to whole antibodies results in faster

penetration into tissues and solid tumors, and speed of clearance of these fragments from blood flow is high and low drug dosages resulted in low side effects and immunologic responses [24].

## 2.2. Therapeutic Applications

Due to the unique Nb features such as access to hidden epitopes and high binding capacity, they can be used as powerful diagnostic and therapeutic candidates. Many studies have been done in the therapeutic areas in the field of inhibition of cancer cells proliferation, inflammation, autoimmune, and infectious diseases. Now also many nanobodies in the treatment of these diseases have reached the clinical trial stages. In the field of diagnostics especially for cancer tumors, nanobodies have more advantages compared to conventional antibodies. Small size compared to whole antibodies results in faster penetration into tissues and solid tumors, and clearance speed of these fragments from blood flow is high and low drug dosages resulted in low side effects and immunologic responses [25].

## 3. Results and Discussiuon

### 3.1. Infectious Diseases

High stability, high solubility, and capability of large-scale production in prokaryotic systems in comparison to conventional antibodies have created suitably applied capabilities for nanobodies. Great efforts have been carried out to develop the production of Nb against specific surface antigens of pathogenic bacteria, surface proteins of

viruses, fungi and also to identify epitopes of parasitic glycoproteins (Table 1). Nb against protein A of *Staphylococcus aureus* as a nano-conjugated particle enables early detection of these bacteria in about ten minutes [26].

Nb against human papilloma virus L1 antigen can bind to the virus with high affinity and this Nb can be considered as a candidate for the treatment of cervical cancer [27].

In the following, Nb was applied against transcriptase enzyme as an effective tool to inhibit replication of HIV virus and because of small size has the ability to identify the hidden

epitopes. Therapeutic effect on inactivation of HIV virus also can be considerable [28].

Inhaled Nb ALX-0171 which is in phase II of clinical trials has great potential in the treatment of respiratory syncytial virus (RSV) infection in newborns. ALX-0171 Nb inhibits the replication of RSV by binding to F protein on the surface of the virus and allows the host immune system to clean up the viruses [29].

ARP1 Nb against Rota virus rhesus monkey is at the end of phase II and is the candidate for the treatment of diarrhea-induced RV that has been successful in the animal rat model

**Table 1.** Nanobodies applications in infectious disease.

| Product name                                   | Target                                   | Disease                                 | Ref  |
|--|--|---|------|
| <b>ALX-0171</b>                                | RSV                                      | RSV <sup>1</sup> infection              | [29] |
| <b>ARP1</b>                                    | Rhesus monkey RV                         | RV-induced diarrhea                     | [30] |
| <b>H5- V<sub>H</sub>Hb</b>                     | H5 hemagglutinin                         | H5N1 influenza                          | [32] |
| <b>Nb D03</b>                                  | HCV E2 glycoprotein                      | HCV                                     | [33] |
| <b>Nb 190</b>                                  | Rev                                      | HIV-1                                   | [28] |
| <b>NbFedF6; NbFedF7</b>                        | Lectin domain F18                        | ETEC <sup>2</sup> and STEC <sup>3</sup> | [34] |
| <b>FlagV1; FlagV6</b>                          | Flagella                                 | <i>Campylobacter jejuni</i>             | [35] |
| <b>Multiple</b>                                | Biofilm-associated protein               | <i>Acinetobacter baumannii</i>          | [36] |
| <b>S36- V<sub>H</sub>H</b>                     | <i>Streptococcus mutans</i> strain HG982 | <i>S. mutans</i> <sup>4</sup>           | [36] |
| <b>Nb 25</b>                                   | TssM protein of type VI secretion system | Gram-negative bacteria                  | [37] |
| <b>Parental and HMR23 V<sub>H</sub>Hs</b>      | UreC subunit of urease                   | <i>Helicobacter pylori</i>              | [38] |
| <b>Nb An46 (lytic) and Nb An33 (non-lytic)</b> | VSG <sup>5</sup>                         | <i>Trypanosoma brucei</i>               | [39] |
| <b>Nb An33-Tr-<i>apoL-I</i></b>                | VSG                                      | <i>T. brucei</i>                        | [40] |
| <b>Nb 392</b>                                  | Paraflagellar rod protein                | Detection of all trypanosome species    | [40] |
| <b>Nb 4218</b>                                 | Myosin tail interaction protein          | <i>Plasmodium falciparum</i>            | [41] |

<sup>1</sup> Respiratory syncytial virus

<sup>2</sup> Enterotoxigenic *E. coli*

<sup>3</sup> Shiga toxin-producing *E. coli*

<sup>4</sup> *Streptococcus mutans*

<sup>5</sup> Variant-specific surface glycoprotein

[30].

Nbs have been used to identify parasitic glycoproteins epitopes, for example, a VHH fragment against Trypanosome strains has been produced for the development of a fast diagnostic flow cytometry-based system to quantitate pathogen concentrations in blood samples [31].

### 3.2. Inflammatory and Autoimmune Diseases

ALX-0061 or vobarilizumab is Nb against the receptor of interleukin -6 (IL-6R) which is in phase II clinical trial is developed for the treatment of autoimmune diseases such as

rheumatoid arthritis and lupus erythematosus of syphilis wound. Blocking of the IL-6 receptor through ALX-0061 inhibits IL-6 cascade and function [42].

Ozoralizumab against TNF $\alpha$  (Tumor Necrosis Factor- $\alpha$ ) is at the end of phase II clinical trial, and this Nb is developed for the treatment of autoimmune diseases focusing on rheumatoid arthritis [43].

Nb ALX-0761 at the end of phase I clinical trial is developed for the treatment of autoimmune diseases (psoriasis). ALX-0761 is a bi-specific Nb that blocks both targets of IL-17A and IL-17F to bind to their receptors [44].

**Table 2.** Application of Nanobody in inflammatory and autoimmune diseases.

| Product name           | target  | disease  | Ref  |
|------------------------|---|--|------|
| ALX-0061               | IL-6R   | RA <sup>6</sup> /SLE <sup>7</sup>  | [42] |
| ALX-0761               | IL17A/IL17F                                     | Psoriasis  | [44] |
| Ozoralizumab (ATN 103) | TNF   | RA   | [43] |
| ATN-192                | TNF   | RA   | [47] |
| ALX-0962               | IgE   | Asthma   | [46] |
| TROS                   | Human TNFR1                                     | TNF/TNFR1-mediated diseases  | [48] |
| Anti-IgGNb             | IgG   | Auto-IgG-mediated diseases   | [30] |
| Nb 14                  | MMP8  | Sepsis   | [49] |
| V <sub>H</sub> H 5G    | <i>N. meningitidis</i> LPS                      | Sepsis   | [50] |
| Nb 2;Nb 3              | Human procalcitonin                             | Sepsis   | [51] |
| C21; C28               | Fc-g-RIII[CD16]                                 | Recruitment of Fc-g-RIII killer cell to boost cytotoxicity of immune cells | [52] |
| Nb12-12                | Kv1.3 ion-channel                               | Autoimmune and inflammatory diseases                                       | [53] |
| Nb DC2.1               | Myeloid cells; immature bone marrow-derived DCs | Immunization against viral, cancer, and autoimmune antigens                | [54] |

<sup>6</sup> Rheumatoid Arthritis

<sup>7</sup> Systemic lupus erythematosus

Nb against CXCR<sub>2</sub> chemokine that is in phase 1 clinical trial has been produced for the treatment of inflammation [45].

ALX- 0962 Nb against immunoglobulin E (IgE), is in pre-clinical phase and can be used to treat asthma (Table 2) [46].

### 3.3. Cancer

Today, cancer is one of the concerns of the health sector in modern societies. Nb application in medical science creates new possibilities for diagnosis, imaging, and treatment of cancer in humans. Extensive researches are being conducted on the use of nanobodies as a smart drug delivery system

that could protect healthy tissue and have lethal properties only on the cancerous cells. In table 3, some of the nanobodies against cancer are listed that are against tumor-specific antigens and tumor-associated antigens and show effective results in inhibition of tumor growth. Anti-angiogenesis Nbs like anti-VEGF(R) family have highlighted results in prevention of growth and metastases of tumor cells in vitro and in vivo models [55-57]. Cancer imaging for monitoring therapeutic procedures through Nbs conjugate with different dyes improved some drawbacks of the whole antibody such as high background and low penetration to tumor tissues. Many of

**Table 3.** nanobody applications in cancers.

| Product name                                | target             | disease                                  | Ref  |
|---|--------------------|--|------|
| ALX-0651                                    | CXCR4              | Multi myeloma and non-Hodgkin's lymphoma | [58] |
| TAS266                                      | DR <sup>8</sup>    | Solid tumors                             | [55] |
| Antagonistic Nbs<br>Ia1; CONAN-1            | EGFR               | tumors<br>Solid EGFR                     | [59] |
| 4NC2  | EGFR               | Gastric cancer                           | [60] |
| Anti-c-MET Nanobody                         | c-MET              | Multiple myeloma                         | [61] |
| NB 4; NB 5                                  | CXCR7              | Head and neck cancer                     | [62] |
| CAPNb2                                      | CapG               | Breast cancer metastasis                 | [63] |
| Nb 2.17                                     | Leptin receptor    | Melanoma                                 | [64] |
| BsFab C21; BsFab C28                        | CEA/Fc-g-<br>RIIIa | Colon cancer                             | [65] |
| cAb-CEA5-b lactamase                        | CEA <sup>9</sup>   | Colon cancer                             | [65] |
| 7D12/38G7; 7D12/9G8                         | EGFR               | Glioblastomamultiform                    | [66] |
| 3VGR19-PE                                   | VEGFR2             | Inhibited tumor metastasis               | [67] |
| 7D12 Nb coupled to<br>twoZHER2:4 affibodies | EGFR and HER2      | EGFR1 and/or HER2 tumor                  | [60] |
| V <sub>H</sub> H7                           | MHC II             | MHCII cells in tumor tissue              | [68] |
| EGa1  | Ectodomain EGFR    | epithelial tumors                        | [69] |
| V <sub>H</sub> H1                           | HER2               | HER2 breast cancer                       | [70] |

<sup>8</sup> Death receptor

<sup>9</sup> Carcinoembryonic antigen

Nbs, which were up-regulated in cancer tissue like HER2, EGFR, and PMSA were considered in cancer imaging [55]

### 3.4. Application of Nanobodies in the Researches

#### 3.4.1. Proteomics

A group of proteins which are expressed in a particular time, under a certain biological condition by a genome, cell, tissue or organism is called proteome. A comprehensive comparative study of proteins in large-scale is the subject of proteomics science. Nanobodies as affinity capture reagents are suitable in this area because their small size and their single domain format provide higher capacity for binding to the surface. Chakravarty et al. in 2014 used the Nb-based capture affinity for the study of protein-protein interactions and analysis of DNA-protein interactions in vivo in genomic scale. Nb was used in the detection of bacterial infections and purification of recombinant proteins [71].

Specific Nb against KDEL is used for trap proteins in the endoplasmic reticulum.

Development of Nbs targeting signal peptide improved mapping of protein trafficking in physiological and pathophysiological conditions. Development of nanobodies against other non-conserved signal peptides may be challenging because these sites are not well conserved or are placed in a flexible region of the protein (i.e. are poorly antigenic). However, Nb-based affinity capture alone or in combination with other proteomics tools may be used in many of the modern proteomic targets [72].

#### 3.4.2. Intrabody (Intracellular Antibody)

Intrabody can be used for gene inactivation such as RNAi, and siRNA techniques. In fact, intrabody increases target specificity to inhibit multiple isoforms of protein. Intrabody can be designed to different targets in the nucleus, cytoplasm, and the endoplasmic reticulum; however, due to the importance of endoplasmic reticulum in protein production many of intrabodies were designed for these organelles. Intrabodies against amyloid- $\beta$  proteins inhibits the accumulation of amyloid-

**Table 4.** Several examples of studied intrabody.

| Functiontargeted   | Targets  | Ref      |
|--|--|----------|
| Oncogenic receptors  | MMP-9, cathepsin L, oncoprotein E7, VEGFR2, ErbB2, EGFR, metalloproteinases MMP-1              | [74, 75] |
| Virus proteins to prevent virus assembly                   | HIV-1, HBV precore antigen, HCV ApoB, HCV core protein   | [76, 77] |
| Knockdown of cellular virus receptors to block virus entry | CCR5, CXCR4  | [78, 79] |
| Receptors of the immune system                             | MHC I, integrins, VCAM-1, NCAM, TLR2,4 TLR9,5 IL-2, CD147, IL-6                                | [80-83]  |
| Nervous system   | Neurotrophin Receptor, b-amyloid protein, b- amyloid precursor protein, cellular prion protein | [84]     |

$\beta$  in Alzheimer's disease in the animal rat model [73].

Other functional intrabodies in the endoplasmic reticulum can be seen in table 4.

#### 4. Conclusion

Considering the natural source of nanobodies and other advantages that are mentioned, nanobodies may have a good situation in the field of health services. In addition, high similarity of Nb to the sequences of human antibodies and also the potential of humanized of the Nb reduce the concern about immunogenic reactions and in further future Nbs will enter the clinic.

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