Transforming nanobodies into high precision tools for protein
function analysis.

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Running head: Expanding the nanobody toolbox repertoire.

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20 **Abstract. (250w max)**

21 Single domain antibodies, derived from camelid heavy antibodies (nanobodies®) or shark variable
22 new antigen receptors, have attracted increasing attention in recent years due to their extremely
23 versatile nature and opportunities they offer for downstream modification. Discovered more than
24 three decades ago, these 120 amino acid (~15kDa) antibody fragments are known to bind their
25 target with high specificity and affinity . Key features of nanobodies that make them very attractive
26 include their single domain nature, small size, affordable high level expression in prokaryotes, and
27 their cDNAs are routinely obtained in the process of their isolation. This facilitates and stimulates
28 new experimental approaches. Hence, it allows researchers to formulate new answers to complex
29 biomedical questions. Through elementary PCR-based technologies and chemical modification
30 strategies, their primary structure can be altered almost at leisure whilst retaining their specificity
31 and biological activity, transforming them into highly tailored tools that meet the increasing demands
32 of current day biomedical research. In this review, various aspects of camelid Nanobodies are
33 expounded, including intracellular delivery in recombinant format for manipulation of i.e.
34 cytoplasmic targets, their derivatization to improve nanobody orientation as a capturing device,
35 approaches to reversibly bind their target, their potential as protein silencing devices in cells, the
36 development of strategies to transfer nanobodies through the blood brain barrier and their
37 application in CAR-T experimentation. We also discuss some of their disadvantages and conclude
38 with future prospects.

39

40 Keywords: nanobody, single domain antibody, VHH, immunomodulation, intrabody.

41 List of Abbreviations

42	% ID	Percent injected dose
43	% ID/cc	Percent injected dose per cubic centimeter of tissue
44	% ID/g	Percent injected dose per gram of tissue
45	AdPROM	Affinity-directed PROtein Missile
46	AiD	Auxin-inducible degron
47	AID	Auxin inducible degradation system
48	AMT	Adsorptive-mediated transcytosis
49	BBB	Blood-brain barrier
50	BBBD	Blood-brain barrier disruption
51	CAR	Chimeric antigen receptors
52	CDR3	Complementary determining region 3
53	CNS	Central nervous system
54	CNT	Carbon nanotube
55	CPD	Cell-penetrating polydisulfides
56	cpDHFR	Circularly mutated bacterial dihydrofolate reductase enzyme
57	CRL	E3 cullin-RING ubiquitin ligase complex
58	CuAAC	Copper(I)-catalyzed azide alkyne cycloaddition
59	deGradFP	Degrade Green Fluorescent Protein
60	degron	Destabilizing domain
61	DiPD	Drug induced protein degradation
62	EgA1	EGFR targeting nanobody
63	FU-BBBD	Focused ultrasound blood-brain barrier disruption
64	GQD	Graphene quantum dots
65	HcAb	Heavy-chain antibody
66	HIF1 α	Hypoxia factor 1 α
67	IAA	Indole-3-acetic acid
68	icv	Intracerebroventricular
69	IN	Intranasal
70	LiPD	Light induced protein degradation

71	mAb	Monoclonal antibody
72	MWCNT	Multi-walled carbon nanotube
73	Nb	Nanobody
74	OBBBO	Osmotic blood-brain barrier opening
75	optobody	Optogenetically activable intrabodies
76	PCNA	Proliferative cell nuclear antigen
77	PDT	Photodynamic therapy
78	PEG	Polyethylene glycol
79	PEI	Polyethylenimine
80	PEST	Pro-Glu-Ser-Thr degron
81	PET	Positron emission tomography
82	POI	Protein of interest
83	PROTAC	Proteolysis targeting chimeras
84	protein-i	Protein interference
85	RMT	Receptor-mediated transcytosis
86	RNAi	RNA interference
87	ROS	Reactive oxygen species
88	SATA	N-succinimidyl-S-acetyl-thioacetate
89	scFv	Single-chain variable fragment
90	SPAAC	Strain-promoted azide-alkyne cycloaddition
91	SPECT	Single-photon emission computed tomography
92	SPOP	Speckle-type POZ protein
93	TIR-1	F-box transport inhibitor response 1 protein
94	UPS	Ubiquitin-proteasome system
95	VCAM-1	Vascular cell adhesion protein 1
96	VHH	Variable domain of heavy-chain antibody or nanobody
97	VHL	Von-Hippel Lindau protein
98	VNB	Vapor nanobubbles
99		
100		

Introduction

As scientists investigate cellular behavior and the profoundly complex interplay between its numerous molecular constituents, they have recourse to a panoply of research tools allowing them to manipulate and perturb normal behavior of biological molecules. Without these tools, scientific progress would proceed at a tantalizingly slow pace. Of the many tools at hand, antibodies have been an extremely useful and important asset, and they have served investigators in different ways: as a research instrument, a diagnostic and a therapeutic.

In the course of the previous decades we have also come to experience that working with antibodies can lead to problems of reproducibility, *inter alia*. Indeed, polyclonal antibodies for instance are available only in finite quantities and producing exactly the same batch of antibodies even in the same animal is virtually impossible (8, 22). This means that many experiments once performed with these antibodies simply can never be reproduced again. It was hoped that hybridoma cell lines, yielding monoclonal antibodies, would solve this problem. Alas, these cell lines can also die or lose their antibody genes. Moreover, some level of aspecificity is inherently present in biology and many tools, including antibodies, suffer from some degree of non-specific interaction, which means they always have to be carefully characterized, in particular new antibodies, and appropriate controls should always be included.

Although discovered nearly three decades ago (60), in retrospect it has taken considerable time before camelid heavy chain antibodies became a familiar concept. Basically, this type of antibody is devoid of light chains and consists of two heavy chains only, each built up of three IgG modules each. The monovalent antigen binding part of such a heavy chain is known as a single domain antibody or nanobody®. A nanobody is barely 15 kDa in size (110-120 amino acids) and obtaining its cDNA in the course of finding antigen specific binders is one of the greatest assets. Yet even today it seems there is still a significant fraction of the research community who are not familiar

with nanobodies and for what purposes they can be used. Industry is lagging further behind although interest is also increasing there after a long lag period. The reasons for this constitute a topic worthy of investigation in itself and will not be covered in this review but very briefly, it is in part related to the fact that raising nanobodies against any given antigen is a labor intensive process and involves a complex recipe although we are witnessing an improvement over the past years (118, 192) as new protocols are being formulated. Second, since the advent of RNAi in mammalian cells in 2000 (45), siRNAs were picked up relatively quickly by the research community and soon evolved into the availability of several siRNAs targeting virtually each and every possible gene of interest. So why follow the path of highest resistance by generating nanobodies?

Today it is estimated that nanobodies have been generated against less than 1000 targets, in so far that they have been reported in literature. This means we still have a long way to go if we want to cover the entire proteome (human, and numerous pathogens such as corona (42, 68, 87), our unpublished observations). It is definitely worthwhile to follow the chosen path further because nanobody research has generated and will continue to generate new applications but they can also yield more information regarding the function or structure of its target, which in many cases is a protein. Indeed, while RNAi is very successful in down-regulating the expression of its target (albeit it mostly incompletely), it results in eradication of the entire protein. Many proteins however are built up in a modular fashion, particularly proteins from higher organisms, with each domain displaying a different function or interaction partner(s). The subtle behavior of proteins obviously can no longer be investigated when the protein has disappeared altogether from cells. Most conventional immunoglobulins are available in protein format which precludes them crossing the plasma membrane if they need to be used for modulating protein function in cells (it should be noted however that variable regions of immunoglobulins can be cloned at high throughput (70, 151)) . Furthermore, the disulfide bridges keeping the light and heavy chains together would be reduced by the millimolar concentrations of glutathione in the cytoplasm and in cellular organelles. A solution to this problem is the single chain fragment variable (scFv) format. Quite often however, scFv fragments

fold incorrectly or cannot bind their antigen when used as intrabodies but a screening method has been developed to identify scFv fragments that are functional (15). Nanobodies can be directed against circulating and transmembrane proteins, but thanks to their single domain nature and cDNA availability they can be transformed, transfected to transduced like any other cDNA. Their interaction with the (endogenous) target may result in blocking distinct functions of the protein or preventing protein-protein interactions, leaving other functions intact, with the target still present in the cell. As such they can be used as silent molecular rovers that blot out regions of their target and thereby reveal the function of that protein fragment. But many other applications exist and the field is still expanding.

This review is not intended to give a comprehensive overview because the field is entering a distinct phase of expansion as more labs become interested in using camelid nanobodies and shark variable new antigen receptor (VNAR) fragments for their research. Also, several aspects are more than aptly covered in recent reviews such as generation, purification, therapeutic, diagnostic, and clinical potential of nanobodies (10, 36, 69, 78, 120). Instead we here propose to highlight topics where we think progress has been made in manipulating camelid nanobodies for various purposes such as intracellular delivery, derivatization, reversible target binding, brain targeting and protein silencing, which is hopefully of interest to a broad audience. It serves to highlight and emphasize how researchers are fully exploiting the potential of single domain antibodies and this will serve as a springboard for the next era of nanobody research.

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175 **Intracellular delivery of nanobodies: ferrying across the plasma**
176 **membrane.**

177 A decade or more ago, raising nanobodies against intracellular targets was sometimes
178 frowned upon by colleagues in the coffee room because 'recombinant nanobodies could never cross
179 the membrane, make it to the cytoplasm and reach their target. The mere physical properties of a
180 hydrophobic-hydrophilic-hydrophobic stratified lipid bilayer preclude spontaneous transport of a
181 broad variety of small and large hydrophilic compounds including antibodies and antibody fragments
182 such as nanobodies. Use of intracellular nanobodies was further compounded by the uncertainty if
183 nanobodies would be stable in the reducing cytoplasmic environment. High glutathion
184 concentrations in the cytoplasm can reduce disulfde bridges explaining why canonical 4 chain
185 immunoglobulins will not retain their native conformation and functionality for extended periods of
186 time in cells. Moreover, translocation of a putative therapeutic agent across the plasma membrane is
187 further hampered by the high negative charge of the glycocalyx (135). The interest spawns from the
188 fact that the cytoplasm is an luring environment because it contains the majority of cellular proteins,
189 many of which have properties we do not yet know entirely because for most of these proteins
190 specific small molecule inhibitors are lacking. Although many of them have been targeted by knock-
191 out or expression modulation strategies (RNAi), much remains to be discovered concerning their
192 function, modifications, changes in subcellular location and their interaction partners.

193 While nanobody cDNAs are routinely obtained in the course of their isolation, or rather
194 *generation* when synthetic nanobodies are considered, researchers have developed various ways to
195 deliver recombinant nanobodies in mammalian cells, thereby bypassing the obstacles presented by
196 the (plasma) membrane. Some reports exceptionally claim that nanobodies can passively traverse
197 the plasma membrane. For instance, Singh et al. (149) obtained high affinity (K_d 22nM) anti-STAT3

nanobodies after camel immunization with GST-tagged STAT3 protein. In MTT experiments and in vivo xenograft studies, STAT3 VHH13 reduced cell proliferation (dose 10-100µg/ml) and tumor growth (1-2 mg/kg intraperitoneal injection) of MDA-MB-231 cells, respectively. Although VHH13 immunoprecipitated STAT3 from cell extracts, no experimental information was presented on the ability of nanobodies to cross the membrane and to interact with endogenous STAT3, nor the mechanism underlying the effects observed on cell proliferation or tumor growth. While such observations are intriguing it would be beneficial to study membrane translocation in more detail. In vitro transcribed mRNA encoding a nanobody can also be transfected and Zhou et al. (189) showed that the anti-GFP nanobody can be detected 3h post transfection (which took 24h after transfection of the plasmid).

Cell penetrating peptides, particularly arginine-rich peptides have been used to good effect to introduce nanobodies into cells. They are usually attached post-translationally since direct attachment in the primary structure of the nanobody by PCR mediated addition of the codons somehow negatively affects protein expression (189). Sometimes the protein ends up in the insoluble fraction. Nischan et al. (124) were able to circumvent this by designing a cyclic azido group containing TAT peptide and subsequently added this to GFP that has an N-terminal homopropargyl glycine residue (insertion of non-canonical amino acids in nanobodies is described in more detail further in this review). A copper catalyzed azido alkyne assisted (CuAAC) reaction yields a covalent bond resulting in a fluorescent GFP protein with an N-terminal arginine-rich TAT peptide sequence. Naturally, unreacted peptide needs to be removed from the reaction mixture before experimentation with cells. In concentrations up to 150 µM, a linear arginine-rich TAT peptide-GFP fusion did not transduce into HeLa cells, which is a bit surprising because in earlier experiments (using different cargos) the peptide proved more successful in other studies (128, 147). Nevertheless, the cyclic TAT peptide-GFP conjugate transduced 50% of the cells at 50 µM concentration, and up to 100% of the cells at 150 µM concentration. The authors argue that uptake is energy-dependent and bypasses the endosomal pathway as evidenced by nuclear localization of the transduced

protein. This was later followed by another study showing that a cyclic Arg₉ peptide conjugated to various nanobodies could not only transduce those binders, ectopically relocate their endogenous target in 3T3 cells, but also traffic other proteins that piggy back onto the protein that is linked to the transducing peptide through the plasma membrane (65). Using *linear* nona-arginine tagged nanobodies against the EGFR tyrosine kinase domain (recovered from the insoluble protein fraction during expression and purification), Taabtimmai et al. (157) were able to trigger cytotoxicity of A459 cells when R9-VHHs were added. Concentrations used were not always clear in each experiment but ranged between 10-100 nM which is fairly low (nanobodies were added for a relatively long period, 72h). Inhibitory effects were observed on cell migration (scratch assay). One nanobody reduced kinase activity but all affected cell migration and survival, suggesting different mechanisms at work if VHHs entered the cell. VHHs were equipped with fluorescein for staining purposes but their affinity was not divulged. This is probably relevant because the VHHs were isolated from a non-immunized animal. Although results are promising overall, a well-known disadvantage of CPP is their cytotoxicity and entrapment in endosomes. Cell-penetrating polydisulfides (CPD) are a synthetic mimic of polyarginine CPP in which the peptide backbone is replaced by disulfide bonds. CPD circumvent problems linked to CPP because they bypass endocytosis and are less cytotoxic as they are degradable by endogenous glutathione (50).

Klein et al. (85) delivered nanobodies in the cytosol using the technology of cell squeezing. Many other methods of physical delivery exist and these are discussed elsewhere (154). In the cell squeezing approach, cells are 'compressed' to half their normal diameter in a microfluidic device which causes physical sheer stress/strain on the plasma membrane, its partial disruption and formation of transient holes through which nanobodies can diffuse in the cytosol. Cell squeezing, the authors argue, bypasses endosomal uptake and requires much lower nanobody concentrations and is compatible with high-throughput delivery, combined with low cell toxicity (less than 10% dead cells which is quite remarkable)(86, 145). Using this route of delivery and coupling different fluorophores

onto different nanobodies by means of maleimide chemistry (targets include Histone 2B, TAP and laminin) they could deliver different nanobodies into the cytoplasm starting from nanomolar concentrations and trace the targets that were overexpressed as GFP fusion proteins for more than 20 hours, suggesting long term stability. By the same approach it proved possible to target and trace endogenous vimentin and laminin in HeLa Kyoto cells. A good signal to noise ratio was observed but considering the high abundance of the targets under study this may not come as a surprise. It will be of interest to investigate if low abundant cellular proteins such as kinases or other enzymes can be detected by this approach. Han et al. grafted a nona arginine peptide *and* a lysosomal targeting sequence (NPGY) onto the C-terminus of the EGFR 7D12 nanobody with sortase A enzyme (61). Clathrin-mediated internalization of the nanobody in A341 cells was followed by flow cytometry and delivery of a microtubuline inhibitor (antibody drug conjugate) was more superior in triggering cytotoxicity and growth reduction of A431 spheroids when both peptides were used in conjunction in comparison to their absence. They further argue that the NPGY tetrapeptide can be considered a tool to direct drugs to lysosomes where they can be released and then diffuse to bind and perturb their target.

Laser-induced photoporation is a recently developed technique that efficiently delivers nanobodies as well as other biologicals into a broad range of mammalian cells (some of which are very difficult to transfect) and that can be combined with live cell imaging (179). Photoporation creates vapor nanobubbles (VNBs) by photothermal effects. Briefly, VNBs arise by heating gold nanoparticles, added to the cells that need to be photoporated, with a high-intensity laser beam. As VNBs collapse, transient pores in the plasma membrane are generated allowing influx of i.e. fluorescent nanobodies. After approximately 1 min the membrane is sealed again. Fluorescent vimentin nanobodies were shown to distinctly co-localize with intermediate filaments (102) whereas an Alexa Fluor® 488C-terminally labelled fluorescent anti-fascin nanobody decorated filopodia in HeLa cells, which could be followed by live cell imaging for 72h. At this point it should be noted that it cannot be excluded that fascin nanobodies and possibly also nanobodies binding to other targets,

by mere virtue of their interaction, artificially prolong or delay target turnover by preventing their timely degradation. More efficient photoporation results can be obtained with graphene quantum dots (GQD) because they are more resistant to high radiation lasers and capable of producing multiple VNBs when submitted to repetitive laser beams. Up to 80% or more of the cells were photoporated with very low toxicity (2%)(100, 101). Graphene-based materials can be further optimised by means of surface modification with polyethylene glycol (GQD-PEG, rGO-PEG) and polyethyleneimine (rGO-PEI) to enhance their colloidal stability and lower aggregation propensity, allowing virtually all nanoparticles to create VNBs (100). As a result, photoporation with rGO-PEI results in up to 90% photoporated cells.

Approaches such as these enable study of virtually any protein in its native cellular environment without causing gross changes in protein concentration and cellular homeostasis, allowing detailed analysis of individual protein function but also of multiprotein networks. Moreover, as these technologies are independent of modulating gene expression, it becomes possible to analyze proteins that are crucial in cell physiology or survival as the nanobody enters the cell and the target can be immediately traced or manipulated. It is not always clear if (cyclic) nona or deca arginine peptides will be equally efficient in directing any nanobody across the membrane as the nanobody itself will also contribute to some extent. Most research groups investigate their in house developed nanobodies and currently few nanobodies are used by others in the field, except for the GFP nanobody or for instance EGFR nanobodies. Cell squeezing or photoporation devices may develop into a more common approach but await commercialization.

One avenue we think deserves further attention as it has not been tested yet is esterification of nanobodies to promote membrane transfer. If successful it can develop into an affordable tool to transfer nanobodies across the plasma membrane. Tessler and co-workers (135) reasoned that protein esterification with 2-diazo-2-(p-methylphenyl)-N,N-dimethylacetamide at pH 5.5 in aqueous acetonitrile would mask most negative charges, hence reducing electrostatic repulsion with the

negatively charged glycocalix and facilitating membrane cross over. Earlier it was shown to work with GFP (115) but with RNase I as a model protein it was possible to investigate if enzymatic activity is retained once the protein ends up in the cytoplasm. RNase I is a polypeptide of 128 amino acids (not including the signal sequence), a size that is very similar to nanobodies. Wild type RNase I was not cytotoxic on HeLa cells up to 100 μ M but esterified RNase I (4-6 out of 13 carboxyl groups esterified) had an IC_{50} of approximately 10 μ M, indicating also that the enzyme was active in the cytoplasm. Interestingly, as demonstrated with FLAG-tagged RNase I and using mass spectrometry, it could be shown that cellular esterases *reverse* the reaction in the cytoplasm, yielding an unmodified and therefore active RNase I. It will be of interest to investigate if this works with nanobodies as well. Given their stability at non-physiological pH values it seems likely they will retain their typical 3D structure and because the reaction appears to be reversible, at least for RNase I, potential modification of critical aspartic or glutamic residues in the nanobody CDRs would not be detrimental to its activity once it enters the cytoplasm.

Nanobody derivatization.

For research, diagnostic or therapeutic purposes, it is frequently desirable to covalently attach a specific reporter molecule to an antibody to visualize its interaction with, or its effect on, the target. Nanobodies are ideally suited for this purpose, based in large part on their single domain nature and cDNA availability which allows coupling to other cDNAs resulting in the formation of fusion proteins in vitro or in vivo. Moreover, site-specific addition of a broad variety of compounds becomes almost straightforward, yielding derivatized nanobodies with exquisite properties and we are now beginning to see the results of such approaches. Immunoglobulins can also be specifically modified (94, 126, 142) and several antibody drug conjugates like Mylotarg (49), Adcetris (105) or Kadcyra (89) are known as FDA approved drugs mainly used for oncotherapies. While drug to antibody (DAR) ratios used to be less easy to control (181) (64), progress has been made in this area

as well (41). Future experiments will determine if the small size of nanobodies and associated high tissue penetration (9) will prove to be advantageous for drug delivery in pathological conditions.

N-hydroxysuccinimide labeling of primary amines (usually lysine residues) and maleimide labeling of a sulfhydryl group in a nanobody have been covered extensively and are not treated in detail here (13, 109, 130). Interestingly, a comparative study between these two methods was performed by van Moorsel et al. (165). They were particularly interested in the stoichiometry of labeling using nanobodies against von Willebrand factor and against fibrin degradation products. Introduction of thiol reactive groups was achieved with N-succinimidyl-S-acetyl-thioacetate (SATA) chemical modification of nanobodies, a reagent that attaches a thiol group onto a primary amine. Deacetylation is required to release the thiol group prior to conjugation. Finally, mPEG-maleimide was used to obtain PEGylated nanobody derivatives. Click chemistry was performed by first coupling a Gly3-Lys-azide sequence with the sortase A transpeptidase onto the nanobodies that were first modified C-terminally with an LPETG sequence. Next, a 20 kDa dibenzycyclo-octyne PEG moiety was coupled to the nanobody by a strain-promoted azide-alkyne cycloaddition (SPAAC) reaction. Not surprisingly, the click reaction proved to be better controllable than maleimide-thiol conjugation. The latter produced diverse reaction products (4 adducts resulting from modification of multiple lysine residues with SATA), probably because SATA modification already yielded multiple adducts. A SPAAC click reaction with the same nanobodies and polyethylene glycol produced uniform VHH conjugates with a predefined stoichiometry and tagging at a specific pre-chosen site. Moreover, the apparent binding affinity was not affected after click chemistry whereas this was effectively the case after maleimide-thiol conjugation. The authors further note that maleimide-thiol conjugation does not require any genetic modification of the nanobody whereas click chemistry necessitates incorporation of the LPETG sequence before the reaction can proceed.

Consequently, click chemistry is considered favorable to produce uniform VHH conjugates with preserved functional antigen binding capacity. In addition, increased sensitivity by a factor of 5

has been demonstrated as a result of oriented immobilization of a nanobody that detects Dengue virus nonstructural protein 1 in serum (5). This approach likely yields more reproducible results not only in diagnostics and detection but also in interactomics for instance where false positives routinely occur. By orienting the nanobody in a defined manner with its paratope free to interact with the antigen, its interaction partner will be captured more efficiently from a heterogeneous pool of proteins and therefore the interaction partner will more abundantly be represented compared to when a randomly oriented nanobody is used. Recent findings reported in a study (183) underscore how advantageous an engineered orientation of single domain antibodies can be. The Gln13 residue of the 7D12 EGFR nanobody was modified into an azidoPhe for downstream click chemistry and a 6-fold improved cell binding was observed compared to the unmodified nanobody. As a side note, modifying Gln13 exquisitely illustrates that non-canonical amino acids can be inserted at any predetermined site in the primary structure of a nanobody. By modifying not only ligand orientation but also PEG linker flexibility and ligand surface density (when the nanobody is coupled onto quantum crystals) they achieved an 8-fold increase in cell targeting compared to a randomly oriented single domain antibody. When the nanobody was randomly oriented on the quantum dots, it displayed a K_D similar to a wild type monovalent nanobody. Optimal orientation on the quantum dots however improved the K_D by a 100-fold compared to monovalent azidoPhe13 nanobody. The authors argue that this resulted mainly from a 400-fold decrease in k_{off} as compared to the monovalent nanobody (183). Hence, synergistic effects contribute to this finding and it demonstrates how this level of control, imposed by the investigator and difficult to obtain with full length immunoglobulins, can improve experimental efficiency and boost the overall quality of scientific data. Combining this with the possibility to improve the affinity of a monovalent nanobody by concatenation (9, 16) leads to generation of semi-synthetic antibodies that approach the affinity of the biotin-streptavidin interaction.

No effect on the affinity of a Her2 nanobody for its target was also reported by Massa et al. (108) when transforming the nanobody into an imaging agent by chelation with various nucleophilic

probes for use in single-photon emission computed tomography (SPECT), positron emission tomography (PET) or with Cy5 for fluorescence reflectance imaging. As the C-terminus of a nanobody opposes its paratope in the 3D structure (56, 114), it is considered a safe site for tagging biologicals onto a nanobody and the possibility to do this site specifically offers the advantage of obtaining a homogenous derivatized population of molecules. High contrast imaging of Her2 positive BT474M1 tumors in mice was obtained (108). While this approach can produce a sophisticated imaging tool in a clinical setting, it requires considerable expertise and some time to generate: pentapeptide synthesis, conjugation with nucleophilic imaging probes, purification by RP-HPLC, sortase coupling onto the Her2 nanobody (determining the optimal nanobody: sortase A: nucleophilic agent ratios), IMAC and size exclusion chromatographies and radiolabeling with ¹¹¹In (SPECT) or ⁶⁸Ga (PET) followed by a final purification step). A similar Her2 nanobody carrying a C-terminal cysteine has been derivatized with [¹²⁵I] BODIPY-but not yet used in biological experiments (174). Other reports also describe successful PET imaging with nanobodies (extensively reviewed in (175), sometimes even with better signal-To-Noise Ratio than 18F-FDG PET/CT (73) or using SPAAC mediated labeling with 18F (190).

The Schultz lab has done seminal work in developing, characterizing and incorporating unnatural amino acids in the primary structure of polypeptides by mutating tRNA:tRNA synthetase orthogonal pairs with the purpose of altering their substrate specificity from a natural amino acid to the non-canonical equivalent carrying a unique chemical functional group (76, 83, 97, 184). Thanks to this work, a broad range of chemical functionalities can be inserted site-specifically, although there are obviously differences in tRNA synthetases and not all of them catalyze reactions with the same efficiency or fidelity as they are derived from wild type enzymes. It seems likely though that future research in this area will develop further and fine tune new modalities of non-canonical amino acid incorporation in nanobodies and proteins in general. Nanobody derivatization can also be an important stepping stone towards rational development of therapeutic molecules where nanobodies are employed either as a drug or as a specificity key carrying a drug. A recent study illustrating the

latter (32) involves doxorubicin and an EGFR targeting nanobody (EgA1). Different aspects need to be borne in mind and an inkling of how such a modular compound could be assembled and transformed into a drug in vivo is demonstrated. The nanobody is located at the C-terminus of the construct and is preceded by a hinge region and elastin like polypeptides, each consisting of a short peptide polymer (VPGXG, X = any residue except proline), that self-assembles into nanoparticles in a temperature dependent fashion. p-acetylphenylalanine is a non-canonical amino acid that is co-translationally incorporated and it precedes this repetitive region. It carries a reactive keto moiety in its side-chain for site specific attachment of doxorubicin, although other molecules are also possible. The *M. jannaschii* aminoacyl tRNA (aaRSTyr) was used for this purpose, which incorporates Tyr analogs at the amber stop codon with > 95 % efficiency (97). The authors also used a special *E. coli* strain (C321.ΔA) where all 321 amber stop codons in the genome are re-coded to the ochre stop codon (72, 91) to further increase the yield of the protein of interest. Doxorubicin, a compound that causes breaks in double stranded DNA, is coupled to the non-canonical amino acid. The overall design is conceived in such a manner that upon heating to body temperature, a self-assembling structure arises that forms micelles with the nanobody on the outside, for targeting, and doxorubicin in the inner core of the micelle. A pH-sensitive linker between doxorubicin and the rest of the molecule releases the drug in lysosomes. The unnatural amino acid was incorporated with 98 % efficiency using the C321.ΔA strain. The EgA1 nanobody promoted a 13-fold higher uptake in A431 cells that express high amounts of the EGFR. A drop in pH was further shown to release 58% of the coupled Dox in vitro. Free Dox had an IC50 of 1.38 μM in A431 cells whereas this was lowered by one order magnitude with the EgA1 nanobody-micelle targeting approach. Dox was found to localize in the lysosomal compartment. Collectively, these data attest to the versatility offered by combining nanobodies with the possibility to introduce unnatural amino acids and hence unique chemical functionalities into nanobodies at a pre-chosen site. This approach can also prolong the circulation time of a drug. Whereas cisplatin is quickly cleared out, conjugation of the drug to a construct that is built up of a biparatopic EGFR nanobody, a gadolinium binding domain for MRI imaging and Cys, for

maleimide directed cisplatin coupling, markedly prolonged circulation time but also reduced the platinum level in liver, kidney and spleen where it is not supposed to act (71). Which coupling strategy is selected in the end depends in a large measure on the biological problem under study. Complex strategies can be technically demanding, are not always desirable and 'simple' NHS-based coupling of a GFP nanobody to quantum dots for example can also be used as a way to obtain interesting data on trafficking of neurotransmitter receptors after they are overexpressed in neuronal cells (116).

Instead of using a nanobody as a drug (carrier), it can also be put to use in photodynamic therapy (PDT) as a series of recent studies has shown. In phototherapy a photo-sensitizer is activated by radiation with light of the appropriate wavelength. Energy is transferred to oxygen with ensuing formation of reactive oxygen species (ROS). These will subsequently damage and degrade proteins, lipids and nucleic acids in a non-selective manner. Not all tumors are susceptible to PDT and the ability of light to penetrate to tumour depth is by far the most limiting factor. However, as pointed out by van Lith and co-workers (164), PDT is not as invasive as surgery and direct cell killing by PDT may prevent development of resistance. The small size of nanobodies is advantageous here as it allows for faster tumor accumulation and tissue penetration is also better (164). Large antibodies can circulate for a long time thereby increasing the risk of phototoxicity caused by exposure of the skin to light (112). IRDye 700DX, a phthalocyanine dye, is a commonly used photosensitizer in these studies mainly because it is more water soluble compared to others and it is not that sensitive to photobleaching. This strategy, with nanobodies randomly or site-specifically modified with photosensitizer, has been applied to Her2- breast, EGFR-melanoma, Met-overexpressing (67) and US28 GPCR glioblastoma cells or spheroids (17). Her2 monovalent or biparatopic photosensitizer nanobodies induced cell death and promoted significant tumor regression of trastuzumab resistant high Her2 expressing tumors (38). NHS-mediated conjugation did not affect the affinity of the nanobodies, presumably because no critical lysines were modified or present in the paratope. The 7D12 EGFR-specific IRDye 700DX conjugated nanobody was tested without or with a cell penetrating

peptide but the latter was less effective in triggering cell death of 3D spheroid cell cultures (164). Mechanistically, Hernández and colleagues discovered that the 7D12- conjugated EGFR nanobody triggered necrosis of cells (17). They further identified certain damage associated molecular patterns (DAMPs) from dying cells, including the presence of HSP70 on the plasma membrane, the cytoplasmic localization of HMGB1 which normally resides in the nucleus and the release of pro-inflammatory cytokines IL-1 β /IL-6. Other photosensitizers include Ruthenium-polypyridyl complexes. Conjugation to a 7C12 EGFR nanobody with Sortase A however yielded ROS upon illumination but no cytotoxicity, probably because the conjugate was not internalized in cells (81).

Nanobodies and CAR-T.

One area where nanobodies could be therapeutically useful in the relatively near future is treatment of hematological cancers using CAR-T technology. Chimeric antigen receptors (CAR) consist of an antibody fragment (i.e. single-chain variable fragment or scFv) connected to co-stimulatory domains from CD27, CD28, or 4-1BB, and a T cell signaling domain such as CD3 ζ ,4 (4). Patient derived T cells can be transduced or electroporated forcing them to express these constructs. When transferred to the patient, the antibody fragment that is usually directed against an antigen that is highly expressed on the surface of cancer cells will act like a magnet and direct the T-cells towards the cancer cells leading to their destruction. A major obstacle obviously is selecting the antigen against which the antibody is directed. CD19 has been used with success (110). Recent studies showed that nanobodies can substitute for scFvs. Their single domain nature, virtually non-existent tendency to aggregate unlike scFvs, the 'ease' to obtain their cDNAs and their high similarity to human VH3 genes may designate them as instruments of choice for CAR-T. An et al. used a CD38 nanobody to target multiple myeloma cells (4). The nanobody was linked to 4-1BB and CD3 ζ as costimulatory and activating domains. Upon activation, the resulting CD38 CAR-T cells produced more inflammatory cytokines including IL-2, IFN- γ , and TNF- α compared to wild type T-cells. The CD38-CAR-T cells lysed various CD38+ cell lines (LP-1, RPMI 8226, OPM2) but also primary cells, in a

CD38-dependent manner. The therapeutic effects in vivo were evidenced by a reduction in tumor size and weight in a multiple myeloma xenograft mouse model. Hambach and co-workers (59) reasoned along the same lines and with their own set of CD38 nanobodies that bind to different epitopes found that CAR-transduced NK-92 natural killer cells were also cytotoxic towards target myeloma cells in vitro. They further duly noted that the affinity of CD38 nanobodies is an important parameter in the therapeutic effect of nanobodies, particularly when considering potential off-target effects caused by antigen expression on cells other than the tumor. Maybe counterintuitively, a relatively low affinity binder in this respect may have a better effect on target cells with high surface antigen levels and disregard other – non-tumor- cells with a lower expression of the antigen. Ideally a high affinity binder will target its antigen that is uniquely expressed on cancer cells but these targets are usually quite hard to find. Hence, considering the affinity may be important.

Lack of specific tumor antigens also applies to solid tumors. ErbB2, PSMA, and B7-H3 have been considered potential CAR targets (178) but their expression is not limited to tumor cells. Potential down stream off-target effects should however be taken seriously as evidenced by cytokine storm induced mortality in a patient treated with autologous T-cells modified with an ERBB2 CAR (117). Xie et al. (178) used a different approach by developing PD-L1 CAR T cells for targeting the tumor microenvironment rather than the tumor itself, arguing that the microenvironment of different tumor share similar characteristics, in addition to EIIIB-specific nanobodies, a splice variant of fibronectin strongly expressed in the tumor ECM and neovasculature (7, 27). In addition to the expected cytotoxicity it was observed that PD-L1 CAR T Cells Slowed down growth of Solid tumors in mice. Also, EIIIB CAR T Cells Slowed B16 Melanoma Growth in Vivo. They also found a way to overcome T cell exhaustion caused by low endogenous expression of PD-L1 in WT T cells by generating PD-L1 CAR T cells in the presence of a saturating dose of an anti-PD-L1 which prevents engagement of the PD-1/PD-L1 axis. Another study showed that expression of a VEGFR2 CAR T construct also led to cytokine production, cytotoxicity and degranulation when cells were confronted with VEGFR2-expressing cells (58). In view of these encouraging findings it is to be expected that

nanobody CARs provide a basis for clinical development of personalized medicine and treatment of different cancers. Nanobody amino acid sequences are > 80% identical to the V_H sequences of human family III immunoglobulins (78). Other studies (84) further support the contention that nanobodies are expected to be only weakly immunogenic when used as therapeutic in humans (82) and a soluble and stable universal humanized nanobody scaffold into which antigen-binding loops from other nanobodies can be grafted has been reported earlier (169). The first FDA approved nanobody drug, Caplacizumab ((143) and ongoing clinical trials with potentially therapeutic nanobodies (78) point in the same direction.

On a different note, Wöll et al. (176) added a PEGylated dimyristyl lipid anchor onto an anti CD11 VHH using sortase A transpeptidation and studied its membrane insertion into liposomes and into the phospholipid bilayer of myeloid-derived suppressor cells and T-cells. Liposomes decorated with the lipidated anchor were directed to antigen-positive cells. Similarly, T-cells carrying the anti-CD11 VHH were directed to CD11b+ myeloid-derived suppressor cells. Such an approach can endow lipid encapsulated drugs with higher specificity or promote desired cell-cell interaction in a therapeutic cells, similar to use of CAR-T cells in killing cancer cells.

Manipulation of intracellular proteins with intrabodies.

Genetic approaches such as RNA interference (including short interfering RNA (siRNA), short hairpin RNA (shRNA), micro RNA (miRNA)) and more recently developed approaches like clustered regularly interspaced short palindromic repeats (CRISPR) and transcription activator-like effector nuclease (TALEN) have momentously contributed to our understanding of gene function and the function of gene products. Even so, many eukaryotic proteins typically consist of different domains, with each domain performing one or more functions. The tyrosine kinase Src for instance is built up of a catalytic domain, an SH2 domain for phosphotyrosine recognition and an SH3 domain for PXXp helix II recognition (19). Genetic approaches will invariably obliterate the entire protein thus

precluding a thorough understanding of the function of its different domains. Ideally, one would have a high affinity and specific inhibitor, cell membrane permeable, inhibiting the catalytic function of a protein domain or its interaction with a protein partner. However, it is time consuming to develop such inhibitors and usually they are not suitable for covering the interface of interaction proteins.

To address this problem, researchers have resorted to antibodies and antibody fragments, commonly referred to as intrabodies when they are delivered in cells or directed to cell organelles. Antibodies can be generated fairly rapidly once the protein (domain) under study can be obtained in suitable (recombinant) format for immunization. Single-chain variable fragments (scFvs) and nanobodies have been used to this end, but also synthetic alternatives are useful such as designed ankyrin repeat proteins (DARPs), affibodies (derived from protein A) or fibronectin folds (monobodies) (96, 113, 187).

When nanobodies are expressed in the cytoplasm of mammalian cells as intrabodies in order to bind and perturb the antigen it can yield important biological information regarding the function of the target in a given setting, particularly when an endogenous protein is targeted as this avoids potential overexpression artefacts. Moreover, unlike siRNA, which eradicates or strongly down-regulates target expression, a nanobody will very unlikely block all functions of its target or all protein-protein interactions. Thus it is possible to address specific biological roles of any given protein by using a nanobody that binds to a particular region in its target.

Nanobodies usually contain a conserved disulfide bond between Cys22 and Cys92 (Kabat numbering) that is required for their stability. Removal of the disulfide bond in an mCherry VHH by mutating Cys to Ala lowered its denaturation temperature, corroborating its importance for stability, but had no significant impact on the strength of the interaction (98). Conversely, Kunz et al. (90) compared dromedary nanobodies carrying a second disulfide bond with a set of llama nanobodies with one disulfide bond and found that the second disulfide bond affects the conformational stability of nanobodies but also reduces nanobody aggregation (90). When nanobodies are expressed as

intrabodies in the cytoplasm of mammalian cells or in organelles, they encounter a strongly reducing environment which could impair their biological activity. Using different bacterial strains, Plainer et al. found that nanobodies (targeting nucleoporins) were predominantly reduced (130) in the cytoplasm of bacteria. Contrary to what one may think though, this did not affect the affinity of the nanobodies for their antigen although differential scanning fluorimetry revealed a decreased thermostability of reduced anti-Nup98 and anti-Nup93 nanobodies, down to 47°C and 57°C, respectively. This interesting observation probably accounts for the numerous instances where nanobodies have been proven to act as bona fide tracers of endogenous proteins under reducing conditions in mammalian cells, although findings that point to the contrary are likely not always reported.

In spite of this possibility, it remains desirable to introduce additional levels of control to fine tune target manipulation and for instance to demonstrate reversibility of any biological effect that may be triggered by a nanobody. This has been achieved by inducible nanobody expression although the 'reversibility' will inevitably be slow as it is dependent on cDNA expression. As a side note, an additional advantage entails that the nanobody can be expressed at the same level as the cognate antigen (163) by using an appropriate doxycycline concentration thereby avoiding nanobody overexpression and minimizing potential off-target effects (when using fine chemicals for targeting intracellular proteins it is not always clear how much of the compound really enters cells). This however requires prior determination of the antigen concentration in extracts by using for instance the recombinant protein as a standard. Even if a nanobody does not block any property of its antigen it can still be used to delocalize for instance the target to different organelles or cellular compartments such as the nucleus, cytoplasm, mitochondria, peroxisomes, ER, plasma membrane (14, 34, 39, 159, 162, 163, 166) and this can also lead to loss of function.

More recent innovations in reversibly regulating interaction between nanobody and its target are found in a number of studies. Farrants et al. (47) inaugurated the ligand-modulated antibody

fragments (LAMA) method. This approach relies on the use of small molecules to reversibly allow a nanobody to bind its target. Basically, they inserted a circularly mutated bacterial dihydrofolate reductase enzyme (cpDHFR) into complementary-determining region 3 (CDR3) of the GFP nanobody (138), which did not disrupt interaction with GFP although CDR3 is generally known to be important for covering a significant part of the epitope:paratope interface. It is only upon binding of the anabolic reducing equivalent and co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH) or the DHFR inhibitor trimethoprim (TMP) that cpDHFR undergoes a conformational change leading to strong reduction in affinity of the nanobody in which it is inserted, leading to disruption of the interaction. Experiments in cells demonstrate that targets could be delocalized to mitochondria indicating intracellular validity of the approach. While the authors demonstrate their method applies also to other nanobodies it is mentioned that having a co-crystal structure of the nanobody-antigen complex significantly facilitates experimental design and activity of the cpDHFR fusion construct. The overall use and applicability for other nanobodies awaits confirmation by other researchers.

Instead of using small molecules and inserting a protein directly into the coding sequence of a nanobody, Yu et al. (47) followed a split-nanobody approach in combination with blue light responsive photoreceptors to generate what they called 'optobodies' (optogenetically activatable intrabodies). By using N65/C66 as a split site they generated two GFP nanobody fragments: the N-terminal fragment contains CDR1/2 and the second has CDR3. Each fragment was then linked to Magnet optical dimerization tools nMagHigh1 and pMagHigh1, respectively, and following expression in cells activated by light. This leads to dimerization of nMagHigh1 and pMagHigh1 which brings to two nanobody fragments together thereby reconstituting functionality. The approach was further extended and shown to work for deGradFP (28), a gelsolin nanobody (162) and the beta 2 adrenergic receptor (152). NIH3T3 cells expressing the gelsolin optobody were affected in their migration when irradiated with light as this nanobody is known to affect cell migration (162). The Nb60 anti-androgen receptor optobody disrupted endogenous β 2AR signaling. Although it is mentioned that intrabodies sometimes aggregate we have experienced this very rarely and the

majority of our intrabodies targeting cytoskeletal proteins do not aggregate. However, when a nanobody is split up in two fragments the overall IgG fold is disrupted and this can lead to self aggregation, particularly when they are highly expressed in cells and this is what these authors observed.

Yet another approach involving nanobody photoactivation was recently reported. In this setup, an inactivated nanobody is first generated by incorporating a non-canonical tyrosine derivative in CDR3 of the nanobody (tyrosine frequently occurs in CDR3 of a nanobody). Upon light activation, the nanobody (photobody) caged tyrosine is liberated and the nanobody is converted to its active form and binds to its target. Unlike the previous two examples described above this procedure is obviously not reversible but allows tight activation control by irradiation with 365 nm UV light. Jedlitzke et al. (74) modified Y37 in the GFP nanobody with *O*-nitrobenzyl-protected tyrosine, genetically incorporated using stop codon suppression technology involving *Methanococcus jannaschii* tyrosyl tRNA synthetase as explained earlier (37). This singular modification caused a massive reduction in affinity (four orders of magnitude) compared to the wild type nanobody. After photodeprotection the nanobody regained its affinity in vitro. Using other nanobodies against Her2 and EGFR, the authors demonstrated that photoactivation can also be executed when the nanobody is added to cells. This approach may develop into a more generally applicable methodology because tyrosine occurs frequently at key positions of the nanobody paratope but requires the ability to deprotect with a light source. Because this procedure generates a photocaged recombinant nanobody it will not be possible to introduce it into cells unless one of the approaches described elsewhere in this review are used such as CPPs, photoporation or cell squeezing. Brigde et al. (23) in a large measure used a similar approach to photocage the 7D12 EGFR binding nanobody and observed that the photocaging group inhibits nanobody binding to EGFR on the surface of cancer cells which is reversed upon irradiation with 365 nm light. Importantly, they observed that not every tyrosine residue in the binding interface will lend itself to photocaging and blocking of binding as Y109

630 photocaging did not affect 7D12-EGFR binding and that some empirical approach will remain
631 necessary when testing nanobodies against other targets.

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Nanobodies harnessing their target into a desired (non-pathological) conformation: structural correctors.

Changes in protein structure, either drastic or subtle, can lead to pathology and disease. Nanobodies can be used as biosensors or molecular rovers, scanning the surface of proteins for (subtle) changes in structure (2) or even act as structural correctors. This does not necessarily qualify them as therapeutic agents but in the first place as tools to study protein dynamics and conformational changes in protein structure causing alterations in protein function. Many diseases are caused by protein misfolding, sometimes resulting in proteolysis and/or aggregation. Examples include Alzheimer's disease and fibrinogen amyloidosis, but also non-amyloid disorders like Parkinson's disease, sickle cell disease, ALS, cystic fibrosis, Alexander disease (GFAP aggregation) and others are associated with proteopathy and aggregation (170-172). We here discuss examples of two diseases where nanobodies have proven instrumental in understanding protein structure and dynamics, and for setting the stage to develop new strategies for treating these and other diseases in the future..

In Parkinson's disease, the LRRK2 leucine-rich repeat kinase 2 and α -synuclein both play a very important role (92). Apart from phosphorylating substrates, LRRK2 also displays GTPase activity. Leemans recently analysed nanobodies directed against a bacterial homologue of LRRK2 which lead to some surprising findings. During cycles of GTPase activity, LRRK2 shuttles between dimerization and monomerization. In the monomeric form GTP hydrolysis is higher whereas in the dimeric form GTP hydrolysis is considerably lower. Some mutations in Parkinson's disease LRRK2 lead to decreased GTPase activity, like L487A which corresponds with I1371V in LRRK2 from humans and this results in stabilization of the dimeric form. A nanobody was identified that shifts the monomer-dimer equilibrium to the monomeric form by an allosteric mechanism as the nanobody does not bind to the interface in the dimer. This effect triggers an increase in GTPase activity and the nanobody actually

boosts GTP turnover rate beyond that of the wild type protein. Interestingly, this effect also equated to a complete rescue of the L487A mutation. It is surmised that the nanobody binds to an epitope that changes conformation between the monomeric GTP bound state and dimeric nucleotide GTP bound or free state. In a more classical approach investigators would seek small compounds that fit in the GTP binding pocket although it is not easy to understand how such a compound should act in this case. Moreover, such a compound is likely to cross react with other GTP binding proteins while the nanobody interface with LRRK2 likely defines a more unique landscape. In effect, the nanobody has pointed to another possibility by allosterically affecting the dimer-monomer equilibrium.

Familial amyloidosis of the Finnish type or gelsolin amyloidosis (Agel) is an incurable orphan amyloid disease caused by a mutation in the gelsolin gene resulting in an aspartic acid residue that is replaced by asparagine or tyrosine in the primary structure at position 187 (35). Since then other mutations have been described (N184K, G167R) (43, 144), all located in the second gelsolin domain. D187 is involved in calcium binding and the mutation prevents this region in gelsolin to bind calcium. As a result, the structure changes slightly (122) and as gelsolin's hydrophobic interior is exposed, the proteases furin and MT1-MMP consecutively cleave gelsolin, generating amyloidogenic peptides and amyloid fibril deposits. Prolonged blocking of these proteases in a chronic disease setting would no doubt trigger deleterious side effects. To circumvent this problem, Van Overbeke et al. used a nanobody that was raised against *wild type* gelsolin (162). It also interacts with D187N mutant gelsolin, albeit with an affinity that is one order of magnitude lower as compared to wild type gelsolin. This Gelsolin nanobody 11 acts as a molecular chaperone and protects gelsolin against furin cleavage in vitro (166), in transfected HEK293T cells (when the nanobody is directed to the ER because the affliction is caused by plasma gelsolin) and in vivo. A transgenic mouse was generated that expresses Gelsolin nanobody 11 from the ROSA26 locus and secretes it into the circulation (166). Back crossing this animal with a diseased mouse in which the proteolytic cascade is recapitulated produced offspring with improved muscle contractility. Mutant human gelsolin in this animal model is secreted from muscle cells and as amyloid fibrils deposit around the muscle fibers, their

contractility is impaired. As nanobody 11 binds to the gelsolin domain where furin cleaves it was assumed that the nanobody causes steric hindrance. After solving the X-ray structure between the nanobody and mutant or wild type gelsolin domain it became clear that the nanobody reduces flexibility of dynamic stretches in gelsolin and decreases conformational entropy of the C-terminal tail of the domain (53). Hence the nanobody basically harnesses mutant gelsolin into its wild type configuration, thus preventing furin proteolysis.

The examples described above both have in common that nanobodies were generated against a wild type protein but have ‘therapeutic’ effects on a mutant. Whether the gelsolin/LRRK2 approach can be applied to other diseases caused by protein misfolding awaits future research.

Nanobodies and the Blood-Brain Barrier (BBB).

The brain, as the center of the nervous system, is armed with a combination of natural barriers, preserving homeostasis and protecting neurons. The blood-brain barrier, the most studied one, consists of 3 distinct layers: endothelial cells rigidly attached to one another by tight junctions, the basement membrane enforced with embedded pericytes, and astrocytes end-feet encircling the endothelial cells (180). It serves as a shield to protect neurons from unwanted contaminants and pathogens whilst serving as a ferry that is actively responsible for the introduction and clearance of different compounds.

Transferring therapeutic or diagnostic agents across the blood-brain barrier (BBB) has been proven to be one of the greatest challenges in treating diseases of the central nervous system (CNS). Systemic intravenous administration of an IgG antibody usually leads to less than 0.1% ID (injected dose) to reach the brain parenchyma, which can sometimes be enough to notice therapeutic effects (18, 129). Although direct intraparenchymal (ip) or intracerebroventricular (icv) injection of the drug is possible, it is very invasive, is associated with high costs and poses safety issues towards patients’

health. As a result, there is a demand and need for non-invasive highly efficient delivery methods (150). Precise delivery of nanobodies is quintessential for successful therapy. For example, a neutralizing anti-leptin receptor nanobody has been proven to be effective in treating melanoma implanted mice but because side-effects are severe and inevitable after systemic injection, direct injection is mandatory (111).

The permeability of a compound is dependent on the balance between para- and transcellular transport. Passive diffusion of ions and gradient-dependent solutes mainly take place paracellularly whilst gaseous substances such as oxygen and carbon dioxide can passively diffuse transcellularly. Small molecules (amino acid, hormones, nucleotides,...) are transported by carrier-mediated transport. On the other hand, peptides and proteins are mainly crossed by specific receptor-mediated (RMT) or by non-specific adsorptive-mediated transcytosis (AMT) which is induced by electrostatic interaction between positive charges on the surface of the agent and the negative charges on the lipid membrane of endothelial cells (103, 131, 191).

BBB Transmigrating nanobodies

Transbodies (defined as nanobodies capable of spontaneously crossing the BBB) have been developed and contain intrinsic characteristics. High alkaline (pI=9.4) nanobodies (anti-GFAP, glial fibrillary acidic protein) would be capable of crossing the BBB by interaction of their positive residues to the membrane of BBB endothelial cells and subsequent AMT by clathrin-mediated endocytosis (95). The authors claimed implementation of these positive charges side chains would lead to 7.8%, 4.3% and 3.75% of the monomeric, dimeric and GFP-fusion nanobody being transported respectively. Sadly, although reasonable and easy to implement, these results have never been confirmed by a different group or with different nanobodies.

Other nanobodies are thought to cross the BBB independent of their net charge. FC5 nanobodies, for example, were created with the sole purpose to bind to, and be internalized by

human cerebrovascular endothelial cells. FC5 transmigrates the BBB by RMT via the $\alpha(3,4)$ -sialoglycoprotein receptor and subsequent actin- and PI3-kinase-dependent transcytosis (63, 119). The nanobodies are transported through the cell by clathrin-coated endosomes and reach the laminal side of the BBB. Dimerisation and N-terminal addition of the Fc region enhanced brain uptake even more. The opioid peptide Dal was linked to the Fc-fusion nanobodies and was shown to be 17% more effective when compared to ICV injection of the peptide itself. The results thus provide potential for these nanobodies to serve as a transport tool for therapeutic loads *in vivo* (48, 63).

Anti-amyloid beta nanobodies have been isolated which have proven to be more effective in crossing the BBB compared to FC5 *in vitro* (139). The group identified three atypical amino acids at position 13, 14 and 16 of framework 1 region to be responsible for their enhanced BBB transport. Subsequent insertion/replacement of these amino acids in a different nanobody also enhanced BBB transmigration. Active transport mechanisms were involved in the transmigration process, yet the receptor for RMT was not identified. Additionally, *in vivo* studies were less effective than the FC5 nanobody (121). This could be due to a lower biodistribution of the receptor in living mice, furthermore stressing the importance of representative *in vitro* BBB models.

Another strategy involves the use of nanobodies against receptors that are enriched at the BBB (136). A very recent example is vascular cell adhesion protein 1 (VCAM-1) against which anti-VCAM-1 nanobodies have been developed (54). Comparable to FC5 nanobodies, extended half-life in the blood increased BBB transmigration. A bispecific bivalent nanobody that additionally targets albumin showed the highest accumulation and reached up to nearly 3% ID/g (percent injected dose per gram of tissue). Furthermore, the bispecific nanobody was tested as a targeting moiety for the introduction of superoxide dismutase (SOD-1) and liposomes, with 1.9% ID/G and 1.16% ID/G reaching the brain compartment, respectively.

It should be noted that most transbodies have not yet been confirmed or employed by other research groups. This poses the question whether the results are unique to the nanobodies tested

and if the strategies involved can be extrapolated for further use. Many studies confirm nanobody presence in the brain by (fluorescent) staining, yet these techniques only demand low amounts of the transbody to obtain confirmatory results. Quantification of the amount of nanobody successfully introduced is often measured by radioactive compounds or estimated by cerebrospinal fluid (CSF) concentration. Yet, different methodologies make it difficult to compare obtained results and bring in the demand for overarching studies which involve common methodology and quantification.

Although all above examples prove to be more efficient in BBB transmigration due to intrinsic properties of the nanobodies themselves, efficiencies are still limited as roughly maximum 5 %ID/g reaches the brain. Transcytosis is likely limited due to a number of factors: receptor expression, competitive binding of natural proteins and lysosomal sorting. When aiming for pharmaceutical effects either by the nanobodies or by a drug load transported by them, more effective methods for BBB transport are required.

Carbon nanotubes (CNT) are carbon hollow tubes of up to a few hundreds of nanometer and have been shown to be promising carriers for drug loads across the BBB both *in vitro* and *in vivo* (57, 79, 133). Nanobodies can be conjugated to these drug carriers and provide them with a targeting moiety (107). Multi-walled CNTs (MWCNT), two or more single-wall CNTs joined together by weak intermolecular forces, have been conjugated with polyethylenimine (PEI) and the antiviral drug ribavirin. As targeting moiety for these MWNCNTs, a chemical reaction covalently links the PEI amines at the outermost layer to anti-PGNNV (pearl gentian grouper nervous necrosis virus) nanobody succinimide active ester, obtained by reaction between the nanobody and butanedioic anhydride (191). This PGNNV nanobody has been tested in a zebrafish model which naturally displays a highly conserved BBB structure and functionality compared to humans (1, 80). Indeed, ribavirin-loaded MWCNT-Nb tended to accumulate specifically in the brain of the larvae and were capable of drastically decreasing mortality in virus-infected larvae, even more powerful than direct ribavirin therapy.

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BBBD + Intranasal delivery

Because hijacking natural transport processes at the BBB is dependent on different factors and crossing efficiencies for the nanobodies mentioned above are low, one strategy is to bypass the blood-brain barrier itself. Intranasal (IN) delivery is possible by unique transport from the olfactory epithelium to the brain by two pathways: the olfactory and trigeminal nerves, either extra- or intracellularly. As IN delivery often leads to the drug reaching the brain within minutes, the extracellular route is emphasized to be the most important as the intracellular route can take hours to days depending on the nerve involved (52). Small molecular drugs have been approved for IN delivery for a variety of applications such as anaesthesia, migraine, therapy-resistant depression (168). Anti-Transthyretin (TTR) and anti-rabies virus nanobodies have been introduced through the IN route and lead to accumulation in all areas of the brain with highest levels in the olfactory bulb, the entry gate to the brain, and in lesser degree the ventral parts of the brain (brainstem, cerebellum) (55). Although quantifiable through ELISA, the administration was inefficient and aside of some maximal concentrations for distinct brain regions, no general efficiency was shown, making it difficult to compare to other methods. Nevertheless, it is estimated that 98% of low molecular and 100% of high molecular (9200 > Da) weight active substances do not reach the CNS, yet optimization can be performed to enhance efficiency (20, 88).

A successfully applied strategy is the creation of blood-brain barrier disruptions (BBBD), temporary openings created for the passage of biologics. A promising technique in development is focused ultrasound in combination with the injection of microbubbles which leads to so-called focused ultrasound BBBD (FU-BBBD). It is non-invasive and precise, lowering the side-effects for non-targeted, healthy brain sites. A multitude of studies across the world are studying the safety of repeated BBBD for different patients (mainly in Alzheimer's Disease). A plethora of agents, ranging from small molecules to full length antibodies to nanoparticles, have been introduced through the FU-BBBD (24, 77, 99). Microbubbles are modifiable and can be applied for ligand-receptor targeting, payload conjugation and biocompatibility. In our opinion, nanobodies could thus potentially be

employed to specifically target microbubbles to the BBB after systemic injection and increase effectivity, likewise to for example echistatin conjugation for angiogenesis (155).

Yet nanobodies in combination with these microbubbles have not been tested yet for their BBB transmigration. Nevertheless, nanobodies have already been conjugated to microbubbles by the biotin-streptavidin bridge method (66). VCAM-1 conjugated nanobodies have been proven to be a feasible option to diagnose atherosclerosis by means of contrast-enhanced ultrasound molecular imaging (132). In oncology, anti-PSMA and anti-G250 nanobodies have been conjugated to microbubbles and visualised in PMSA-positive LNCaP and C4-2 cells and G250-positive 786-O cells, HeLa cells and tumour tissue slices, respectively (46, 185). Moreover, these microbubbles were loaded with a drug load, albeit not extremely effective (5%), and have been proven to precisely reduce renal cell carcinoma in mice (186). Similar to BBBD, the microbubbles were introduced and the carcinoma area was submitted to ultrasound for microbubble destruction, leading to drug exposure at a specific site.

A more chemical approach is the administration of mannitol to trigger BBBD or osmotic BBB openings (OBBBO). The osmotherapy is based on two effects: plasma expansion and osmotic action. Administration of mannitol is not without risk to the patient's health, making its use currently limited to intracranial hypertension patients (141). Nevertheless, bevacizumab has successfully been introduced in mice brain without side-effects, reaching concentrations up to 23% ID/cc. Key is the combination of a mannitol catheter and administration in the carotid artery, making it possible to site-specifically introduce biological compounds to the brain. This opens a window of opportunities and a proof of concept study recently demonstrated that a gelsolin nanobody can be introduced into the brain via this route (93): G4 PAMAM (polyamidoamine) dendrimers and gelsolin nanobody, both lacking a bona fide biological endogenous binding target inside the brain, were tested. Interestingly, although almost identical in size, the dendrimers only displayed marginal transmigration. The nanobody on the other hand reached accumulations and retention of up to 60 %ID/cc (percent

835 injected dose per cubic centimeter of tissue). As introduction to the cortex was scarce (72), recent
836 optimisation has been accomplished by occlusion of the contralateral carotid artery. This lead to
837 passage of bevacizumab to on average 34 %ID/g in the cortex and 17 %ID/g in the subcortical
838 structures whilst the cortex was rarely part of the field of introduction when this occlusion wasn't
839 included (30). As nanobody accumulations were already 2.5 times larger compared to bevacizumab
840 without occlusion (72), this demands the question how this occlusion would enhance effective
841 delivery of a nanobody with a specific brain target allowing retention. is dat een eigen conclusie?
842 60% is hoog maar kan altijd beter

843 Overall, methods hi-jacking natural transcytosis in the BBB promote mostly lower
844 transmigration compared to BBBD. Additionally, as these methods rely on RMT and AMT, they may
845 require healthy BBB functioning for adequate efficiency. Nonetheless, BBB breakdown has been
846 linked to several neurodegenerative disorders and often leads to functional and structural changes in
847 the blood vessels (156). Transcytosis efficiency thus depends on different factors in different stages
848 of the process such as competitive ligand-receptor interactions, receptor expression and lysosomal
849 sorting. BBBD techniques have the advantage that they are independent of these biological variables
850 and have been shown to direct high quantities into a particular brain area due to specific localization
851 of the disruptive agents that are responsible for BBBD.

852 Nanobody-induced Protein silencing

853 Gene/protein depletion is often employed to study protein function and
854 potential therapeutic effects. Classically, RNA interference (RNAi) has been applied to knock down
855 essential gene products. Effectiveness ultimately relies on the turnover rate of the protein of interest
856 (POI) but degradation is often incomplete. In general, an effect can require up to 2-3 days of
857 treatment and even longer for low turnover rate proteins. Additionally, the knockdown is regularly
858 associated with off-target effects. Since the arrival of CRISPR/Cas9-mediated gene knock-outs, it is
859 possible to specifically prevent protein expression after deletion of the start codon or the

introduction of missense mutations. Nevertheless, unlike RNAi, the technique is limited to non-essential proteins for cell proliferation and survival. Additionally, the knock-out sometimes is incomplete due to truncation of the protein still being translated. Also, individual cells with complete gene knock-out are prone to genetic compensation leading to altered expression patterns of related proteins (44, 137).

To overcome these limitations, researchers have looked for new techniques directed at targeted protein proteolysis to directly manipulate a protein of interest rather than regulate its expression at the nucleic acid level. To achieve this, several ingenious systems have been developed by researchers over the past decade. These however are covered in more detail in recent reviews (137, 167) and will not be dealt with here. Of note, when nanobodies are used to obtain protein silencing, it offers the advantage of targeting endogenous proteins directly and the protein of interest does not have to be tagged which may potentially affect its biological activity or subcellular localization. Employing the built-in protein homeostasis machinery and more specifically the ubiquitin-proteasome system (UPS) has been quite successful to attain targeted protein degradation. A great example are Proteolysis targeting chimeras (PROTAC). These heterobifunctional nanomolecules of approximately 10 nm are capable of recruiting proteins to the UPS for degradation and can even be cell-permeable (26, 104). On one side the bifunctional molecule binds the protein of interest (POI, the protein that needs to be degraded) and through the other side it binds an E3 ligase. A ternary complex is thus formed leading to ubiquitination and degradation of the POI (140).

Modifications like the implementation of a destabilizing domain (degron) into the POI sequence can be sufficient for inducible degradation by drugs or even light (21, 134). Yet, the introduction of the PEST (Pro-Glu-Ser-Thr) degron into an antibody fragment sequence can also already lead to degradation of the POI (113). Nanobodies offer exceptional possibilities due to their small size and specificity, and could allow easy modifiability by simply swapping nanobody. A PEST-nanobody fusion against alpha-synuclein, for example, performed higher degradation rates

885 compared to a well-studied anti-alpha-synuclein VH14-PEST, yet accomplished lower survival rates
886 (25, 29).

887 During the last decade, multiple nanobody-based systems for targeted proteolysis have been
888 developed in different animal models (Fig. 2). Introduction of the nanobody takes place by
889 replacement (30, 146) of or coupling (51, 173) to the substrate recognition domain of E3 cullin-RING
890 ubiquitinating ligase (CRL) complexes. Most approaches remain focused on the degradation of
891 fluorescently tagged proteins. Fluorescently tagged proteins may be more easy to monitor because,
892 unlike their endogenous counterpart, they are less involved in protein-protein interactions and when
893 overexpressed can result in a protein population that is 'free floating' in the cytoplasm and therefore
894 more easily accessible for degradation. There are however model organisms for which large
895 collections of strains with endogenous genes tagged with GFP are available such as *D. melanogaster*
896 (Kyoto Stock Center, Vienna Drosophila Resource Center), *C. elegans* (Caenorhabditis Genetics
897 Center) and *D. rerio* (Zebrafish International Resource Center). Yet, when unavailable, homozygous
898 introduction of the GFP-fusion protein can be tiresome and interfere with natural expression and
899 functioning of the POI.

900 The first nanobody based system for targeted proteolysis to be introduced was deGradFP and
901 consisted of an anti-GFP nanobody fused to the F-box domain of the *Drosophila melanogaster* Slmb
902 protein which associates with the CUL1 protein complex (Fig. 2a) (28). deGradFP has been tested in
903 leaves of *Nicotiana tabacum* and leads to GFP degradation sufficient to reach levels virtually
904 undetectable on western blot (11). Likewise, in *Caenorhabditis elegans*, a similar system coupling an
905 anti-GFP nanobody to the SOCS-box adaptor protein ZIF1 protein of SOCS-CUL2 CRL complex lead to
906 specific degradation of GFP-tagged knockin POIs (173). Interestingly, as the genome of *C. elegans* is
907 well-studied, the use of tissue-specific promoters was tested and obtained target-dependent, tissue-
908 specific, rapid and efficient degradation of GFP-MAD-1, GFP-PP1, GFP-b-tubulin and GFP-DLG-1.
909 Overall, the degradation was comparable to the genetic introduction of the ZF1-degron (ZIF1 target)

910 and auxin-inducible degron (AiD) to the POI (6, 173, 188). The AiD requires the activation of a
911 degradation system unique to plants, but is partially conserved in other eukaryotes (see further). On
912 the other hand, degradation of ZF1-degron carrying proteins naturally occurs by the same ZIF1-SOCS-
913 CUL2 CRL complex machinery and is essential in *C. elegans* during early embryogenesis. This limits its
914 use as it cannot be used for proteins essential during embryogenesis and later developmental stages.

915
916 Although functional in the study of Caussinus (28), deGradFP seemed poorly effective in a
917 stable cell line expressing histone H2B-GFP (146). For this reason, a new system for mammalian cells
918 and *Danio rerio* embryos was developed to allow proteasome-dependent degradation of specific
919 nuclear proteins. The protein interference (protein-i) was created by replacement of the substrate
920 recognition domain of Speckly-type POZ protein (SPOP) by an anti-GFP nanobody (Fig. 2b). Targeted
921 proteolysis of H2B-GFP by protein-i was more efficient compared to RNAi treatment. Additionally,
922 fluorescent staining of the stable cell lines showed that protein-i is active in the nucleus as nuclear
923 proteins (Cdk4-GFP, Elf3-GFP, cMyc-GFP and Pin1-GFP) were depleted, but only in that compartment.

924 In theory, any of the systems should be capable of degrading (endogenous) proteins by
925 simply replacing the anti-GFP nanobody by a nanobody of choice. The affinity-directed PROtein
926 Missile (AdPROM) system consists of the fusion of an anti-GFP nanobody to the Von-Hippel Lindau
927 (VHL) protein and subsequent proteolysis by the CUL2-E3 UB ligase (Fig. 2c). It was shown to degrade
928 both GFP-tagged cytosolic and endosomal proteins PAWS1 and VPS34, respectively. Most
929 interestingly, the group has swapped the anti-GFP nanobody by a nanobody targeting either ASC or
930 SHP2, two ubiquitously expressed proteins. The system triggered complete proteolysis of
931 endogenous SHP2 in multiple human cancer cell lines and of ASC in K-562 cells. The expression of the
932 AdPROM system does not seem to have an effect on VHL's natural substrate hypoxia factor 1 α
933 (HIF1 α), by western blot analysis. Nevertheless, aside from HIF1 α , the impact on other endogenous
934 VHL targets and downstream biology has to be further determined.

In plants, proteins carrying the auxin-inducible degron (AiD) bind auxin hormones, leading to subsequent degradation. The auxin inducible response is unavailable in other eukaryotes as auxins are hormones exclusive to plants and the F-box transport inhibitor response 1 (TIR-1) protein is lost during evolution. Nevertheless, the remainder of the necessary SCF degradation pathway is conserved as the SCF-TIR1 E3 ligase complex is still present. This opens opportunities for the controllable exploitation of auxin inducible degradation (AID) by expression of the TIR1 protein in non-plant cells and treatment with auxin hormones such as indole-3-acetic acid (IAA) or the synthetic 1-naphthaleneacetic acid (NAA) (Fig. 2d) analogue (33, 123). Addition of the AiD degron to the POI is sufficient for degradation, yet again, implementation of a nanobody system carrying the AiD degron is more versatile. Furthermore, unlike other studies, the research group had shown ubiquitination of the nanobody, thus lowering the efficacy of the entire system. For this purpose, lysine mutagenesis was performed to preserve anti-GFP nanobodies during proteolysis and assure that one nanobody-AiD can target multiple POI molecules (33). All studies utilized this lysine-less anti-GFP nanobody and lead to efficient degradation for a variety of POI, tagged with several GFP-like fluorescent proteins at a variety of subcellular locations such as venus-tagged Lamin A, venus-tagged cyclin D1, GFP-tagged inner centromere protein and venus-tagged ANAPC4, a subunit of the APC/C complex. Additionally, the system was introduced in *Danio rerio* for the degradation of GFP-tagged proliferative cell nuclear antigen (PCNA), lamina-associated polypeptide 2, utrophin and myosin light chain 12 genome duplicate 1. Safety with cell lines or organisms has to be tested as the off-target effects of TIR1 expression in non-plant cells are unknown. Furthermore, high auxin concentrations (minimum of 100 μ M) are required for induction and subsequently lead to toxicity by IAA oxidation. Stronger auxin-TIR1 interactions could offer a solution for this problem (182).

Trim-Away is a system for targeted proteolysis, hi-jacking the naturally occurring intracellular immunity by TRIM21 (Fig. 2e) (31). The protein is an E3 Ub ligase that recognizes the Fc-region of antibodies. These are co-endocytosed with pathogens and subsequently auto-ubiquitinated leading to proteosomal degradation of the TRIM21:Ab:virus complex (106). Trim-Away is capable of

depleting POI levels after micro-injection of TRIM21 mRNA and POI-specific antibody with a half-life for degradation as short as 9 min (31). The POI can vary from cytosolic to membrane-bound proteins or even nuclear proteins in balance with the cytosol through the nuclear pore, such as NLS-GFP. Only proteins retained inside the nucleus such as H2B-GFP were not degraded at first. To resolve this issue, anti-GFP-nanobody-Fc fusion proteins were tested and proven to be successful in rapid degradation. Nevertheless, the system requires the necessary scale-up for mRNA and protein introduction to be used for phenotypical effect and pathway studies compared to the single cell analysis by microinjections and is yet to be used for nanobodies against endogenous, not GFP-tagged, proteins. The researchers have employed electroporation for bulk introduction of the necessary mRNA (TRIM21) and proteins (antibody or nanobody-FC) for trim-Away and have shown similar efficiency in degradation to micro-injection. Yet, electroporation can be costly and extrapolation to large scale is tiresome. This furthermore stresses the need for efficient and elegant intracellular delivery of nanobodies.

Most techniques employ drugs to induce expression of the necessary components or to activate the ubiquitination process. Nevertheless, some drugs can have negative effects especially when high doses have to be administered as for example case with auxin in AID. One research group tackled this problem by the introduction of light induced protein degradation (LiPD) (40). In short, the group tested several E3 Ub ligase complexes to deplete GFP-tagged CXXC4-type zinc finger protein 4. An anti-GFP nanobody was fused to several E3 Ub ligase domains and the RING domain of LNX1 protein functioned most effectively (>95% degradation). For the implementation of LiPD, a light sensitive heterodimerization module is required, in this case CIBN/CRY2. By the emission of blue light, the light sensitive protein (CRY2), carrying the anti-GFP nanobody, undergoes conformational changes and allows heterodimerization with CIBN, carrying the E3 Ub ligase (Fig. 2f). This brings the GFP-tagged POI in close proximity of the E3 Ub ligase and permits ubiquitination and subsequent degradation (GFP-CXXC4 levels to below 10% initial cell concentration, 30 min half depletion time).

For larger cell populations, the group created a LED lightbox with different illumination programs and reached dose-dependent depletions ranging from 20 to 70% of the initial value. Chess board pattern induction was even tested, allowing side-by-side phenotypical comparison with neighboring non-depleted cells.

A similar dimerisation strategy for drug induced protein degradation (DiPD) was created in the same study by combining the FKBP/FRB pair with rapamycin induction (Fig. 2g). After valorisation with GFP-tagged proteins, the system was tested for the depletion of untagged, endogenous POI and led to efficient depletion of LMNA/C protein and PCNA. For the latter, the efficient depletion also led to a total loss of function as it plays a central and essential role in DNA replication. Furthermore, the DiPD system was also applicable in *C. elegans* and led to complete depletion of CED3-GFP within 6 hours after induction and which gradually recovered afterwards. Additionally, aside from multiple proteins (GFP, LMNA/C and PCNA by DiPD) being depleted simultaneously by one system, both LiPD and DiPD can be induced simultaneously to force combinatorial depletion of proteins (GFP-CXXC4 by LiPD and LMNA/C by DiPD).

In conclusion, quite a few different nanobody-based systems for targeted proteolysis have been developed for eukaryotic cell lines and model organisms such as *Drosophila melanogaster*, *Danio rerio* and *Caenorhabditis elegans*. The systems allow fast induction and reversible degradation of target proteins to near complete levels for most targets tested. Induction can be as simple as switching on light as with LiPD or drug administration, allowing easy implementation. Some research groups have taken into account possible drawbacks to their own system and have found solutions which have not been tested in others. An example is the implementation of lysine mutagenesis to prevent ubiquitination and degradation of the nanobody itself (33). Considering that nanobodies as such are not easily transferrable through membranes (cf supra), several hurdles would need to be taken for this approach ever to develop into a clinically useful methodology. Rather, we believe that a combination between nanobodies and PROTAC would be worthwhile to explore, as their

advantageous properties could lead to the development of compounds with improved specificity. Even though two PROTACs, ARV-110 and ARV-471, have entered the clinical setting (125), there is still considerable margin for improvement. As organic compounds are difficult to develop against undruggable (i.e. structural, non-enzymatic) proteins and are frequently inefficient in binding their target with high affinity and specificity, it has been argued that, with the rapid development of structural biology, peptides could substitute for small compounds (75). Currently however most protein-protein interactions have not yet been charted by X-ray crystallography. As nanobodies are conducive to protein crystallization and structure determination (62, 127, 158), they could help in developing aptamers for designing so-called peptide- or p-PROTAC. Such peptides however can also be poorly cell-permeable, or trigger non-specific degradation of the target in normal cells. To improve specific targeting, nano-carriers may be used such as micelles, polymersomes and polyplexes (75, 160). An encapsulated PROTAC in a nano-carrier could be targeted more specifically to diseased cells by decorating the carrier with a nanobody (3, 78, 177) against for instance a receptor on the surface of those cells. Very recently, Simpson et al. (148) combined nanobody and HaloPROTAC technologies to achieve inducible degradation of GFP-tagged proteins and endogenous Ras in cells.

General conclusion

Research on single domain antibodies is steadily increasing but there is still a long way to go as relatively few targets have been under investigation. The field is expected to grow further as researchers come to discover their ease of use and ample opportunities to turn them into a surgeon's scalpel of precision. It is anticipated that as yet unknown properties of proteins will surface in the future because directly targeting a resident protein in its native environment with a single domain antibody (achievable by transfection, transduction, genome insertion of a nanobody cDNA) is an approach that differs substantially from gene/cDNA overexpression, gene expression down-regulation by RNAi or gene ablation by CRISPR/Cas9. Only by using the complete repertoire of

technologies will we obtain a full picture of how genes and their products operate in cells and organisms. Moreover, by using different approaches we can better filter out disadvantages and off-target effects, which very likely also apply to nanobodies by the way, something that has thus far not been looked at in detail. For instance, a GFP nanobody is frequently used as a control in experiments and usually it does not have any effect as compared to the actual nanobody targeting protein X under study. That does not necessarily exclude off-target effects triggered by the nanobody under investigation. Usually, the intrinsic high affinity of an average monovalent nanobody (K_d 10^{-8} - 10^{-10} M) is perceived as a token of specificity. But off target effects are practically inherent in every tool that is used to perturb activity of proteins, or their interaction with other polypeptides. It may for instance be more suitable to mutate the nanobody CDRs and when the affinity and its activity have dropped dramatically in concert with the biological effect that is under study, such a nanobody could be a more appropriate control instead of the GFP nanobody. Inevitably this involves more experimental work but researchers should be aware that nanobodies are not devoid of off-target effects. Single domain antibodies are expected to trigger additional interest in the future and develop further as an instrument of choice in certain applications because they are also generated faster than small compounds which in many cases are difficult to obtain, particularly for structural ('undruggable')(12, 14, 153, 161) as these are not endowed with a small catalytic cleft where small compounds usually bind. A potential disadvantage of nanobodies, when used as intrabodies, is that they artificially extend the lifetime of their target protein, resulting in findings that can be misleading, unless it would be intentionally triggered. Hence, imaging a protein for extended periods of time (24 hrs or longer) could trigger artificial data. Although there is currently scarcely evidence in support of this contention, it is not inconceivable that interaction between a 15 kDa polypeptide and target protein occludes ubiquitination sites or prevent physiological interaction between ubiquitinase and substrate/target protein. The reverse potentially also holds true, namely that the interaction partner stabilizes its cognate nanobody. It is not impossible to investigate but it is not routinely done in most

1062 studies. Using different nanobodies that target different non-overlapping regions in the target
1063 protein could be one way to solve this question.

1064 This review also demonstrated that nanobodies can be derivatized and molded into a high-
1065 precision, complex and multitasking compound. We therefore think that single domain antibodies
1066 will in some cases oust regular immunoglobulins in view of their broader versatility and ease of
1067 (recombinant) production in bacteria. A disadvantage is that, although nanobodies can be generated
1068 relatively fast, usually more than one is obtained, and their characterization in terms of affinity and
1069 biological properties remains a labor intensive process as screening tests need to be set up for
1070 identification of nanobodies with interesting biological properties.

1071 Given the relatively short period of time in which nanobody applications have been
1072 investigated by the research community at large, it is amazing how much progress has been made.
1073 Yet we believe that the field has currently not yet reached maturation, as researchers are still looking
1074 for the best way to use nanobodies in protein silencing approaches or the best route to introduce
1075 them into cells. A generally useful approach to tackle one type of problem would probably promote
1076 dissemination of the technology. To address common questions we need overarching, generally
1077 applicable methodologies, and viewed from that perspective it is not surprising that many
1078 investigators still gravitate towards genetic approaches for studying gene and protein function
1079 because the protocols and tools are relatively easy to execute, more harmonized, and, commercially
1080 available in most cases. It is only when we can take these hurdles that nanobodies will become part
1081 of the generally established tool kit that most researchers can draw from. Looking however at the
1082 current pace of nanobody research we surmise that the next period of exploration will provide new
1083 tools, and with that in mind we think that the best is yet to come.

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Figure legends:

Figure 1. Conventional and heavy chain only antibodies. Representation of a heavy-chain antibody (HcAb) and its antigen binding domain, called single domain antibody or nanobody. Unlike the monoclonal antibody (mAb) depicted on the left, the heavy-chain only antibody consists solely of two heavy chains (CH1-CH2-VHH), whilst the mAb consists of two heavy chains (CH1-CH2-CH3-VH) and two light chains (CL-VL). The Fc (c=crystallisable) region is responsible for triggering the immune system. The antigen binding domain in mAb is represented by two variable domains, one of the light chain (VL) and one of the heavy chain (VH). Antigen binding fragments can be obtained from both structures. For mAb there are several options for which the most well-known are depicted. Antigen-binding fragment (Fab) consists of one constant and one variable domain of each of the heavy and light chain. Single-chain variable fragment (ScFv) is a fusion between both variable domains (VH & VL), most often linked by a serine-glycine linker. The variable fragment (Fv): a single variable domain, either VH or VL. For HcAb, antigen recognition is performed by one domain and the isolated domain is called a nanobody (Nb) or VHH.

Figure 2. Nanobody based protein silencing. **(a)** DeGradFP: a GFP-specific Nb replaces the substrate recognition domain of the E3 complex binding domain Slmb and the fusion protein is expressed in cells. POI-GFP fusion protein is recognised and recruited to the CUL1-RBX1-Skp1 complex, resulting in ubiquitination and degradation of POI. **(b)** Protein-i: a GFP-specific Nb replaces the substrate recognition domain of the E3 complex binding domain SPOP. POI-GFP fusion protein is recognised and recruited to the CUL3-RBX1 complex, resulting in ubiquitination and degradation. **(c)** AdPROM: a GFP/POI specific Nb is fused to the substrate recognition domain of VHL and is expressed in cells. POI is recognised and recruited to the CUL2-RBX1-ElonginB/C complex, resulting in ubiquitination and degradation. **(d)** AID: the F-box protein TIR-1 is expressed in cells together with a lysine-less GFP-specific Nb carrying AiD. The addition of auxin, enables interaction between TIR-1 and AiD-Nb and

thus POI-GFP fusion protein. Next, the CUL1-RBX1-Skp1 Complex enables ubiquitination and degradation of POI. **(e)** TRIM-Away recognises the fused Fc region of a GFP-specific Nb which eventually leads to the ubiquitination and degradation of POI. **(f)** LiPD: By the induction with blue light, CRY2 undergoes conformational changes, allowing interaction with CIBN. This brings the GFP-specific Nb carrying POI-GFP fusion protein in close proximity to the Lnx1 ubiquitinase complex for ubiquitination and degradation of POI. **(g)** DiPD: By chemical induction with rapamycin, FRB and FKBP are dimerized. This brings the POI-specific Nb carrying the POI in close proximity to the Lnx1 ubiquitinase complex for ubiquitination and degradation of POI. E2 = ubiquitin conjugating enzyme, Ub = ubiquitin

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