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Use, Applications and Mechanisms of Intracellular Actions of Camelid VHHs

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Abstract

The discovery of heavy-chain-only antibodies (HCAbs) in *camelids* and *sharks* led to the rise of a new research field in which single-domain antibodies are used for various applications. Single-domain antibodies are the antigen-binding fragments derived from HCAbs showing several beneficial properties (e.g., small size, specificity, stability under extreme conditions, cost-effective production, and ease of engineering). Importantly, they are stable in reducing cytoplasmic environment, which allows their use as an intrabody to target a wide range of intracellular targets. In this chapter, we discuss both the therapeutic potential of camelid single-domain antibodies (nanobodies) and their use as a research tool with the main focus on its intracellular employment. Targeting intracellular proteins using nanobodies as a therapeutic per se is, up to now, limited due to its incapacity to traverse the cellular membrane. They can however serve as a stepping stone to small compound development, since they directly target a resident, endogenous protein, similar to how a conventional drug acts. In addition, nanobodies are highly adaptable tools and possess interesting properties for more fundamental research objectives like the elucidation of protein function, the tracking and visualization of endogenous proteins in an in vivo setting, and the assessment of protein-protein interactions.

Keywords: VHH, single-domain antibody, nanobody, intrabody, therapy, research tool

1. Nanobodies: a concise introduction

In 1993, Hamers-Casterman discovered the presence of heavy-chain-only antibodies in the sera of Camelidae and assessed that these antibodies are still capable of recognizing an extensive repertoire of antigens despite the absence of the light chain. Single-domain antibodies from camels are called nanobodies. They stated that this discovery could be of inestimable

value to the development and engineering of soluble V_H domains or new immunological molecules for diagnostic, therapeutic, and biochemical purposes [1]. This discovery gave rise to a whole new research field in which single-domain antibodies are used for a wide range of applications. Some of these will be reviewed in the current chapter.

The structural properties of conventional IgG antibodies are well known. These consist of two heavy-chain polypeptides and two light-chain polypeptides, each of which is folded into four and two domains, respectively. A variable domain is situated at the N-terminus of both chains (VH and VL) and, as the name suggests, its sequence diverges between IgG antibodies. Paired VH-VL domains make up the variable fragment (Fab) and are responsible for the recognition and binding of the target antigen. The sequence of the other domains is well conserved between IgGs, which led to the designation of these domains as constant domains. Heavy-chain-only antibodies differ from conventional IgG antibodies by the lack of a light-chain polypeptide and the first constant domain of the heavy-chain polypeptide (CH1). Consequently, the antigen-binding fragment of heavy-chain antibodies from camels consists of one single domain, termed the VHH domain. This unit forms the functional and structural equivalent of the Fab in conventional IgG antibodies [2]. The smallest antibody fragment that can be produced from conventional IgG antibodies is a short-chain variable fragment (ScFv, ± 27 kDa), which consists of a VH and VL domain linked via a polypeptide. In the continuous search for smaller antibody formats, HCABs were a thrilling novelty, because their discovery allowed researchers to produce an even smaller antibody fragment of only ± 15 kDa. This antibody format derived from camels consists of an isolated VHH domain also known as a single-domain antibody or a nanobody (Nb) (**Figure 1**). In addition, human single-domain antibodies VH and VL have been engineered from human conventional antibodies [3–5], and sharks develop heavy-chain-only antibodies (HCABs) too [6].

The structural features of nanobodies are quite similar to those of the variable domain of conventional IgGs. The core structure of the immunoglobulin domain is formed by four framework regions (FR), whereas antigen binding occurs through three complementarity-determining regions (CDRs). The latter are located in loops in between β -strands that form the variable immunoglobulin domain. Importantly, FR2 of the VHH domain often contains amino acid substitutions of residues that are involved in hydrophobic interactions between the VH and the VL domains of conventional IgGs (V37 \rightarrow F/Y, G44 \rightarrow E/Q, L45 \rightarrow R, and W47 \rightarrow G/F/L; Kabat numbering). These substitutions lie at the heart of the single-domain nature of nanobodies because they reduce the hydrophobicity of the former VL interface and improve the nonstickiness of the domain. There are other examples of amino acid substitutions that frequently take place, but these appear to be of less importance [7, 8]. Since nanobodies only consist of one domain, one might wonder whether nanobodies have a diverse antibody repertoire. After all, they lack the VH-VL combinatorial diversity in the antigen-binding site. Nanobodies have counterbalanced the absence of the three hypervariable loops of the VL domain by an *extension* of the hypervariable loops in the VHH domain. These loops show substantial variation in both conformation and length compared with the corresponding loops of the VH domain. This implies that a larger structural repertoire and thus a sufficient diversification in antigen-binding sites can be obtained [9]. More specifically, the introduction of additional Cys residues in the CDRs creates extra disulfide bridges within the VHH domain,

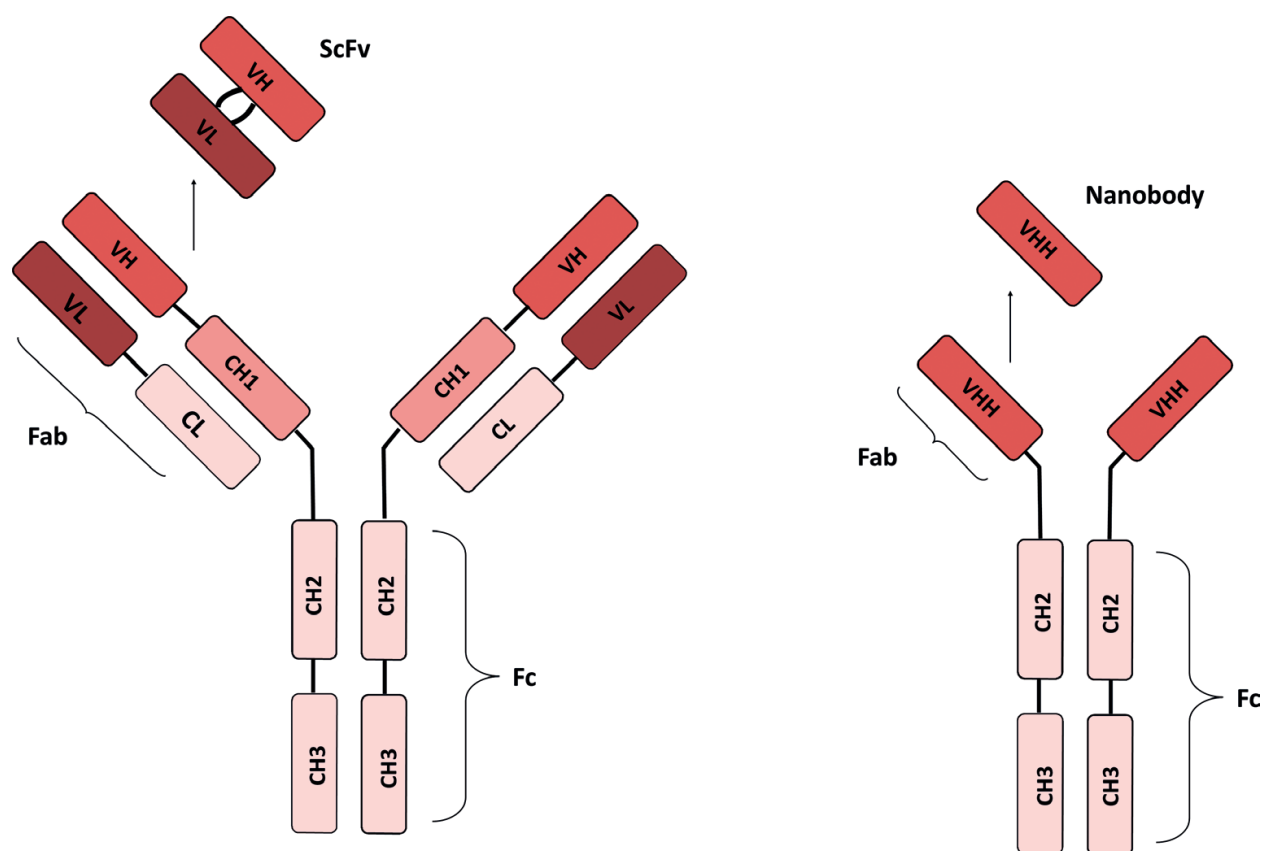


Figure 1. Schematic representation of a conventional IgG antibody and a camelid heavy-chain-only antibody (HCAb). For both antibody formats, the smallest producible antibody fragment is depicted: a short-chain variable fragment (ScFv, ± 27 kDa) and a nanobody (Nb, ± 15 kDa), respectively.

and these are in part responsible for the diversification of the structural repertoire. The disulfide bridges crosslink the antigen-binding loops, resulting not only in the stabilization of the domain but also in the induction of constraints in CDR1 or CDR3. These constraints probably lead to novel loop conformations and thus in an increase in paratope repertoire. Furthermore, VHHs are more prone to insertion and deletion events near or within the paratope compared to VHs. This is translated into an increase of the surface area of the hypervariable regions and contributes to the structural variation [10].

2. The therapeutic potential of extracellular and intracellular nanobodies

Several monoclonal antibodies (mAbs) have already been approved for clinical use [11], but some limitations are still present despite their success. This includes their large size, relative instability, which imposes restrictions on the administration route and their relative expense of manufacturing. The potential of nanobodies as a therapeutic agent was rapidly recognized as they overcome some of the aforementioned limitations of mAbs. The small size of nanobodies in combination with their extended CDR loops allows them to bind into clefts and cavities, whereas mAbs preferably recognize flat and concave surfaces. Many biological

interactions take place in clefts, and nanobodies can target these otherwise inaccessible surfaces and thus function as neutralizing agents or antagonists of protein-protein interactions. More specifically, this property is advantageous when it comes to therapeutically targeting infectious diseases since the essential epitopes of pathogens are often hidden. When cancer has to be targeted, the small size permits a better tissue penetration and thus a significant improvement of the effective antibody concentrations that can be reached in solid tumors. Unfortunately, this advantageous property comes with a price. The small size of nanobodies results in their rapid clearance from the human body and thus in a limited *in vivo* half-life (a few hours). Therefore, nanobodies are often linked to a serum albumin-binding monomer to prolong serum half-life. The monomeric nature of nanobodies simplifies antibody engineering. For instance, nanobodies can be assembled into multimers, thereby increasing their potency due to avidity effects. The development of therapy resistance can also be curbed by creating bispecific nanobodies. The creation of a targeted drug delivery vehicle is also possible, since nanobodies can easily be linked with drugs [12–17]. Additionally, the outstanding stability of nanobodies under extreme conditions opens the possibility to more patient-friendly routes of administration. In general, mAbs are administered via injections, but due to the extraordinary stability of nanobodies, they can be administered orally, topically, and even via inhalation [15, 18, 19]. The cost of nanobody production lies several times below those of mAb production. The fact that nanobodies are efficiently produced in microbial systems keeps the expenses low. Considering the fact that immunotherapy involves administration of relatively high doses of antibody during prolonged periods, this is a factor that should not be underestimated [7, 8, 20]. Currently, there are no nanobody-based products approved as therapeutic agent, but several products are in the pipeline or in advanced clinical trials. Their value in treating envenoming [21], infections [22], amyloidosis [23, 24], cancer, and other pathologies [25, 26] has already been proven. The research concerning the use of nanobodies as a therapeutic agent is mainly performed on extracellular targets. Nonetheless, nanobodies can also aid in identifying intracellular targets since they directly target a resident, endogenous protein, similar to how a conventional drug acts. RNAi-based approaches rather eradicate, or at least downregulate, expression which is quite different from the mode of action of an average drug. Hence, caution is warranted when making predictions regarding the therapeutic value of a given protein target using this approach. Moreover, nanobodies retain their functionality in the reducing intracellular environment. The major stumbling block toward a successful clinical implementation however is their inability to traverse the cell membrane. For that reason, most experiments are limited to cell cultures and transgenic animals. In the following paragraph, the use of nanobodies to target intracellular proteins with possible therapeutic implications will be described.

2.1. Intracellular nanobodies and antiviral therapy

2.1.1. Hepatitis

About 500 million people worldwide are chronically infected with hepatitis B virus or hepatitis C virus (HBV or HCV). Infection induces an acute and chronic inflammatory liver disease, which puts the patient at risk of developing liver cirrhosis and possibly hepatocellular

carcinoma. Currently, there is no vaccine available against HCV, and there is also no cure for most people who are already infected with HBV [27]. Initially, therapy existed of a strict and intensive treatment with ribavirin and PEGylated interferon. This regimen was however associated with severe side effects [28], emphasizing the importance of further research into the pathogenesis of the viruses and how they evade our immune systems. Extensive research led to the discovery of direct-acting antivirals (DAAs), which are small compounds targeted against viral enzymes. The second-generation DAAs are highly potent in treatment, show less side effects, and have a less intensive treatment regimen [29]. However, during infection a large number of viral variants are continuously produced, resulting in the presence of *quasi species* within the patient. This heterogeneity implies that not all therapeutic agents will be equally effective and that drug resistance may develop. Therefore, the search for other therapeutics remains useful. Sarrazin et al. mention that compounds targeting a more conserved region of viral proteins have a better chance of efficacy. Importantly, compounds targeting the active site of the viral polymerase show a high barrier to the development of resistance, due to the fact that mutations at this site often result in loss of polymerase function [30]. As stated previously, nanobodies can bind epitopes localized in clefts, like the active site of an enzyme, and thus are perfect candidates for antiviral drug development.

The potency of nanobodies for counteracting HCV infection has been evaluated. Several non-structural HCV proteins have been targeted via nanobodies: NS5B, NS3, NS4A, and NS4B [28, 31–33]. NS5B functions as an RNA-dependent RNA polymerase. NS3 has a dual function and displays serine protease activity in its N-terminal region and helicase activity in its C-terminal region. The helicase is involved in the replication of the viral genome and is also thought to increase the translational efficiency of the polyprotein by melting highly stable secondary structures in the HCV RNA [34]. The N-terminal serine protease domain is, together with NS4A, involved in downstream processing of the HCV polyprotein with the formation of mature proteins [32]. NS4B plays a major role in HCV replication by inducing an ER membrane web on which HCV replication takes place [31]. Nanobodies against the different nonstructural proteins were obtained via screening of a VH/VHH library constructed from peripheral blood mononuclear cells of an 8-month-old naïve male dromedary. Recombinant nanobodies reduced NS5B activity by two-thirds (ELISA) [28], and NS3 helicase activity was inhibited up to 88–100% in the presence of the nanobodies, depending on the assay used [33]. Cell-based assays were performed on Huh7 cells (human hepatoma cell line) transfected with RNA (genomic replicon of heterologous HCV). The nanobodies also led to a significant reduction of HCV RNA levels in Huh7 cells transfected with the JFH1 genotype 2a strain, both inside the cells as in the culture medium, when compared to control conditions. Overall, the nanobodies were capable of eliciting responses of a magnitude similar as seen for conventional therapeutic strategies (ribavirin + PEGylated interferon or telaprevir) [28, 31–33]. Furthermore, treatment of cells with the nanobodies against NS3/NS4A and NS4B induced the expression of genes involved in the innate immune response (IRF3, IL28-B, and IFN- β). This is interesting because the innate immune response signaling is interrupted by the virus [31, 32]. In general, these studies lack a detailed insight into the molecular mechanisms behind the observed effects. The authors used computerized modeling to make an assumption about which epitopes are recognized by the nanobodies. Crystallization studies would allow a more

detailed view and could help to resolve remaining questions. Even more, the crystal structures could lay the foundation for small molecule development and thus be invaluable. The nanobodies certainly have potential, but there are some remaining questions that need to be resolved before continuing with animal experiments.

As mentioned earlier, currently the biggest obstacle for using nanobodies as a therapeutic against intracellular targets is their inability to traverse cellular membranes. In the aforementioned articles, it was reported that coupling the nanobody with a cell-penetrating peptide (penetratin) seemingly promoted cellular uptake of the nanobody with efficiencies of roughly 80% [28]. However, some caution is warranted here, since the mechanisms behind the internalization of CPPs are still a matter of debate. Even more, in the highly cited article of Richard et al., it has been shown that experiments used to detect the occurrence of CPP internalization are sensitive to artifacts. It appeared that even mild fixation protocols used for fluorescence imaging can induce an artifactual redistribution of these peptides in the nucleus. Furthermore, the highly cationic nature of, for example, penetratin peptides lead to their strong binding to the overall negatively charged plasma membrane [35]. It is thus of crucial importance to remove membrane-bound peptide before analyzing cellular uptake of the construct. Initially, it was thought that CPPs allowed the delivery of biomolecules without relying on endocytosis. Adaptations of the used protocols, however, gave rise to data supporting an active process of cellular internalization involving endocytosis. Applications with CPPs and the controversial issues regarding their internalization mechanisms are elaborately reviewed and will not be discussed in detail [36, 37]. Internalization via endocytosis is however associated with a major drawback, since the delivered biomolecule needs to escape from the endosomal vesicles before it traffics back to the plasma membrane for recycling or it fuses with lysosomes. This might strongly limit the bioavailability of the compound, thus curbing its efficacy. Finally, the nonspecificity of CPP-conjugated constructs imposes a risk of drug-induced toxic effects on normal tissues [37]. In conclusion, a meticulous evaluation of intracellular uptake of the bioactive molecule and of possible toxic effects on normal tissues is warranted, before taking any further steps in its development as a therapeutic agent.

The genome of hepatitis B virus (HBV) is translated into HBV surface proteins, polymerase protein, X protein, or core and pre-core proteins [38]. Targeting the hepatitis B surface antigen (HBsAg) and the hepatitis B core antigen (HBcAg) with antiviral drugs to, respectively, reduce viral secretion and replication is a feasible strategy. HBsAg is the major component of the viral envelope. HBcAg, on the other hand, is the structural unit of the nucleocapsid that encloses the viral genome within a viral particle [38, 39].

To obtain nanobodies against the aforementioned proteins, a llama was immunized with recombinant HBcAg and HbsAg. The peripheral blood cells and cervical lymph node cells were used to construct a VHH library. Both immune and naïve libraries are good sources for retrieving antigen-specific binders. However, in general, superior binding affinities are observed for nanobodies originating from immune libraries, since they were subjected to *in vivo* affinity maturation. On the other hand, naïve libraries offer an elegant solution for those cases where immunization is difficult due to the lack of an antigen, low immunogenicity, or toxic antigens [39]. The nanobodies against HBsAg were cloned in frame with an ER-targeting

signal and an ER retention signal. Co-transfection of these nanobodies and a HBV-expressing plasmid in HepG2 cells induced the intracellular accumulation of HBsAg and caused a reduction of HBsAg particle secretion of approximately 80–90%. The *in vivo* potential of the HBsAg nanobodies was examined in a SCID mouse. The mouse model for HBV infection was created using a hydrodynamics-based transfection method. Remarkably, measured HBsAg levels in the plasma decline in the presence of the nanobody, and this reduction goes hand in hand with an increase in intracellular HBsAg levels. This observation implies that less virions are secreted. The researchers assume that the observed effects are either due to the disruption of the interaction between the nucleocapsid and the S-type of viral membrane proteins or due to the prevention of the interaction between individual proteins in the ER [40].

2.1.2. HIV

The current anti-HIV treatment strategy, known as highly active antiretroviral therapy (HAART), has changed the field and has turned HIV into a chronic manageable disease. However, patients are lifelong bound to this regimen, its associated side effects, and drug-drug interactions. Sometimes, treatment fails due to multidrug resistance which warrants research for alternative drugs [41]. Nanobodies could serve as a useful purpose in the treatment of HIV infection and have been successfully raised against Rev and Nef. Rev is involved in the nuclear export of late viral mRNA to the cytoplasm. Rev multimers form a higher affinity complex with RRE (Rev-response element), and this affinity is a determining factor for the efficiency of RNA export [42, 43]. The idea of targeting Nef with antivirals came from the observation that a limited amount of patients, infected with Nef-deleted HIV, presented a lack of disease progression. The Nef protein exerts multiple functions: CD4 downregulation, major histocompatibility complex downregulation (MHC1), activation of p21-activated protein kinase (pak2), and enhancement of virion infectivity. These functions can be targeted each independently from one another since the activities are genetically separable. Interfering with Nefs' capacity to downregulate CD4 appears to be the most effective strategy [44].

Vercruysse et al. produced nanobodies against the N-terminal multimerization domain of Rev, because its ability to form multimers is essential for its function. One nanobody is capable of efficiently inhibiting Rev multimerization in cell-based assays. The nanobody induces a cytoplasmic delocalization of Rev, that is similar to that observed for Rev mutants incapable of multimerization. In addition, the nanobody is able to suppress the Rev-dependent expression of late viral mRNAs and consequently also *de novo* virus production [42]. Further experiments were performed to elucidate whether the nanobody displays a broad-spectrum anti-HIV activity. This was examined by infecting several cell lines, expressing the nanobody in a stable manner, with different HIV-1 subtypes. Virus replication was monitored 5 days post infection by measuring cytopathogenic effects and the presence of virus-associated p24 levels in the supernatant. The nanobody strongly reduced p24 levels for infected cells compared to a control nanobody. More specifically, p24 levels were reduced by >10 folds for subtype A, >100 folds for subtypes C and G, and >10,000 folds for subtypes B, D, and H [45]. The cells proved to be resistant to viral replication and survived infection. These results are relevant, considering the fact that subtypes A, B, and C are the most prevalent genetic forms on a global scale [46].

Bouchet et al. picked Nef, a HIV-1 nonstructural protein, as target for antiviral therapy. Using cell-based assays and in vivo assays, it was established that the Nef-specific nanobody efficiently counteracted Nef-induced CD4 downregulation and p21-activated protein kinase (pak2) activation. The functional effects of the nanobody are thought to result from its interference with the interaction between Nef and other cellular partners [47]. Nef-induced CD4 downregulation in infected cells is important to prevent interaction between the envelope protein (Env) of the budding virion and CD4 of the host cell, since this interaction might impede the formation of fully infectious particles [44]. The nanobody is capable of reducing the rate of Nef-induced CD4 internalization back to levels measured in uninfected cells. The biological relevance of this observation was tested in a mouse model (CD4⁺/HIV Nef Tg mouse) that presents a downregulation of cell surface CD4, an altered thymic CD4 T-cell development, and a profound peripheral CD4 T-cell depletion. The nanobody rescued the Nef-mediated thymic CD4⁺ T-cell maturation defect and reversed the downregulation of cell surface CD4 in vivo. T-cell receptor signaling normally leads to profound actin cytoskeleton rearrangements. The Nef-pak2 complex, however, halts these rearrangements by deregulating cofilin, an actin-severing factor. Actin polymerization in infected T cells is thus strongly disturbed. The nanobody disrupts the Nef-Pak2 complex and counteracts as such the inhibition of actin remodeling in a dose-dependent manner. Finally, it was also observed that the inhibition of specific Nef functions by the nanobody resulted in the reduction of virus infectivity of new progeny virions by 80% (molar ratio of 1:1) [47].

Current HAART targets four different steps in the HIV-1 replication circle: the conversion of viral genomic RNA into dsDNA, the maturation of budding viral particles, the entry of the virus into new target cells, and the insertion of viral DNA into a host cell chromosome. Although current strategy is effective, it remains important to explore novel treatment strategies. The development of compounds that inhibit less explored drug targets would be of benefit, and structural biology can aid in defining new drug targets [48]. Nanobodies targeted against both Rev. and Nef appear to have pronounced effects on pathogeny of HIV-1. Crystallization studies to elucidate the exact binding epitopes for both nanobodies are thus of paramount importance since they could aid in new small compound design.

2.2. Intracellular nanobodies as a means to suppress toxins

There exists a multitude of antimicrobial drugs, but compounds capable of neutralizing the produced toxins are often lacking. The question whether or not antibodies hold great potential as toxin-neutralizing agents has been investigated by several researchers. Examples of studies where monoclonal antibodies are used as antitoxins are listed in the review of Chow et al. [49]. Several researchers have exploited nanobodies as a means to neutralize toxins. Intrabodies have been employed to counteract following toxins: ricin, *Salmonella* SpvB protein, and botulinum neurotoxin.

2.2.1. Ricin

Ricin is a naturally occurring toxin derived from the castor bean plant and a well-known type 2 ribosome-inactivating protein. It achieves an inhibition of eukaryotic ribosomes by the

depurination of a specific adenine in the 28S ribosome resulting in cell death. Exposure might be lethal, and unfortunately current treatments are mainly of a symptomatic and supportive nature [50]. Herrera et al. constructed a bispecific nanobody, named JJX12, consisting of a VHH targeted against the enzymatic subunit of ricin coupled with a VHH targeted against the galactose-binding subunit [51]. JJX12 fully protects mice against a ricin challenge (molar ratio of 4:1). The protective effects observed for the bispecific construct are superior to those observed for an equimolar mixture of the nanobodies and are the result of both extracellular and intracellular effects. JJX12 promotes aggregation of ricin in solution and makes cell-bound ricin-JJX12 complexes more resistant to dissociation as shown by ricin competition assays with lactose [51]. In the presence of these complexes, further ricin binding to the cell surface is reduced by shielding cell surface receptors for the galactose-binding subunit of ricin [52]. The presence of aggregates changes the internalization and intracellular trafficking of ricin. Internalization of the aggregates occurs via a macropinocytosis-like mechanism rather than via receptor-mediated clathrin-dependent and clathrin-independent endocytosis, which is normally observed for ricin. Furthermore, biochemical and live cell imaging techniques showed a 54% reduction of the retrograde transport of ricin to the trans-Golgi network and the accumulation of ricin in late endosomes in the presence of JJX12, which probably targets ricin for degradation [51].

2.2.2. *Salmonella SpvB protein*

Salmonella bacteria are Gram-negative enterobacteria associated with human enteric fever. The systemic virulence of the bacterium is largely dependent on the SpvB gene, encoding an actin ADP-ribosylating toxin that is secreted into the host cell cytosol. The toxin interferes with actin polymerization resulting in apoptotic cell death. Nanobodies targeted against the SpvB protein are capable of blocking its enzymatic and cytopathic effects. By means of in vitro radioactivity and fluorescence assays, it was demonstrated that the nanobody completely rescues actin polymerization from the inhibitory effects of the SpvB toxin at a molar ratio of 1:1. Cell-based assays, performed on RAW macrophages and Vero cells, confirmed these observations, since cells exposed to the toxin presented no signs of cell rounding or actin cytoskeleton disintegration in the presence of the nanobody [53].

2.2.3. *BoNTs*

Botulinum neurotoxins (BoNTs) produced by the Gram-positive bacterium *Clostridium botulinum* can cause flaccid paralysis in humans, which can last for several months. The toxins deliver their light chain, possessing a protease function, to the motor neurons. The protease cleaves SNARE proteins and as such prevents the release of acetylcholine from presynaptic nerve terminals at the neuromuscular junction causing a neuromuscular blockade [54, 55]. Two different strategies were used to suppress botulinum neurotoxin intoxication. Tremblay et al. investigated the potential of using nanobodies as a protease inhibitor per se [55], whereas Kuo et al. implemented the nanobody as a part of a targeted F-box agent to induce accelerated degradation of the protease [54]. A nanobody with nanomolar affinity ($K_d \sim 1$ nM) for the light chain of BoNT serotype A (A-Lc) was used for both strategies.

Serotype A is associated with the longest persistence and is thus most relevant for therapeutic intervention. The nanobody allows a near stoichiometric inhibition of BoNT-A function as shown by an in vitro FRET-based SNAP25 cleavage assay [55]. The production of cells expressing the nanobody in a stable manner could offer an elegant solution to problems associated with transient transfection techniques and was consequently implemented in the studies performed with the targeted F-box reagent.

Kuo et al. made a fusion protein between a nanobody and a truncated F-Box protein (TrCP) that is capable of associating with Skp1 and Cullin1, with the formation of the SCF complex. This complex, called targeted F-box (TFB), functions as an E3 ubiquitin ligase, thus targeting BoNT proteases for proteasomal degradation. Two constructs were made in which a nanobody targeted against either A-Lc or B-Lc was incorporated. The TFB fusion proteins reduce A-Lc and B-Lc levels with 65 and 50%, respectively (capture ELISA experiments), and decrease the half-life of the A-Lc protease (from ~3.7 to ~1.5 days). Application of MG132, a proteasome inhibitor, results in the accumulation of poly-ubiquitinated BoNT protease and eliminates the effect of the TFB fusion proteins on its steady-state levels. This indicates that the observed effects are due to the increased degradation of the BoNT protease. Furthermore, in the presence of the TFB fusion proteins, cells are less sensitive to BoNT-A intoxication and also recover 2.5 times faster [54].

2.3. Camelid intrabodies: a ministering angel for patients suffering from protein misfolding diseases?

Proteins exert crucial roles in a variety of cellular processes. Each of these proteins has to adopt its native tridimensional structure to acquire the functional biological state and thus to act faultlessly. However, sometimes proteins fail to either fold correctly or to maintain the native state due to the presence of mutations or increased protein levels. When these proteins escape the inherent quality control systems, serious diseases can develop. These disorders can be characterized by the deposition of misfolded peptides or proteins in the nervous system or other tissues and organs resulting in pathological and insoluble aggregates.

Preventive and curative treatments are often lacking. These therapeutic approaches are feasible when using nanobodies as a tool: increasing the stability of the correctly folded proteins, neutralization of toxic protein/peptide species, and inhibiting or reversing the aggregation of misfolded proteins into oligomers or fibrils [56]. Several research groups have already exploited the use of nanobodies for targeting protein misfolding diseases [57–59]; however, most of the time, they aim at extracellular targets. We will focus on the intracellular application of nanobodies.

2.3.1. Oculopharyngeal muscular dystrophy

Oculopharyngeal muscular dystrophy (OPMD) is an autosomal dominant disease characterized by an extended N-terminal poly-alanine stretch of polyadenylate-binding protein nuclear 1 (PABPN1). The poly-alanine stretch is extended from 10 to 12–17 alanines. The mutant protein forms aggregates in skeletal muscles, and this phenomenon is, at least in part, responsible for

the disease, although the exact pathological mechanisms are still poorly understood. PABPN1 is a multifunctional protein and is involved in pre-mRNA polyadenylation, transcription regulation, and mRNA nucleocytoplasmic transport [56].

Verheesen et al. screened a nonimmune VHH library for PABPN1-selective binders. Panning yielded six nanobodies with affinities ranging between 5 and 57 nM. Initial experiments were performed with nanobody 3F5 ($K_d = 5$ nM), which binds PABPN1 at its N-terminal coiled-coiled domain. Co-transfection of mutant PABPN1 and nuclear targeted 3F5 (3F5-NLS) in HeLa and COS cells showed a dose-dependent reduction in the formation of aggregates (37% \rightarrow 10% in HeLa cells, $P < 0.01$). The expression of the nanobody neither induces cytotoxic effects (MTT assay) nor has any effects on mutant PABPN1 expression levels [60]. A more in-depth analysis on how the formation of intranuclear inclusions is prevented revealed that the nanobody reduces the formation of oligomers of mutant PABPN1 but not of insoluble aggregates [61]. The in vivo efficiency of the six nanobodies was tested in a *Drosophila* model of OPMD in which the expression of mutant PABN1 and the nuclear targeted nanobodies is induced with the muscle-specific driver Mhc-Gal4. Nanobody 3F5-NLS showed the best in vivo efficacy and alleviated several symptoms of OPMD in the *Drosophila* model, including prevention of degenerative effects on flight muscles and the restoration of muscle fiber ultrastructure (Z and M bands). Transcriptome analysis performed to evaluate thorax gene expression patterns demonstrated that 3F5-NLS induced a partial or complete rescue of 58% of the genes deregulated by the presence of mutant PABPN1. These effects are the strongest in the early stages (day 2 after induction) but persist during the life span [62].

2.3.2. Gelsolin amyloidosis

Gelsolin amyloidosis is an autosomal dominant disease for which currently only symptomatic treatment strategies exist. A point mutation in the GSN gene (G654 A/T) is responsible for the incorrect folding of the secondary domain of mutant gelsolin (D187 N/Y) that adopts a protease-sensitive conformational state. A pathological proteolytic cascade involving furin and MT1-MMP like proteases leads to the secretion of amyloidogenic 8 and 5 kDa peptides in the extracellular matrix and thus to the formation of extracellular deposits [63]. Van Overbeke et al. used gelsolin nanobodies to shield mutant plasma gelsolin (PG) from aberrant furin cleavage [24]. Furin is a membrane-associated pro-protein convertase that is ubiquitously expressed. It cleaves mutant PG as it passes through the trans-Golgi network (TGN) and generates a C-terminal 68 kDa fragment (C68) that is secreted into the extracellular matrix [63]. This initial step in the amyloidogenic cascade is targeted using a Nb that binds mutant PG near the furin cleavage site with low nanomolar affinity (10 nM, in the presence of Ca^{2+}). In vitro experiments demonstrated a dose-dependent decrease of mutant PG cleavage. The C68 signal intensity is reduced by 76% ($P < 0.001$) when a twofold molar excess of Nb is added. In cell-based assays, the nanobody drastically reduced secretion of C68 in the cell medium. The in vivo efficiency of the nanobody was further analyzed in a gelsolin amyloidosis/nanobody double-positive mouse model expressing human mutant PG. The Nb not only positively affects transgenic mutant gelsolin proteostasis in skeletal muscle tissue but also attenuates the decrease in contraction speed of the extensor digitorum longus in an 8-min fatigue protocol

[24]. Using adeno-associated virus as a vehicle, a bispecific nanobody was introduced in these mice that protects against both furin and MT1-MMP, yielding similar effects on muscle contraction speed [64, 65]. Inhibiting the enzymatic activity of furin could be an alternative strategy, and noncompetitive furin-inhibiting nanobodies have been identified although they have not been tested for treatment of gelsolin amyloidosis [66]. However, despite the involvement of furin in several pathological processes, some considerations have to be made regarding its use as a therapeutic target. Although a complete/partial cleavage redundancy of furin toward several substrates was observed in the liver of an interferon-inducible Mx-Cre/loxP, furin knockout mouse model and obvious adverse effect were absent; a complete knockout of furin in a mouse model resulted in embryonic lethality at day 11 [67, 68]. This observation probably precludes their use in chronic treatments because it is rather unlikely that the long-term inhibition of furin does not go hand in hand with severe adverse effects. Therefore, shielding mutant PG from aberrant cleavage seems to be the better strategy. Moreover, this approach is already successfully implemented in the treatment of early-stage familial amyloid polyneuropathy caused by amyloidogenic variants of transthyretin, thus highlighting its feasibility [69].

3. Camelid intrabodies: a versatile research tool

Over the years, nanobodies have earned their mark as a research tool. A variety of extracellular and intracellular applications using nanobodies exist, and the latter will be discussed here. Intrabodies are often used to unravel protein functions and to gain insight into their dynamics. The versatility of nanobodies and the ease by which they can be engineered allow researchers to use different lines of approach (**Figure 2**). Chromobodies, consisting of a nanobody fused with a fluorescent protein, allow researchers to recognize and trace endogenous proteins in living cells [70]. Since they are already well known, they will not be discussed in detail here.

3.1. Pinpointing protein functions

Nanobodies are an attractive tool for the determination of endogenous protein function. They not only complement well-known RNAi and CRISPR/Cas9 techniques but also allow a more detailed insight by pinpointing specific functions with “surgical precision” by targeting individual protein domains (rather than eliminating the entire protein altogether) and protein conformations, which cannot be achieved by expression modulation. In other words, nanobodies can be of inestimable value to deepen our knowledge of several biological pathways. Researchers have employed several strategies for assessing the functionality of proteins or protein domains, and the different options will be discussed here.

As stated earlier, nanobody cDNAs are available, and these are easily engineered. This implies that the addition of a delocalization tag is fairly straightforward. A variety of targeting sequences are available and can be used to induce the enrichment of both nanobody and its target at specific (ectopic) subcellular compartments. This strategy allows researchers to assess the interaction between the nanobody and its target in the strongly reducing intracellular environment

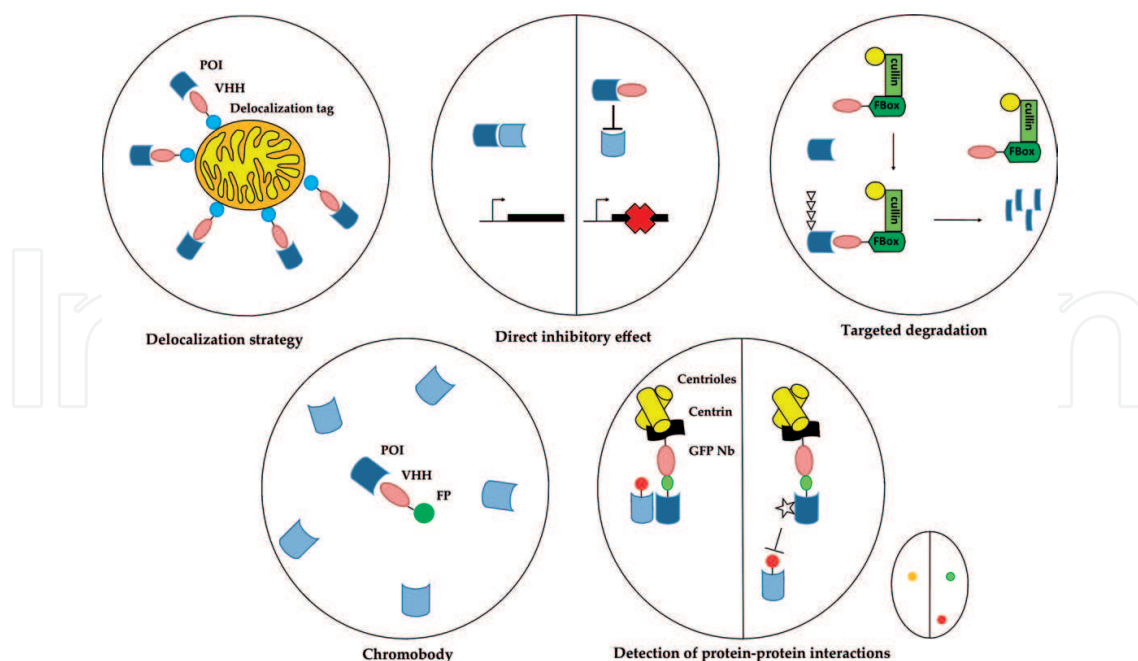


Figure 2. Schematic overview of nanobody-based applications in research. Upper panel. *Left:* Nanobodies can be equipped with a delocalization signal sequence, which allows the enrichment of a protein of interest (POI) at a specific subcellular localization, such as mitochondria. *Middle:* Nanobodies can exert a direct inhibitory effect which induces a protein knockout. *Right:* Nanobodies can be used to target a POI for proteasomal degradation. For this purpose, the nanobody is fused with a cullin-RING E3 ubiquitin ligase. Lower panel. *Left:* A chromobody, consisting of a fusion between a nanobody and a fluorescent protein (FP), allows the visualization and tracing of endogenous proteins in a background of contaminating proteins. *Right:* This nanobody-based application allows one to determine whether or not two proteins of interest interact. Both proteins are labeled with a different fluorophore. One protein, labeled with GFP, is recruited to a specific location via a GFP Nb coupled with a targeting signal. If the second protein, in this example labeled with RFP, is an interaction partner, both fluorescent signals will co-localize. When a compound interferes with a specific protein-protein interaction, the co-localization is absent.

and thus to confirm the *in vivo* functionality of nanobodies. Moreover, in this way, one can also disturb protein function by restricting free diffusion of the protein and limiting its availability at places where it is needed [71]. Considering that the paratope of the nanobody is located at its N-terminal end, it is safer to fuse the tag at the C-terminal end of the nanobody. Otherwise, a substantial risk at disturbing antigen binding exists [2], although there are examples where a long tag is added to the nanobody N-terminus without disturbing its functionality [72]. Beghein et al. elegantly demonstrated how effectively nanobodies can delocalize their target protein to a variety of subcellular organelles. A survivin Nb (Kd ~ 1 nM) was capable of guiding endogenous survivin in or out the nucleus (*nuclear localization sequence tag* and *nuclear export sequence tag*), capturing survivin on the outer membrane of mitochondria (*mitochondrial outer membrane tag*) and even at the intermembrane area (*mitofilin tag*) which probably required (partial) unfolding of the nanobody and possibly chaperone-assisted entry into mitochondria. This had not yet been investigated. Also, transport of survivin in the peroxisomes (PST-1 tag) was demonstrated [72]. Since interaction between the nanobody and survivin apparently did not perturb survivin functionality, the tagged nanobody is a perfect research tool for further elucidating survivin biology. This strategy also provided evidence that only actin-free gelsolin is able to migrate to the nucleus (in contrast to the actin-gelsolin complex) to potentially act as a nuclear cofactor for

the androgen receptor [73], that fascin plays a role in the formation of filopodia/cell spreading and is also involved in MMP9 secretion, and that the SH3 domain of cortactin directly regulates MMP secretion [74].

Some nanobodies exert a direct inhibitory effect, resulting in a functional knockout of the protein. These nanobodies can help researchers to define the biochemical activities of proteins. For example, mechanistic insights in podosome formation were revealed by two inhibitory nanobodies targeted against L-plastin (LPL). LPL Nb5 is capable of blocking the actin-bundling activity of L-plastin, and LPL-Nb9 locks LPL in an inactive conformation. Experiments involving these nanobodies revealed the participation of L-plastin (LPL) in podosome formation and stability [75]. Furthermore, L-plastin is a component of cancer cell invadopodia and contributes to matrix degradation and cancer cell invasion. These effects are mediated by the actin-bundling activity of L-plastin and its bundling independent role in MMP9 secretion and activity, as revealed by the differential effects observed in the presence of LPL Nb5 and LPL Nb9 [76]. One can also interfere with signaling pathways by specific inhibition of the transcriptional activity of proteins, like beta-catenin and p53 [77, 78]. These nanobodies can be used to elucidate the impact of cofactors and post-translational modifications on the targeted protein and allow us to broaden our understanding of the respective signaling pathways. Insight into pathological mechanisms, which might result in the identification of druggable targets, can also be obtained. For example, nanobodies were used to investigate the role of two enzymatic domains of TcdB, a toxin produced by *Clostridium difficile*. Using specific inhibition of the effector glycosyltransferase activity or the cysteine protease, it was, among other things, established that the TcdB-cytopathic effects are mainly mediated by the glycosyltransferase activity [79].

Finally, nanobodies are known to stabilize certain protein conformations and are often used as an aid in crystallization experiments [2]. This property also comes in handy when one wants to study the mechanisms by which cellular receptors translate extracellular cues into intracellular responses. Depending on which conformation the receptor adopts after ligand binding, certain downstream signaling events can be either activated or inhibited. Staus et al. have identified nanobodies that preferentially recognize and stabilize the $\beta 2$ adrenergic receptor in its active or inactive conformation resulting in a variety of functional effects [80]. These experiments indicate that nanobodies, by acting as an allosteric modulator of receptors, can help us to understand receptor biology.

3.2. Depleting endogenous proteins through proteasomal targeting

An alternative way to determine the function of a protein of interest (POI) in an in vivo setting is to selectively induce their degradation and study the resulting knockout phenotype. To achieve this goal, three different groups have exploited a combination of nanobodies and the endogenous ubiquitin proteasome pathway, a system that is responsible for selective protein degradation in eukaryotes [81–83]. Caussinus et al. were the first to use the ubiquitin pathway for targeted degradation by making adaptations of an E3 ubiquitin ligase, more specifically the cullin-RING 1 (CLR1) E3 ligase complex. For this purpose, a fusion between the F-box domain of *Slmb* and a GFP Nb (VHH GFP4) was made. *Slmb* is part of an F-box protein, responsible for substrate recognition that is expressed in *Drosophila melanogaster*. When this construct,

called DeGradFP, was expressed in mammalian cells or *D. melanogaster* embryos, certain GFP-tagged proteins were depleted. DeGradFP was also capable of phenocopying specific loss of function mutations. In spite of these successful results, treatment with DeGradFP was not always followed by the degradation of the targeted protein (e.g., GFP) [81]. In addition to that, a broader application of DeGradFP is still to be demonstrated.

Just like DeGradFP, the cullin-RING E3 ubiquitin ligases were used as the framework for synthetic E3 ligase design. In an attempt to enhance the E3 activity, however, the GFP Nb was fused directly to a truncated adaptor protein instead of the substrate recognition protein. The best results were obtained with Ab-SPOP, a synthetic version of the CLR3 E3 ligase complex, displaying a 10-fold stronger signal reduction of a GFP-tagged protein compared to DeGradFP (50-fold vs. 5-fold). Importantly, the construct degrades only nuclear proteins, and possibly in the future, similar constructs may become available that degrade cytoplasmic proteins. The in vivo effectiveness of Ab-SPOP was confirmed in zebra fish embryos. Ab-SPOP-induced depletion of Hmg2a-citrine, a protein responsible for the modulation of nucleosome and chromatin structure, resulted in various early developmental defects [83]. Fulcher et al. tailored the von Hippel-Lindau (VHL) protein as an affinity-directed protein missile, called AdPROM. Under normoxic conditions, this substrate recognition protein recruits the hypoxia-inducible factor (HIF1 α) to the CLR2 E3 ligase. AdPROM is composed of a fusion between the VHL protein and a GFP Nb. It was of crucial importance that the GFP Nb was positioned at the C-terminus of the VHL protein in order to obtain a proper orientation of the target proteins to the CLR2 E3 ligase complex. Since the paratope of a nanobody is localized at the N-terminal end, one should definitely check for potential detrimental effects of this fusion on the binding capacity of the nanobody itself. However, the affinity-directed protein missile was competent in inducing the specific degradation of GFP-tagged VPS34 and PAWS1 proteins in human cell lines, which was further substantiated by the observation of functional effects. Interestingly, during these experiments the researchers observed the co-degradation of UVRAG which is a regulatory component of the VPS34 kinase complex. This suggests that AdPROM has the potential of destroying protein complexes although only individual proteins are targeted [82]. Targeted degradation of proteins of interest by the use of nanobodies holds great potential and might be the perfect complement to CRISPR/Cas systems or RNAi in the elucidation of protein function. The tunability of this system is a huge benefit. Future experiments should point out whether the GFP Nb can be replaced by highly selective nanobodies targeted against specific proteins. In this way, one could investigate the functions of the protein of interest in a more direct manner, without the requirement of protein tags.

3.3. Detection of protein-protein interactions

Nanobodies can be utilized for the detection of protein-protein interactions in cell-based assays. There is a large supply of in vitro methods which can be used for the detection of protein-protein interactions. These methods are widely used and highly efficient for high-throughput screenings but are limited by the fact that they don't operate in intact mammalian cells. Screening for interaction between proteins in their native environment guarantees their proper folding and the presence of necessary cofactors or regulatory proteins. Both

nanobody-based methods rely on the interaction between a GFP Nb and a GFP-tagged protein. Herce et al. covalently linked a GFP Nb with a protein that accumulates at a specific subcellular location. In mammalian cells, this protein could be, for example, laminin B1 or centrin, which results in the delocalization of the GFP Nb to the nuclear lamina or the cytoplasmic centrioles, respectively. Subsequently, a GFP-tagged protein will be recruited to a specific location. If the second protein of interest, labeled with another fluorophore, interacts with the first protein of interest, the fluorophores will co-localize at a discrete spot. This interaction can be visualized by a single-fluorescence snapshot. Interestingly, this technique also allows screening for inhibitors of protein-protein interactions [84]. Another recently developed technique uses biocompatible engineered upconversion nanoparticles (UCNPs) conjugated with GFP Nbs. Visualization of the interaction between two proteins of interest is based on the lanthanide resonance energy transfer (LRET). As a proof of concept, they probed for the indirect interaction between the mitochondrial proteins TOM20 and TOM7. The latter was expressed as a fusion protein with EGFP and the former as a fusion protein with dsRed and a Halo tag. This Halo tag was subsequently labeled with tetramethylrhodamine (TMR), while the EGFP was recognized by the GFP Nb-labeled UCNPs. Co-localization of both proteins results in the detection of LRET-sensitized TMR emission. Remarkably, TOM7 and TOM20 are spatially separated by TOM40. The capacity of this technique for reporting indirect long-distance interactions might be of interest to unravel cellular protein complexes [85].

4. Concluding remarks

Nanobodies are highly versatile tools with interesting biochemical properties, which result in their application in various fields ranging from basic research and diagnostics to therapy. In this chapter, we aim to shed light on their multifunctionality and in this way encourage other researchers to include this technology in their future projects. Since their discovery in 1993, the numbers of publications wherein nanobodies are employed are gradually increasing which indicate that their merit has been proved. Here, we have shown that nanobodies have a high therapeutic potential and form an ideal stepping stone to drug development. Despite isolated cases, nanobodies are not capable of traversing the cellular membrane, preventing their direct use as a therapeutic. The effects observed with nanobody treatment are established through multiple mechanisms. Nanobodies can act as an inhibitor of enzymatic activity, interfere with specific protein-protein interactions, and shield a protein of interest from aberrant cleavage, or they can be used as a tool to target proteins for proteasomal degradation. We believe that effects triggered by nanobodies *in vitro* or *in vivo* are a faithful representation of what to expect with conventional pharmacological drugs, since both compounds directly target the resident endogenous protein. However, since current experiments are often limited to cell-based assays, animal experiments are warranted to confirm their effectiveness. Furthermore, nanobodies have a lot to offer as a research tool. They can help researchers to elucidate protein functions and thereby gain insight in biological pathways. Several strategies are possible, ranging from subcellular delocalization to the induction of protein knockouts. Last but not least, nanobodies may represent an adequate answer to problems encountered with (conventional) antibody reproducibility

[86, 87]. Indeed, particularly polyclonal antibodies run out of stock at some point in the future, making experimental verification impossible. Because nanobody cDNAs are readily obtained and researchers all over the world can use exactly the same nanobody in their experiments, problems of reproducibility can be reduced. In the future, we hope to stimulate a closer consultation within the nanobody field and by doing so taking the research to the next level.

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