Nanobodies as Probes for Protein Dynamics in Vitro and in Cells*

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Nanobodies are the recombinant antigen-recognizing domains of the minimalistic heavy chain-only antibodies produced by camels and llamas. Nanobodies can be easily generated, effectively optimized, and variously derivatized with standard molecular biology protocols. These properties have triggered the recent explosion in the nanobody use in basic and clinical research. This review focuses on the emerging use of nanobodies for understanding and monitoring protein dynamics on the scales ranging from isolated protein domains to live cells, from nanoseconds to hours. The small size and high solubility make nanobodies uniquely suited for studying protein dynamics by NMR. The ability to produce conformation-sensitive nanobodies in cells enables studies that link structural dynamics of a target protein to its cellular behavior. The link between in vitro and in-cell dynamics, afforded by nanobodies, brings the analysis of such important events as receptor signaling, membrane protein trafficking, and protein interactions to the next level of resolution.

In addition to the conventional IgG antibodies, camelids and sharks produce unusual antibodies that lack light chains (Fig. 1, A and B) (1). These heavy chain-only antibodies retain full antigen specificity and binding affinity and can be further truncated without significant loss of their antigen-recognizing properties to produce isolated variable domains (VHH).2 The VHHs from camelids were initially trademarked as Nanobodies, but VHHs from llama are now more commonly employed for research. VHHs are also known as single domain antibodies.

Several unique properties of VHHs define their potential as research tools distinct from the conventional antibodies. First, nanobodies are much smaller. Although the IgG antibody consists of four chains with multiple domains and has a molecular mass of about 150 KDa, the nanobody is a single domain with a molecular mass of only about 15 KDa. Second, nanobodies can be easily screened for affinity and specificity using a wide spectrum of approaches ranging from phage display to NMR. Most importantly, nanobodies can be cloned, genetically or chemically modified, and produced in a recombinant form in various cells, and, potentially, in live organisms. Bacterial expression systems enable generation of purified nanobodies in milligram quantities per liter of culture, offering an unlimited supply of a selective reagent with consistent properties.

Importantly, nanobodies can be used to trace in real time and manipulate localization and activity of target proteins in eukaryotic cells (2–4). This allows correlating protein structural dynamics in vitro with the behavior of proteins in cells. The focus of this review is on the novel nanobody applications that span the full temporal and spatial scales of protein dynamics, from nanoseconds to hours, from isolated domains to whole cells. Such applications range from studies of fast protein dynamics by NMR, to detection and stabilization of functionally important transient protein conformations, to manipulating protein trafficking in the cell.

Generation of Nanobodies

A detailed review of nanobody production (Fig. 2) has been published recently (5). Briefly, llamas (or camels) are immunized with the antigen protein. When immune response develops, mRNA is isolated from lymphocytes, and a cDNA library of variable heavy chain domains is created by reverse transcription. The cDNA is used to express VHH as fusions with phage coat proteins (phage display), and the nanobodies are enriched by one or more rounds of panning against the immobilized antigen. Routinely, the selected nanobodies are expressed in Escherichia coli with a hexahistidine tag added to allow purification by nickel–nitritotriacetic acid affinity chromatography, and a secretion signal sequence inserted to direct the expressed protein to the periplasm for easier purification and to enable disulfide bond formation.

Shark heavy chain antibodies IgNAR have also been used to derive single domain antibodies, which possess similar antigen binding mode to camelid VHHs (6), and have many of the same advantages as research tools and potential therapeutic agents (7).

Nanobodies Versus Fragments of the Conventional Antibodies as Tools for Structural Biology

The conventional antibodies, exemplified by the most common isotype IgG, consist of two heavy and two light chains (Fig. 1A) associated through the disulfide bonds and non-covalent interactions. The binding properties of the antibodies are defined by the six complementarity-determining regions (CDRs) located within the variable domains of the...
FIGURE 1. Domain structure of IgG antibodies (A) and heavy chain only camelid antibodies (B). The isolated variable domain of the latter is called nanobody.

FIGURE 2. Schematic representation of the nanobody generation process, starting with the immunization of a camelid. PBMC, peripheral blood mononuclear cells; Ni-NTA, nickel-nitrilotriacetic acid; Nb, nanobody; IMAC, immobilized metal affinity chromatography; SEC, size exclusion chromatography.
heavy (V\textsubscript{H}) and light (V\textsubscript{L}) chains. The large size, multichain composition, and requirement for essential disulfide bond formation complicate production of the recombinant IgGs. Two smaller antibody fragments have been developed. The F\textsubscript{ab} has a molecular mass of about 50 KDa and consists of the light chain and a truncated heavy chain, including the two variable domains. The smallest practical derivative of a conventional antibody, scFv (\textasciitilde25 KDa), was produced by connecting V\textsubscript{H} and V\textsubscript{L} with an artificial peptide linker.

Because the association between the variable domains of the light and the heavy chains depends on the hydrophobic interactions, production and applications of the single isolated variable domains are hampered by poor protein solubility and aggregation. Although it is possible, in principle, to modify the variable domains to eliminate the hydrophobic interactions, production and applications of the single isolated variable domains are hampered by poor protein solubility and aggregation. However, this is possible, in principle, to modify the amino acid sequence of V\textsubscript{H} or V\textsubscript{L} to eliminate the hydrophobic patches on the protein surface (8), such work is time-consuming and may be derailed by the unintended effects of such mutations. The heavy chain-only camelid antibodies, where variable domains (VHH) do not interact with any other domains, offer a much more practical starting point for producing the single-domain antibodies. Indeed, unlike the isolated human V\textsubscript{H} domains, nanobodies are highly soluble. In turn, solubility and folding properties of the human V\textsubscript{H} domains can be improved by introducing a few mutations at the contact surface with the V\textsubscript{L} domain based on the sequence comparison to the camelid VHH (8, 9).

The antibody-derived fragments, in particular F\textsubscript{ab}, have been widely used in structural biology to assist crystallization of difficult proteins, including several by now classic structures (10–12). Antibodies can promote crystallization by inducing order in the flexible regions of the target protein, improving crystal contacts, and increasing the hydrophilic surface area of the complex. Nanobodies can be used to assist protein crystallization essentially in the same way. Examples of the protein structures solved with the help of nanobodies include several membrane receptors and transporters, proteins of bacterial secretion systems, and others (13–20).

Nanobodies with known binding sites can be employed to map location of individual domains or proteins by cryo-electron microscopy (21). When the epitope is located at the interface of the interacting domains or subunits of a protein, the nanobody can be used to disrupt domain interactions (22). The main advantage of the nanobodies in such conventional applications is that a large panel of nanobodies can be easily screened to identify preferred epitopes or the best co-crystals between a nanobody and the target protein, or to reveal and stabilize unknown target conformers (23).

**Nanobodies Versus Specific Binding Proteins Designed on Non-antibody Scaffolds**

Natural antibodies inspired design of engineered proteins that bind to their targets with high affinity and specificity. In these constructs, a small protein domain with a high natural propensity for protein interactions is used as a scaffold for the target-specific binding sequences similar to the CDRs of the natural antibodies. Initially, a large library of potential binders with a partially randomized amino acid sequence in the binding site is created. The high-affinity binders are then selected by phage display or, recently, by ribosome display (24). Examples of such scaffold-protein affinity reagents (SPARs) (25) include DARPins (designed ankyrin repeat proteins) (26), monobodies, designed on the scaffold of human fibronectin III domain 3 (27), and also Affibody molecules (28) and anticalins (29).

Crystal structures of various SPARs in complex with their targets have been solved (30, 31), revealing the binding mode and illustrating their potential applications in structural biology. DARPins (32–34) and monobodies (35, 36) have been used to solve x-ray structures of such challenging targets as membrane transporters. Nanobodies and SPARs share many of the same advantages, such as small size, single domain composition, and the ease of producing recombinant proteins. Production of SPARs does not involve animal immunization, a cost-saving factor. On the other hand, nanobodies have inherently high affinity, whereas to achieve comparably high SPAR affinity, much larger synthetic libraries have to be generated and screened, a potentially daunting task. Some knowledge of the binding mode for the given target would allow using smaller biased libraries, but such knowledge is often unavailable for complex membrane proteins from mammalian cells. Another consideration in choosing between various SPARs and nanobodies is the preference for different epitope architectures, which is to a large extent determined by the shape of the scaffold protein (30). This aspect is discussed below in more detail for the nanobodies.

**Structural Basis of the Distinctive Binding Properties of the Nanobodies**

The VHH domain is composed of a folded \(\beta\)-sheet with three loops in the regions homologous to the CDRs of the IgG V\textsubscript{H} domains (Fig. 1). The length of CDR3 loop in the VHH can exceed 20 amino acid residues, as compared with the typical length of 9 (mouse) or 12 (human) in the conventional antibodies. The longer CDR3 loop can insert into the newly partially buried binding sites, as first seen in the structure of lysozyme in complex with a nanobody (37) (Fig. 3) (structure diagrams were generated using MOLMOL (38) and PyMOL (39)), and then in some other nanobody complexes (40–43). However, the binding mode of the nanobodies and the length of CDR3 loop can vary greatly (44, 45).

A longer CDR3, the convex shape of the antigen-binding site, and the small size allow nanobodies to access epitopes that may be cryptic and non-antigenic for conventional antibodies. In fact, unlike the conventional antibodies, which more often detect linear or planar epitopes, many nanobodies bind to concave and discontinuous epitopes that only form in the folded protein (45). This property makes nanobodies valuable tools for probing conformational states of the target proteins, both in vitro and in cells. Nanobodies that bind discontinuous epitopes can be selected from the initial panel by competition with linear peptides or with an unfolded protein or by using the masked selection technique (46). Such nanobodies are particularly useful for stabilizing folded intermediates and specific protein conformations.

An interesting consequence of the preference for the discontinuous epitopes has been observed in the structure of the nanobody complex with a heterodimer of editosome proteins.
A3 and A6. These two proteins have overall low level of sequence identity, but a similar constellation of the essential residues in two different binding pockets, which caused nanobody binding to both A3 and A6 (19). This phenomenon can be exploited to select nanobodies against substrate- or ligand-binding sites, which usually have a high percentage of conserved residues across various species.

The stability of the nanobodies is on par with the more stable of the VH domains of the conventional antibodies, but a truly remarkable property of VHH is its ability to efficiently refold with full restoration of its antigen binding properties after thermal denaturation (47). This property opens interesting and largely unexplored possibilities for the nanobody use in protein folding studies. Because thermal denaturation of nanobodies is reversible, conceivably, nanobody binding could be turned on or off in the NMR tube by simply raising or lowering sample temperature.

**Nanobodies Capture Transient Protein Conformations**

In 2010, Kirchhofer et al. (44) published an exciting “proof-of-concept study” that demonstrated the utility of nanobodies for detecting and regulating conformational transitions of proteins in vitro and in cells. The authors identified two nanobodies, dubbed the Enhancer and the Minimizer, which had opposite effects on GFP fluorescence. Subsequent structural analysis revealed that each of the nanobodies induced subtle changes in the chromophore environment, thus modulating the absorption properties of GFP. This ability to manipulate GFP fluorescence enabled higher sensitivity and improved spatial resolution in studies of GFP-fused proteins in living cells. Recent development of nanobody applications for super-resolution microscopy combined with inventive methods of nanobody derivatization with fluorescent labels opens new and exciting possibilities for visualizing intracellular processes (48–52).

The ability to identify and trap specific protein conformations using nanobodies has already found creative uses in elucidating the mechanisms of important cellular events, such as receptor-mediated signaling, trafficking, and protein complex assembly. In recent studies of epidermal growth factor receptor (EGFR) (53–55), the nanobodies enabled detection of a functionally silent EGFR heterodimer, which is distinct from the active ligand-bound conformer. EGF binding to the extracellular domain of EGFR triggers conformational changes and homodimerization, or heterodimerization with the other members of the EGFR family, such as ErbB2, initiating the signaling cascade. The existence of an inactive “tethered” dimer was predicted (53, 54), but its conformational status was difficult to define. Nevoltris et al. (55) isolated nanobodies selective either for the ligand-free or for the ligand-bound EGFR. Using these conformation-sensitive nanobodies and homogenous time-resolved fluorescence measurements, the authors demonstrated the presence at the plasma membrane of the EGFR/ErbB2 “pre-dimers,” which were structurally and functionally distinct from the activated dimers. Biological relevance of this finding was further demonstrated in cells using the wild-type ErbB receptors.

Inhibitory conformation-sensitive nanobodies were used to investigate the contribution of L-plastin to the formation of immune synapse (56). L-plastin is an actin-binding protein, which redistributes to the immune synapse following interaction between the T cells and the antigen-presenting cells. Nanobodies that trapped L-plastin in an inactive conformation affected several distinct steps in the formation of the synapse, including IL-2 secretion and T cell proliferation. Nanobodies against various domains of L-plastin further helped to dissect its interactions with the other proteins involved in the synapse formation. Thus, nanobodies enabled functional studies of L-plastin at the level of detail typically achieved by mutagenesis.
but without potentially complicating the effects of mutations on protein folding or stability.

Of particular interest are nanobodies that trap the target protein in a defined conformational state and can be used to manipulate its activity in the cell. For example, mouse P-glycoprotein (Pgp) has been crystallized in complex with a nanobody that bound to one of the two nucleotide-binding domains in the Pgp dimer. This binding stabilized the inward-facing conformation of the Pgp dimer and inhibited Pgp catalytic cycle by interfering with the interaction of the two nucleotide-binding domains (57). The ability to modulate intracellular activity of Pgp, an ABC-type transporter involved in cancer cell resistance to chemotherapeutic drugs, suggests the potential for developing a conceptually new therapeutic strategy to fight drug resistance in cancer cells in the future.

A significant advantage of nanobodies lies in their amenability to further optimization for binding to the desired conformer by repeated rounds of selection from the initial cDNA libraries or by directed evolution of the previously selected nanobodies. This property was used extensively to assist crystallization of the active conformation of the β2-adrenergic receptor (BAR) by the Kobilka group. Crystallization of GPCRs in the active form had been a major challenge due to the conformational plasticity of these states. Multiple optimizing mutations, fusions with a helper protein, and agonists with very high binding affinity had been previously employed to stabilize GPCRs in the active conformation (58–60). A conformation-selective nanobody offered an elegant alternative to these methods.

A nanobody that showed preferential binding to BAR in the active state (61) was further optimized for binding affinity. A library of nanobody variants with partially randomized binding sites was subjected to multiple rounds of positive selection for stronger binding to BAR in the active conformation stabilized by a strong agonist. A round of negative selection against BAR bound to an inverse agonist removed variants with reduced conformational specificity. This selection process produced a nanobody with a 10-fold higher affinity, which was used to stabilize and successfully crystallize the active conformation of BAR in complex with adrenaline (62). A similar strategy was used to select the conformation-specific variants of a nanobody against the agonist-bound muscarinic acetylcholine receptor and led to a high-resolution structure of this GPCR (63).

One biologically important facet of the conformation-selective nanobodies is that by stabilizing the active conformation, they emulate the effect of the natural interacting partners of their target protein. Thus, the Nb80 nanobody used to stabilize the active state of BAR increased its affinity to the agonist isoproterenol by about 100-fold, perfectly matching the effect of the cognate G-protein, which shows strong binding cooperativity with the receptor agonists (61), and also stabilizes the active state of the receptor. In fact, crystal structures of the β2-adrenergoreceptor in complex with either Nb80 or the Gs protein were found to be almost identical (61, 64).

The conformation-selective nanobodies help to investigate receptor-mediated signaling in live cells with an unprecedented insight into protein dynamics by bridging protein conformational transitions and complex intracellular functions (65). A recent elegant study employed a conformation-sensitive nanobody to demonstrate signaling by the activated and internalized β2-adrenergic receptor from the early endosomes (66). This study revealed that the internalized receptors contribute to the cellular cyclic AMP response within several minutes after agonist application, thus providing previously unavailable spatial and temporal information. Selectivity of nanobodies for the conformational, non-linear epitopes is also likely to reduce nonspecific “off-target” effects in intracellular applications.

**Nanobodies and Intrinsically Disordered Proteins**

Nanobodies have been used as crystallization chaperones for proteins with a high extent of intrinsic disorder, such as antitoxin MazE, a component of the programmed cell death system in bacteria (67). Remarkably, only 45% of amino acid residues were ordered in that structure. An x-ray structure of the human prion protein represents another interesting example of using nanobodies to reveal structural propensities of intrinsically disordered proteins (68). This structure shows expansion of a β-sheet in the usually disordered N-terminal region of PrP(C), the normal form of the prion protein. Although formation of the additional β-strands was likely caused by the nanobody, this effect may mimic a step in the naturally occurring structural transition that leads to the increase in the β-strand content, and consequently to PrP(C) aggregation, which culminates in amyloid fibril formation. Interestingly, the discontinuous nanobody epitope in PrP(C) includes the connecting loop leading to the newly formed β-strand, but not the strand itself, suggesting that the nanobody may have triggered a natural transition between the two energetically close conformations, rather than forced an artificial structure formation. This work suggests the possibility of using large nanobody panels to explore the dynamic conformational landscapes of the intrinsically disordered proteins to identify physiologically relevant transitions.

**Nanobodies as Probes of Fast Protein Dynamics**

Nanobodies open new opportunities for correlating fast protein dynamics with the function of protein in the cell. NMR spectroscopy is the most powerful method for studying protein dynamics on the nanosecond scale. For protein NMR, the small size of the nanobodies offers unique advantages and truly sets them apart from the conventional antibodies or their derivatives. Unlike x-ray crystallography or electron microscopy, solution NMR is increasingly difficult to use with larger proteins. This is mostly a consequence of the relationship between the rate of molecular tumbling of the protein molecule and NMR relaxation rates. The slower tumbling of larger proteins leads to fast relaxation, which produces broad NMR lines that result in significant peak overlap, deterioration of signal intensity, and eventually major loss of spectral information. Although the size of protein envelope accessible by high-resolution NMR has been constantly expanding, proteins above 40 KDa remain challenging targets. Therefore complete IgG antibodies (150 KDa) and Fαb, fragments (50 KDa) are not well suited as tools for protein NMR. In contrast, nanobodies (12–15 KDa) can be successfully applied to studies of protein conformation and dynamics in solution by NMR.

Nanobodies can be used as NMR invisible probes, while the target proteins are isotopically labeled to record two-dimen-
Nanobodies have been used to study the folding of the amyloid-forming variants of human lysozyme by NMR (70), as well as the interactions with the monomeric and fibril forms of α-synuclein (71, 72). These studies nicely illustrate the high quality and high information content of the NMR spectra that can be obtained for the protein-nanobody complexes. The binding sites of the nanobodies can be accurately mapped by NMR. Signals of the residues located at the contact surface with the nanobody show intensity loss, or chemical shift change, depending on the nanobody binding parameters. Crystal structures of several nanobody complexes with dihydrofolate reductase showed good agreement between NMR mapping and the x-ray structures (73).

The potential of nanobodies to analyze dynamics of multidomain proteins is illustrated in the recent studies of Wilson disease protein (ATP7B), a transmembrane copper transporter powered by ATP hydrolysis (Fig. 4A) (74). In cells, activity and localization of ATP7B are regulated by copper (75). The available data point to copper-dependent interactions between the six ferredoxin-like metal-binding domains (MBDs) located in the cytosolic N-terminal portion of ATP7B (Fig. 4A) as a basis of this regulation. However, analysis of the domain-domain

![Diagram](image-url)
interactions proved to be difficult due to their transient nature and the largely independent motions of MBDs revealed by NMR studies (76).

NMR offers access to fast protein dynamics through the analysis of relaxation rates, $R_1$ and $R_2$, and heteronuclear NOEs. These parameters contain information on the degree of structural order (order parameter $S^2$), the rate (rotational correlation time $\tau_c$), and the extent of anisotropy (diffusion tensor component ratio $D//D_\perp$) of the molecular motions. Transient interactions between protein domains will affect their mobility, and thus will be reflected in NMR relaxation parameters. However, to extract this information, reference data for non-interacting domains are required. By virtue of their uniquely small size, nanobodies can be used to selectively disrupt domain-domain interactions in the context of a full-length multi-domain protein.

Two nanobodies were used to probe the dynamics of the 600-amino acid-long chain of six metal-binding domains of ATP7B (MBD1–6, Fig. 4B), one binding to MBD3, and the other to MBD4. The effects of the two nanobodies on the domain dynamics were strikingly different. Nanobody binding to MBD3 predictably increased its correlation time $\tau_c$ (slower tumbling), but decreased the correlation time of MBD1 and MBD2 (faster tumbling), to values close to those observed for the isolated MBD2. Accelerated molecular motions of MBD1 and MBD2, in response to nanobody binding to MBD3, pointed to breaking interactions between the three domains through displacement (Fig. 4C). Thus, the anti-MBD3 nanobody revealed transient interactions of the domains, which were not detectable from the relaxation data without this differential approach (77). In contrast, the anti-MBD4 nanobody only caused deceleration of its target domain without any significant effect on the dynamics of the others (Fig. 4D), indicating that MBD4 does not significantly interact with the other domains.

The effect of nanobody binding to the MBDs was further studied in the cells where a stable expression of the nanobody facilitated trafficking of ATP7B from the trans-Golgi network to vesicles, mimicking the effect of copper binding to MBDs. To put domain dynamics into the context of protein function in the cell, nanobodies can be used to mimic interactions of multidomain proteins with their physiological partners, in the same key as described above for the GPCRs and their cognate G-proteins. In the case of ATP7B, the nanobody may mimic the effect of Atox1, a cytosolic copper chaperone protein that delivers copper to ATP7B in the cell (78). Atox1-Cu binding and subsequent copper transfer to MBD2 (79) may break interactions between MBD1, MBD2, and MBD3, in the same fashion as the nanobody binding to MBD3, producing a similar open conformation of MBD1–6 that triggers ATP7B trafficking.

In summary, nanobodies undoubtedly hold great potential for mechanistic studies of protein dynamics. A particularly exciting avenue is combining nanobody-assisted structural studies in vitro with the manipulation of the protein properties, activity, or localization in the cell, using the same nanobody expressed endogenously. Another promising set of applications is nanobody derivatization. Heavy metals for x-ray crystallography and small-angle x-ray scattering, paramagnetic labels for protein NMR, and fluorescent tags for in-cell work can be conveniently attached to the nanobodies, and this is in many cases preferable to the corresponding modifications of the protein of interest. A largely unexplored area is the use of nanobodies for protein folding studies, and their use in fast protein dynamics studies by NMR is just beginning. Many other nanobody applications that at this point escape the imagination of the authors will undoubtedly be developed in the near future.

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