Nanobodies: Chemical Functionalization Strategies and Intracellular Applications

Dominik Schumacher, Jonas Helma, Anselm F. L. Schneider, Heinrich Leonhardt, and Christian P. R. Hackenberger*

Keywords: antigen-binding proteins · cellular delivery · molecular biology · nanobodies · site-specific functionalization

= fluorophore, tracer, biotin, drug....
Nanobodies can be seen as next-generation tools for the recognition and modulation of antigens that are inaccessible to conventional antibodies. Due to their compact structure and high stability, nanobodies see frequent usage in basic research, and their chemical functionalization opens the way towards promising diagnostic and therapeutic applications. In this Review, central aspects of nanobody functionalization are presented, together with selected applications. While early conjugation strategies relied on the random modification of natural amino acids, more recent studies have focused on the site-specific attachment of functional moieties. Such techniques include chemoenzymatic approaches, expressed protein ligation, and amber suppression in combination with bioorthogonal modification strategies. Recent applications range from sophisticated imaging and mass spectrometry to the delivery of nanobodies into living cells for the visualization and manipulation of intracellular antigens.

1. Introduction

Antibodies are the core molecules of the immune system for identifying, targeting, and clearing pathogens from the infected organism. Immunoglobulin G (IgG), a 150 kDa protein consisting of two heavy and two light chains is the predominant antibody type found in nature.[1] Since antibodies were used for the detection of rhesus factor immunization and to quantify the amount of insulin present in blood plasma, uncountable analytical applications have been developed.[2] The ability to generate humanized and monoclonal antibodies highly specific to almost any antigen of interest has intensified this development and laid the foundation for the targeted therapeutic use of antibodies.[3] While early therapeutic concepts exclusively relied on the function of the antibody itself, more recent development combine the target specificity of antibodies with the effectiveness of small drug molecules in so-called antibody-drug conjugates (ADCs).[4] For this, a drug molecule is covalently linked to a functional group within the antibody, which requires selective chemical methods for attachment without interfering with antibody function.[5] The same trend of attaching functionality holds true for analytical and diagnostic antibodies. While many of the established methods rely on indirect detection modes like radioative labelling of the antigen, oxidation by horseradish peroxidase, or the enzyme alkaline phosphatase (in enzyme-linked immunosorbent assay, ELISA),[6] more recent developments have made use of small fluorescent labels that are covalently bound to the primary antibody.[7]

The generation, production, functionalization, and intracellular application of full-length antibodies can be challenging. Antibodies are posttranslationally glycosylated proteins and their function has been shown to be dependent on the attached glycans.[8] Furthermore, antibodies have a complex structure involving inter- and intramolecular disulfide bonds, which is vulnerable towards environmental changes, the reductive milieu of the intracellular environment, and the attachment of payloads. Moreover, conventional IgGs contain a highly conserved loop length for the antigen-binding domain (complementary determining regions, CDRs), which evolved to bind convex paratopes, thereby limiting the scope of potential antigens.[9] For instance, the receptor-binding domains of various pathogens have evolved as cavities, which prevents the binding of full length IgGs.[10]

Consequently, novel classes of recombinant antigen-binding proteins that lack these limitations are on the rise.[11] Besides their reduced size and structural complexity, many recombinant antigen-binding proteins (binders) can be produced in high amounts using eukaryotic and prokaryotic cells,
and based on their increased stability towards reductive conditions, can be applied within cellular environments.\[12\]

This opens avenues for live-cell detection and the manipulation of important intracellular processes with minimal impairment to the cell. In contrast, the use of full-length antibodies is often limited to extracellular targets and fixed or permeabilized tissues. These promising properties have led to the development of various classes of binders that are either immunoglobulin-derived or synthetic derivatives of completely different protein classes. Nanobodies are noteworthy examples of recombinant antigen-binding proteins that are distinguished by unique physical properties and binding specificity.\[7,13\] They are defined as single-domain variable fragments of camelid-derived heavy-chain antibodies (hcAb). Nanobodies are introduced in Section 2 and discussed in comparison with other formats of recombinant binders. In Section 3, an overview of nanobody generation and selection procedures is given, and in Section 4, selected applications of genetically encoded nanobodies in cellular biology and imaging are depicted. In Section 5, techniques for the chemical functionalization of nanobodies will be highlighted. Recent developments allow the generation of homogenous nanobody conjugates that have increased binding affinity and beneficial in vivo properties compared to their randomly functionalized equivalents.\[14\]

Finally, in Section 6, advances in the cellular delivery of nanobodies and other binders will be reviewed.

2. Recombinant Antigen-Binding Proteins: Nanobodies and Others

IgGs are the predominant isotype of immunoglobulins and consist of two identical heavy and two identical light chains that are covalently linked through disulfide bonds.\[3\] The antigen is recognized through an interplay between the variable N-terminal domains of the heavy (\(V_\text{H}\)) and the light (\(V_\text{L}\)) chain and six CDRs (Figure 1a).\[8\] Binders derived from IgGs can be classified as antigen-binding fragments (Fab, ca. 50 kDa), single-chain variable fragments (scFv, ca. 25 kDa), and heavy- or light-chain single domains (\(V_\text{H}\) or \(V_\text{L}\), ca. 12.5 kDa). Fab and scFv binders consist of both the \(V_\text{H}\) and \(V_\text{L}\) domain of the parental IgG, and retain the size and affinity of the area binding the antigen. Due to their reduced size compared to regular IgGs, they show enhanced pharmacokinetic properties for in vivo applications.\[10a\] \(V_\text{H}\) and \(V_\text{L}\) are covalently linked by artificial amino acid linkers or disulfides and associated through strong hydrophobic interactions. Ward et al. were able to demonstrate, that functional single \(V_\text{H}\) domains of mice can be secreted from \(E. \text{coli}\), and they hypothesized that their reduced size should enable binding to the cavities of pathogens.\[15\] However, these expectations
have not been fulfilled. The antigen-binding area of isolated VH domains is bisected and their binding affinity significantly reduced compared to the parent antibody. Moreover, the hydrophobic amino acids that are essential for VH/VL interaction in full-length IgGs are solvent-exposed, thus leading to aggregation and poor solubility.

In the early 1990s, an exceptional class of IgG immunoglobulin was detected in the sera of Camelidae. These so-called heavy-chain antibodies (hcAb) from Camelidae lack the CH1 and CL domain of conventional antibodies. They recognize their antigen through a single variable domain, VH (dark gray). The X-Ray structure of a nanobody binding its antigen GFP (green) is shown (PDB ID: 3G9A). A fibronectin-based monobody binding the SUMO protein (PDB ID: 3RWZ). An affibody based on Protein A, binding to HER 2 (PDB ID: 3MZW). A libocalin derived anticalin binding to the Alzheimer’s disease (AD)-relevant amyloid-ß (PDB ID: 4MV1). A designed ankyrin repeat protein (DARPin) in complex with human interleukin-4 (PDB ID: 4YDY). Antigens are shown in blue, antigen-binding proteins in gray.

Figure 2. Comparison of the binding regions and surface structures of nanobodies and human-derived VH domains. a) A VH (nanobody) from Camelidae with GFP as the antigen (PDB ID: 3C9A). A human derived variable domain (VH) with vascular endothelial growth factor as the antigen (PDB ID: 2FJF). VHs contain a significantly enlarged CDR3 framework (black), thus ensuring high binding affinity. Several hydrophobic amino acids that are highly conserved in conventional VH domains are mutated within nanobodies, which increases their solubility (orange).
derived single V_{H} to increase their binding affinity and stability while maintaining their low immunogenic potential in humans. Alternatively, a humanized nanobody scaffold has been engineered, which facilitates CDR grafting from other nanobodies for the development of nanobody based therapeutics. Along these lines, pharmaceutical companies like the Belgian company Ablynx nv have a growing number of pre-clinical and clinical programs in development that are based on proprietary nanobody technology, thus further emphasizing their potential.

In addition to immunoglobulin-derived binders, non-immunoglobulin-based proteins have been engineered to specifically bind antigens with similar affinity compared to conventional antibodies. Small proteins that are involved in tight protein–protein interactions serve as scaffolds for the generation of such binders. The specific binding surface of these scaffolds is randomized and high-affinity binders selected through an in vitro display technique. Prominent examples are monobodies anticalins, affibodies, and designed ankyrin repeat proteins (DARPins) Figure 1 b–e).

Monobodies are recombinant antigen-binding proteins based on human fibronectin III. They are structurally similar to immunoglobulin binders but devoid of intramolecular disulfides, thus making them ideal for intracellular applications. The cell wall protein Protein A present in Staphylococcus aureus serves as the basis for affibodies. In nature, Protein A binds the crystalizable (Fc) region of immunoglobulins, preventing phagocytosis triggered by an immune response of the host organism. Mutagensis of the binding area resulted in a number of efficient affibodies that bind targets like human insulin or the cytokine TNFα. Anticalins are derived from the β-barrel-structured lipocalins, a diverse class of proteins responsible for the transport, storage, synthesis, and sequestration of small hydrophobic molecules. Lipocalin-based libraries have enabled the selection of anticalins against various targets with up to picomolar affinities. Finally, DARPins are based on natural ankyrin repeats that are involved in protein–protein interactions. In contrast to other engineered binders, DARPins are characterized by a modular assembly of consecutive repeats engineered to bind a specific target. The different classes of engineered recombinant antigen-binding proteins share many advantageous properties, including high stability and small size. However, the effort needed in generating non-Ig-derived recombinant antigen-binding proteins with sufficient binding affinity is high, thus limiting the applicability of these binders.

3. Nanobody Generation and Selection Procedures

Nanobodies (and other immunoglobulin-based recombinant antigen-binding proteins) can either be generated by immunizing the respective animal with the antigen of interest or by further evolving an existing naïve library. In the case of immunization, up to six injections of around 0.5 mg antigen or immobilized antigen (e.g., BSA conjugate) are performed within a time course of several weeks. The mRNA is isolated from lymphocytes and its complementary DNA (cDNA) synthesized using reverse transcriptase. Next, the specific segment encoding the V_{H} domain is amplified and potent binders isolated or further engineered using a polypeptide display technique. Phage display is the most common display technique for in vitro binder selection and is capable of screening up to 10^{15} sequences per library.

Here, the V_{H} encoding part is fused to a viral coat protein, leading to the library being displayed on the surface of bacteriophages. Since each phage displays a single V_{H} variant and contains its genetic information, the most efficient binders can be selected by challenging the library with the immobilized antigen followed by nucleotide sequencing. Alternative screening strategies include yeast and bacterial display, in which binders can be selected by multiparameter and quantitative flow cytometry, as well as mRNA and ribosome display, which are well suited for the selection of large libraries of up to 10^{15} sequences. Once a potent nanobody is selected, it can be readily expressed in high yields of up to several g L⁻¹ in E. coli, S. cerevisiae, P. pastoris or human cells by using a periplasmic leader sequence. Secretion to a non-reducing environment during expression is advisable, since nanobodies harbor up to two disulfide bridges. An overview of the nanobodies presented within this review, together with their targets, functionalization approaches, applications, and known affinity values is given in Table 1.

4. Genetically Encoded Nanobodies in Cellular Biology and Imaging

As already mentioned, nanobodies have advantageous properties for advanced applications in molecular biology. They feature high thermal and conformational stability and retain their binding activity after prolonged incubation at elevated temperatures, high salt concentrations, and under different pH conditions. Moreover, they are able to efficiently refold and fully restore their antigen affinity after thermal denaturation, thus opening novel opportunities for studying the dynamics of protein folding.

These robust properties allow them to capture their respective antigens in vitro as well as in vivo, one example being the widely used GFP binder. A visual example for antigen capture in living cells is the recently developed fluorescent-3-hybrid (F3H) assay to monitor dynamic protein–protein interactions (Figure 3a). Here, a green fluorescent protein (GFP)-binding nanobody is fused to the Lac repressor, resulting in the recruitment of GFP fusion proteins to artificial LacO DNA repeats. As soon as a second protein labelled with another fluorescent molecule interacts with the GFP fusion, it will be co-recruited to the anchor site, thereby enabling time-resolved visualization of protein–protein interactions.

In recent years, nanobodies have been used to analyze protein function in living cells and organisms. The reversible genetic knockdown of proteins by interfering RNA is a prominent method for elucidating protein function. However, such systems rely on the fast depletion of the target protein. An alternative method to study protein function by
Table 1: Overview of the nanobodies discussed in this review.

| Target Application | Functionalization | Generation | $K_d$ [nm] |
|--------------------|-------------------|------------|-----------|
| GFP Protein immobilization \[46\] & Solid support, randomly attached (NHS-chemistry), \[1] TTL mediated chemoenzymatic biotinylation & Immunization & 0.23 \[27\] |
| Detection of PPIs \[44a\] & Lac repressor, genetic fusion & – & – |
| Protein degradation \[49\] & F-box domain of Slmb, genetic fusion & – & – |
| Imaging \[27, 45, 66, 72\] & FPs, genetic fusion; fluorophores and gold nanoparticles randomly attached (NHS-chemistry), chemoenzymatic TTL \[3\] mediated site-specific attachment & – & – |
| Intracellular protein discovery \[120\] & Polycationic resurfacing, FPs, genetic fusion & – & – |
| Cellular delivery, live cell immunostaining \[118\] & Linear and cyclic cell-penetrating peptides via EPL \[31\] & – & – |
| L-Plastin Trapping of inactive conformation \[70\] & VS-tag for purification, genetic fusion & Immunization & 40-80 \[25\] |
| P-glycoprotein Inhibiting drug-efflux-based multidrug resistance \[73\] & – & Immunization & 520 \[73\] |
| HypeE Inhibiting or stimulating AMPylation \[32\] & Fluorophore, biotin, Sortase A mediated & Naïve phage display library & N/A |
| MazE Crystallization chaperone \[35\] & – & Immunization & N/A |
| β2-microglobulin Crystallization chaperone \[56\] & – & Immunization & 1.6 \[35\] |
| Eps1:Epsj Crystallization chaperone \[33\] & – & Immunization & N/A |
| human lysozyme Studying protein folding using NMR \[31\] & – & Immunization and grafting CDRs to stable nanobodies & 460 \[41\] |
| Proclacitonin High-throughput assay \[70\] & Chitosane-graphene nanocomposite, randomly attached (glutaraldehyde) & Immunization & 6.2-24.5 \[25\] |
| PCNA Imaging DNA replication \[27, 40\] & FPs, genetic fusion & Immunization & N/A |
| β-catenin Imaging of β-catenin \[71\] & FPs, genetic fusion & Immunization & 1.9-44 \[71\] |
| HIV-1 capsid protein Imaging HIV-1 \[32\] & FPs, genetic fusion & Immunization & 0.16 \[72\] |
| Nuclear lamina Imaging the cytoskeleton \[37\] & FPs, genetic fusion & Immunization & N/A |
| Target Application | Functionalization | Generation | $K_d$ [nm] |
| HER2 Biomarker for breast cancer \[74\] & Radiolabel, engineered C-terminal cysteine & Immunization & ~6 \[126\] |
| Biomarker for breast cancer \[74\] & engineered maleimide chemistry & – & – |
| Nanobody based activation immunotherapeutic \[71\] & Radiolabel, fluorophore, chemoenzymatic attachment by the use of Sortase A & – & – |
| Intracellular protein discovery \[120\] & Dinitrophenyl moiety, chemoenzymatic attachment by the use of lipoic acid ligase & – & – |
| CAIX Biomarker for breast cancer \[74\] & Polycationic resurfacing, FPs, genetic fusion & – & – |

*Angew. Chem. Int. Ed.* 2018, 57, 2314 – 2333 © 2018 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim www.angewandte.org
reversible knockout makes use of nanobodies that mediate ubiquitin-dependent protein degradation of the bound protein (Figure 3b). This technology was used against a GFP fusion to show that the myosin II regulatory light chain $\text{Sqh}$ is required for dorsal closure in the fruit fly $\text{Drosophila}$. Moreover, nanobodies have been used to sense and trap specific conformations of proteins. Nanobodies that bind $\lambda$-plastin, an actin-binding protein involved in immune regulation, that trap the protein in an inactive conformation revealed that $\lambda$-plastin plays an important role for immune synapse formation and T-cell proliferation. Moreover, a nanobody that was shown to inactivate the intracellular ATP hydrolysis activity of Pgp, an ABC-type transporter, could potentially serve as the basis for the development of new therapeutics to overcome drug resistance during cancer therapy (Figure 4). Furthermore, nanobodies have been shown to modulate the abundance of posttranslational modifications on proteins in living cells. Truttmann et al. were able to engineer V$_h$Hs that either inhibit or stimulate Huntington associated protein E (HYPE)-mediated AMPylation of proteins and used these tools to identify histones H2, H3, and H4 as new targets for HYPE.

Another important application of nanobodies is their use as crystallization chaperones for intrinsically disordered proteins and large molecular complexes. The production of protein crystals with sufficient quality for X-ray crystallography of such proteins can be highly challenging, and co-crystallization with nanobodies has been shown to improve the crystallization behavior significantly. In this way, the structures of disordered proteins like the addiction antidote MazE and the amyloidogenic $\beta_2$-microglobulin, as well as the structure of a large protein complex of EpsJ and EpsI that is involved in the secretion of proteins, have been solved.

Finally, nanobodies have even been shown to bind challenging epitopes such as small molecules. This has led,
for example, to the development of various nanobody-based high-throughput assays to identify and quantify clinically relevant biomarkers, including testosterone and proclacitoin.\[53\]

The fluorescent labelling of proteins has become one of the most important tools for visualizing and understanding cellular structures and intracellular processes. Fluorescent proteins (FPs), like GFP, are the most frequently used biosensors, and their fusion to a protein of interest enables the dynamic visualization of proteins of interest in living cells.\[54\] Even though genetic fusion to FPs is straightforward, their fluorescence properties, as well as a tendency towards photobleaching, limit their spectroscopic use, and their possible impact to the biological function of the protein of interest is often underestimated.\[57\] To improve the spectroscopic properties of FP-tagged proteins of interest, Kirchhofer et al. developed GFP-binding nanobodies that are able to modulate the absorption properties of GFP by inducing structural changes in the environment of the chromophore (Figure 5a). These changes stabilize GFP fluorescence in living cells and resulted in higher fluorescence sensitivity and spatial resolution.\[57\]
Due to their low production costs and long-term stability, nanobodies have become powerful alternatives to conventional detection reagents composed of primary and fluorescently labelled secondary antibodies to high linkage errors and loss of resolution since the actual dye is removed from the target structure (up to 30 nm). Nanobodies have a diameter of 2.5 nm and a height of approximately 4 nm, which makes them better suited for high-resolution imaging of cellular structures.[24] Guizetti and co-workers used a GFP-binding nanobody to elucidate the abscission stages of human cells and identified contractile filament helices with a diameter of 17 nm to be a central component of intercellular bridges.[64] The gain in resolution was demonstrated with super-resolution imaging of microtubules.[65] This concept of using nanobodies for better fluorescence signal and resolution in imaging experiments has subsequently been further developed by labelling nanobodies with small organic fluorophores and gold nanoparticles.[66,67] In recent years, a number of such methods to facilitate the development of binders with advanced properties have been developed. An overview is given in the following section, and applications of the resulting functionalized nanobodies are discussed.

5. Chemical and Enzymatic Functionalization of Nanobodies: Concepts and Applications

The chemical labelling of nanobodies with fluorophores, their immobilization on solid supports, or their functionalization with recognition motifs, delivery agents, and other chemical groups broadly expands their applicability for imaging, proteomics, and novel therapeutic tools. While traditionally, nanobodies used in imaging are expressed fused to FPs like GFP and RFP, the labelling of nanobodies with small organic probes is expanding their utility as tools for biological research, including in super-resolution imaging.[65,66] Due to their low production costs and long-term stability, numerous nanobodies have been immobilized on different matrices and been used for immunoaffinity chromatography.[67] This is of particular interest for state-of-the-art mass spectrometry (MS)-based proteomics since such technologies require the efficient enrichment of defined targets from complex protein mixtures. In this context, immobilized nanobodies that specifically bind fluorescently labelled proteins has allowed the combinatorial analysis of protein–protein interactions, DNA methyltransferase activity, and histone-tail binding by fluorescent microscopy and mass spectrometry (Figure 6).[27,64,68]

Lys-Selective Functionalization

In first proof-of-principle studies, nanobodies were randomly labelled at solvent-exposed lysine residues by N-hydroxysuccinimide (NHS) ester containing fluorophores to give heterogeneous protein mixtures (Figure 7a).[65,66] Similarly, NHS-functionalized matrices have been used to covalently attach and immobilize nanobodies.[66,67] Even though
the unselective lysine labelling of nanobodies has proven valuable, it has been shown to affect the CDR loops, leading to a significant reduction in epitope recognition.\[14b]\n
Moreover, these conventional bioconjugation technologies have proven unfavorable for in vivo therapy and diagnostics since unselective functionalization of antigen-binding proteins leads to variations in the number of probes attached, which, in combination with alteration of epitope recognition, can lead to impaired pharmacokinetic properties and stability.\[4b,c,71]\n
Therefore, the site-specific attachment of tracers and drugs to nanobodies to give homogeneous conjugates with a defined number of probes per binder can offer advantageous properties. Platanova and co-workers gained higher conjugation control by genetically adding a poly-lysine stretch to the C-terminus of GFP- and RFP-binding nanobodies (Figure 7b).\[72]\n
Incubating the nanobodies with NHS-activated fluorophores resulted in a labeling ratio of 1.0–1.5 fluorescent molecules per nanobody. However, whether the poly-lysine stretch prevented fluorophore conjugation to \(\varepsilon\)-amino groups within the nanobody sequence was not shown and seems unlikely. In recent years, a number of methods for the site-specific functionalization of proteins have been applied to homogeneously modify nanobodies. These methods include selective modification of unpaired cysteine residues, chemoenzymatic systems, expansion of the genetic code, and expressed protein ligation (EPL).

**Labeling of (Unpaired) Cysteine Residues**

Increased homogeneity of protein conjugates can be achieved by addressing cysteine residues, which are less abundant in comparison to lysine.\[73]\n
In this case, the free thiol group of a reduced cysteine is converted with a cysteine-selective chemical entity carrying the probe or drug. Among others, maleimides are the most common functional groups used for the labelling of cysteines.\[74]\n
However, since proteins often contain several cysteine residues that are involved in the formation of interchain disulfide bonds, an additional reduction step is required and the resulting conjugates are once again heterogeneous mixtures. The introduction of an additional cysteine into the protein of interest is a possible way to circumvent this limitation and has found widespread application in nanobody functionalization.\[14a,75]\n
In most cases, the cysteine has been introduced at the C-terminus of the nanobody, thus ensuring that the conjugation site is most distal from the antigen-binding interface (Figure 8a). Massa et al. used an engineered cysteine nanobody to produce homogeneous biomarkers for human epidermal growth factor receptor 2 (HER2)-expressing cancer cells.\[14a\]

Along these lines, single cysteine nanobodies against carbonic anhydrase IX (CAIX) and prostate-specific membrane antigen (PSMA9), labelled with an infrared dye and a radiolabel, respectively, have been applied to the in vivo diagnosis of breast and prostate cancer (Figure 8b).\[75a,d]\n
In 2012, Vugmeyster and co-workers covalently attached branched and linear polyethylene glycol (PEG) linkers to the C-terminus of single cysteine nanobodies to prolong their in vivo circulation time.\[75e]\n
Pharmacokinetic and biodistribution profiles in three different species showed that the site-specific attachment of PEG chains successfully protected the...

---

**Figure 6.** FP-binding nanobodies like the GFP-binding GBP enable the combinatorial analysis of proteins and their interaction partners through imaging and mass spectrometry-based proteomics. A protein of interest (Protein 1) expressed as an FP fusion can be imaged using conventional methods. A nanobody that binds the FP is chemically immobilized on a solid support, which facilitates enrichment of the target protein and any interacting protein (Proteins 2 and 3). Subsequent MS analysis enables the identification and assignment of the co-enriched interacting proteins.

**Figure 7.** Random labeling of nanobodies. a) NHS-activated probes/drugs are reacted with the nucleophilic \(\varepsilon\)-amine of a solvent-exposed lysine residue, resulting in heterogeneous nanobody conjugate mixtures with partly reduced binding affinities. b) The C-terminal fusion of a poly-lysine stretch to nanobodies is intended to prevent unselective NHS-based labeling of Lys residues within the CDR loops responsible for antigen binding. The nanobody is depicted in gray, the introduced functionality (e.g., fluorophore, drug, tracer) in red. The crystal structure of a GFP-binding nanobody is used in (a) and (b) [PDB ID: 3G9A].\[73]
nanobody by masking sites related to cellular uptake, proteolysis, and other clearance pathways (Figure 8c). Moreover, DNA-binding polyethyleneimine-maleimide has been conjugated to a single cysteine nanobody against MUC1-overexpressing cancer cells to selectively induce apoptosis by polyethylenimine/DNA delivery.[75c] However, the C-terminal attachment of cysteine residues often results in dimerization of the nanobodies and glutathione capping of the unpaired cysteine, thus making an additional reduction step prior to functionalization unavoidable.[14a] Therefore, Pleiner et al. analyzed the tertiary structure of a nanobody binding the Xenopus nuclear pore complex and engineered a cysteine at the surface of the nanobody framework region that is less prone towards capping and protein dimerization.[14b] In general, however, introducing additional cysteines into a nanobody can result in reduced expression yields.[14a, 71]

**Chemoenzymatic Labelling:**

Ever since the bacterial biotin ligase (BirA) was repurposed to site-specifically biotinylate a protein of interest, different labelling methods have been developed that are built upon the reinterpretation of a naturally occurring enzyme.[76] In vivo biotinylation by BirA was one of the first chemoenzymatic methods applied to nanobodies. Here, the C-terminus of the nanobody is genetically fused to a short biotin acceptor domain (BAD) and co-expressed in human cells with BirA. The enzyme activates biotin through monophosphorylation and transfers the biotinyl moiety to the target protein during expression. This system has been applied to generate nanobody-based ELISA and immunoarrays for the rapid detection of influenza and apolipoprotein (Figure 9a).[77] However, being limited to the biotinylation of proteins restricts the applicability of this method.

Transglutaminases are another family of enzymes that have been adapted to site-specifically modify proteins. In nature, they play an important role in the crosslinking of proteins and catalyze the formation of an isopeptide bond between the γ-carbonyl amide group of glutamines and the ε-amine group of lysines.[78] In principle, transglutaminases...
can target any glutamine residue within a protein of interest as long as it is positioned in a disordered or highly flexible region of the biomolecule. Since nanobodies do not contain such glutamine residues, transglutaminases were successfully applied for their site-specific modification by placing a glutamine-containing c-myc-tag (EQKLISEEDL) at the protein C terminus (Figure 9b). Although this was only used to biotinylate nanobodies, Fabs and other antigen-binding proteins have been functionalized with different entities, including fluorophores and drugs, and in principle, these findings should be applicable to nanobodies too.

The transpeptidase Sortase A from Staphylococcus aureus specifically recognizes the consensus sequence LPXTG (Sortag) that can be placed at the C terminus of a protein of interest. A nucleophilic attack of the thiol at C148 on the enzyme cleaves the amide bond between glycine and threonine of the Sortag, leading to a thioacyl intermediate. A second nucleophilic attack by an incoming glycine peptide carrying a payload of choice results in the site-specific functionalization of proteins through a native amide bond. Moreover, Sortase A has been applied for the N-terminal modification of proteins and shows substantial promiscuity with respect to nucleophilic substrates. Witte and co-workers used Sortase A to generate nanobody dimers through C-to-C fusion, as well as bispecific nanobodies against GFP and mouse class II MHC products in yields of up to 90%.

In addition to that, Sortase A has been used to site-specifically attach fluorophores and radiotracers for single photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging in vivo. In a recent study, Sortase A labelling was combined with an engineered unpaired cysteine to achieve double functionalization of nanobodies and site-specifically fluorescently labelled nanobody dimers. A drawback of using Sortase A to functionalize nanobodies is the reversibility of the amide-bond formation and the resulting need for a high substrate excess to drive the reaction towards completion.

The lipoic acid ligase (LpIA) is an enzyme that recognizes a 13 amino acid peptide tag, the lipoic acid acceptor peptide (LAP, GFEIDKVWYDKADA), and ligation a lipoic acid derivative to the side chain of a lysine residue. By introducing mutations in the lipoic acid binding pocket of the enzyme, mutant enzyme variants could be generated that accept unnatural substrates instead of lipoic acid. The enzyme has since been used in numerous applications to attach biotin, fluorophores, or other labels to the peptide tag. In this case,
Previously generated mutant of the enzyme was used that can attach a bioorthogonal aryl-aldehyde handle to the acceptor peptide. Gray et al. applied this to the modification of an anti-HER2 nanobody with a dinitrophenyl moiety. By incubating HER2-positive cancer cells with the nanobody and an anti-dinitrophenyl IgG antibody, the authors could trigger an immune response leading to antibody-dependent cytotoxicity (Figure 11b). The functionalized nanobody serves as an immunotherapeutic that mediates between the cancer antigen and the immune system.

Recently, our research groups have added to the toolbox of site-specific chemoenzymatic protein functionalization with Tub-tag labeling. This is versatile method that allows the C-terminal attachment of small unnatural tyrosine derivatives using the recombinant enzyme tubulin tyrosine ligase (TTL) (Figure 12a). The derivatives carry unique chemical entities like azides, aldehydes, and iodides, thus making Tub-tag compatible with several well-established bioorthogonal reactions. In detail, the TTL recognizes a 14 amino acid recognition sequence (Tub-tag, VDSVEGEVEEEGEE) fused to the C-terminus of a protein of interest and covalently attaches the tyrosine derivative of choice in an ATP-dependent reaction. Moreover, based on the enlarged catalytic cavity formed by the enzyme during the catalytic cycle (Figure 12b), we were able to significantly broaden the substrate scope of Tub-tag labeling. This allowed us to advance the versatile two-step method to a fast one-step labelling strategy that makes use of fluorescent coumarin and biotin derivatives as TTL substrates (Figure 12c, d). Since the C-terminus of nanobodies is most distant from their antigen-binding region, we envisioned Tub-tag labeling as a well-suited technology for nanobody functionalization. Therefore, we recombinantly expressed a number of Tub-tagged nanobodies and generated nanobody-based immunoprecipitation tools and super-resolution probes with minimal linkage errors.

Amber Suppression

Another prominent technology for the site-specific labeling of proteins is amber suppression. Here, an unnatural amino acid carrying a unique chemical entity (a bioorthogonal group) is incorporated into a random site within the sequence of the target protein using an engineered expression machinery. In a second step, a payload of choice is site-specifically attached to the bioorthogonal group. One of the major advantages of amber suppression is the high variety of unnatural amino acids that can be incorporated using the system. Recently, the unnatural amino acid AmAzZLys was incorporated into a nanobody against the epidermal growth factor receptor (EGFR). AmAzZLys is a lysine derivative that contains a benzylc amine and azide, which allowed subsequent double functionalization of the nanobody with a fluorophore and a 5 kDa PEG chain (Figure 13a). Moreover, the benzylc azide was used to perform photo-induced crosslinking to EGFR upon antigen binding (Figure 13b). However, amber suppression is technically demanding and results in a significantly lower expression yield compared to the wild-type nanobody, thus limiting the usage of amber suppression for nanobody functionalization.
In principle, while EPL facilitates the C-terminal functionalization of proteins, it is not without its drawbacks. In addition to the functionalization of nanobodies and other antigen-binding proteins, this method also has its limitations. One of the main challenges is the requirement for engineered cysteines, which can be difficult to introduce into the target protein. Furthermore, the site-specific functionalization of nanobodies has proven beneficial to fully maintain protein function, the site-specific C-terminal functionalization of nanobodies has proven beneficial compared to unselective labeling strategies. Owing to reduced engineering effort and high conjugation yields, the labeling of unpaired cysteine residues and chemoenzymatic functionalization strategies are particularly suitable for achieving this goal.

In addition to the functionalization of nanobodies and other binders, their subsequent cellular delivery is of high interest to the scientific community. Recent achievements are discussed in the next section.

6. Cellular Delivery of Small Antigen-Binding Proteins

The direct cellular delivery of nanobodies and antigen-binding proteins is a long-standing goal in cell biology and medicine, since it would offer a non-integrative way to analyze and manipulate cellular processes, protein–protein interactions, and protein function. Therefore, intense research has been invested within recent decades to develop general methods to achieve this goal. In principle, intracellular functional antigen-binding proteins can be obtained by transfecting cells, for example with the use of intrabodies that are optimized for intracellular expression.

Expressed Protein Ligation and Protein trans-Splicing

Expressed protein ligation (EPL) is a technology that is based on the naturally occurring splicing of proteins. A protein of interest is expressed as a fusion with a mutated intein that generates a highly reactive C-terminal thioester upon activation with a reducing agent like 2-mercaptoethanol or dithiothreitol. In a following ligation reaction to a peptide carrying an N-terminal cysteine, a new peptide bond is generated through S-to-N acyl transfer. Most of the time, the cysteine peptide is synthesized by solid-phase peptide synthesis (SPPS), which enables the straightforward incorporation of functional probes and payloads. Therefore, EPL has broad application for the site-specific functionalization of proteins and nanobodies. An alkyne-modified cysteine was recently incorporated to a vascular cell adhesion molecule 1 (VCAM-1)-binding nanobody by EPL and further modified with biotin by a subsequent copper-catalyzed click reaction.

Optimizing the expression and ligation protocol allowed the generation of decent amounts of nanobody with high functionalization yields of up to 100%. In another study, EPL was used for the double functionalization of a vascular-tumor-targeting nanobody with polymersomes for drug delivery and biotin. While EPL facilitates the C-terminal functionalization of proteins, Bachmann et al. recently made use of the GOS-terL intein and a protein trans-splicing reaction to functionalize a GFP-binding nanobody with a synthetic fluorophore at its N terminus. However, when choosing EPL or intein-fusion strategies, refolding from inclusion bodies is often required to achieve functional proteins, which significantly increases experimental effort.

In recent years, huge efforts have been made to broaden the toolbox of conjugation methods for the functionalization of nanobodies and other antigen-binding proteins. While all of the methods discussed in this review have their pros and cons, the methodological versatility enables the interested scientist to pick one of the validated techniques according to the requirements of the individual application. Nevertheless, to fully maintain protein function, the site-specific C-terminal functionalization of nanobodies has proven beneficial compared to unselective labeling strategies. Owing to reduced engineering effort and high conjugation yields, the labeling of unpaired cysteine residues and chemoenzymatic functionalization strategies are particularly suitable for achieving this goal.

In addition to the functionalization of nanobodies and other binders, their subsequent cellular delivery is of high interest to the scientific community. Recent achievements are discussed in the next section.
Supercharged proteins are a class of engineered nanobody (nanobodies) carrying a high net charge. While the net charge of a single molecule like GFP-binding nanobody to bind its antigen, it is difficult to estimate what effect the high net charge could have on the nanobody structure or the ability of the nanobody to "resurface" the nanobodies, that is, mutated several amino acid residues on the surface of the proteins to basic residues, resulting in net positive charges of +14 and +15 for the nanobodies overall. These polycationic nanobodies could then penetrate cells (incubated with 250–500 nm nanobody) and showed localization in the cytosol and not in endosomes (Figure 14a). While they demonstrated that the engineering does not impact the nanobody structure or the ability of the GFP-binding nanobody to bind its antigen, it is difficult to estimate what effect the high net charge could have on localization and antigen binding in a generalistic manner. Besides making the proteins directly cell-permeable, delivery of proteins can also be achieved using biophysical methods like microinjection and electroporation and by using one of the increasingly important carrier-based delivery systems. In principle, the carrier-based delivery of cargo to cells through the use of a) charged surface mediated transduction, b) endocytosis, or c) cyclic cell-penetrating peptides (CPPs). The nanobody is depicted in gray, the introduced functionality in red. d) Thiol-containing mesoporous silica nanoparticles (MSNs) were conjugated to a maleimide-functionalized nitrotetrazolic acid (NTA) linker. Activation with a metal ion (shown for Ni) facilitated binding of a His6-chromobody. The MSN-chromobody complex showed endosomal entrapment upon cellular incubation, thus necessitating endosomal escape triggers like the peptide INF7 to enable cytosolic distribution of the chromobody. e) A cell delivery system based on the anthrax lethal toxin. Recombinant antigen-binding proteins (shown for a monobody) are expressed as a fusion with the 30 kDa N-terminal domain of the toxin enzyme lethal factor (LF31). The protective-antigen (PA)-based pore-forming transporter (PA oligomers shown in green) is bound to a host-cell receptor. The binder-LF31 forms a complex with the transporter (step 1) and endocytosis is initiated (step 2 and 3). Due to the acidic environment in the endosome, the PA oligomers form a transmembrane pore, unfolds the binder-LF31 fusion protein and initiates its translocation to the cytosol (step 4). The nanobody is depicted in gray, RFP (PDB ID: 1GEX[18]) in red, and the monobody in blue (PDB ID: 3RZW[10]). The crystal structure of a GFP-binding nanobody is used in (a), (b), (c), and (d) [PDB ID: 3G9A][37].

However, the functional cytosolic expression of binders remains challenging and their functionalization with small molecules like affinity tags, fluorophores, and drugs is not possible when using such techniques.[11h,c,104a,105]

In this sense, the delivery of functional antigen-binding proteins into living cells would vastly expand the methodological repertoire of antigen-binding proteins for intracellular use.[107] Supercharged proteins are a class of engineered proteins that are able to penetrate mammalian cells.[108] In 2016, Bruce et al. made use of this concept and successfully engineered nanobodies against GFP, HER2, and β-lactamase in order to make them cell-permeable.[109] To accomplish this, they “resurfaced” the nanobodies, that is, mutated several amino acid residues on the surface of the proteins to basic residues, resulting in net positive charges of +14 and +15 for the nanobodies overall. These polycationic nanobodies could then penetrate cells (incubated with 250–500 nm nanobody) and showed localization in the cytosol and not in endosomes (Figure 14a). While they demonstrated that the engineering does not impact the nanobody structure or the ability of the GFP-binding nanobody to bind its antigen, it is difficult to estimate what effect the high net charge could have on localization and antigen binding in a generalistic manner. Besides making the proteins directly cell-permeable, delivery of proteins can also be achieved using biophysical methods like microinjection and electroporation[106] and by using one of the increasingly important carrier-based delivery systems.[104a] In principle, the carrier-based delivery of cargo to cells follows two main pathways; endocytosis-dependent uptake (Figure 14b) and the transduction across the cell membrane (Figure 14c). Endocytosis is the predominant pathway described for the delivery of biomolecules.[111] Here, extracellular macromolecules pass through the plasma membrane via encapsulation in vesicles and become trapped in endosomes, which can lead to lysosomal degradation. To be available inside the cell, the biomolecule then needs to escape from the endosome. This can be achieved through lipid or osmotic pressure mediated destabilization of the membrane or translocation of the cargo through transmembrane pores.[111–112] In 2016, Chiu and coworkers developed large-pore mesoporous silica nanoparticles (MSNs) that are functionalized with nitrotetrazolic acid (NTA) groups at the internal surface (Figure 14d).[113] Activation of the complex with various metal ions enabled the covalent attachment of a His6-tagged GFP-chromobody. Incubation of living cells with these complexes at a concentration of 25 nm resulted in endocytic uptake and endosomal entrapment of the conjugate. Although a small amount of chromobody was able to escape the endosomes (1–2%), probably due to a proton sponge effect generated by the His6-tag, the use of endosomal-escape triggers like fusogenic peptide INF7, acidity, DMSO, or chloroquine was necessary to obtain a decent amount (~17%) of chromobody within the cytosol. Importantly, intracellular co-localization of the
incubation of the encapsulated nanobodies with the MSN-NTA complex allows combination with any His-tagged protein. One year later, Röder et al. combined fluorescently labelled nanobodies (via NHS-esters) with a number of different nanoparticle-forming oligoaminoamides equipped with succinyl triethylene pentamine units that trigger endosomal release.\textsuperscript{[114]} Incubation of the encapsulated nanobodies with HeLa cells at 3.6 $\mu$m concentrations resulted in either receptor-specific or non-specific endocytic uptake. Through this strategy, they were able to achieve high co-localization with the antigen and intracellular availability of the nanobody of up to 60%. Nevertheless, endosomal escape remained a bottleneck, as indicated by a significant amount of nanoparticles entrapped in cellular vesicles.

In the past few years, a receptor-dependent delivery system based on the anthrax lethal toxin has been developed and applied for the delivery of a number of biomolecules.\textsuperscript{[115]} In nature, protective antigen (PA) binds to anthrax receptors on human cells and oligomerizes to form heptamers or octamers. Once lethal factor (LF) binding occurs, the whole complex is endocytosed. Subsequently, the acidic milieu of the endosomes initiates a rearrangement of PA, leading to the formation of a PA pore in the membrane that allows translocation of the LF to the cytosol. Liao et al. made use of this system and chemically attached LF to an affibody, a DARPin, and a monobody by using Sortase A (Figure 14e).\textsuperscript{[116]} In this way, they were able to achieve delivery of these recombinant binders to the cytosol with maintained antigen-binding properties (cells were incubated with pm–nm concentrations of recombinant binder and 20 nm PA). Nevertheless, translocation through the PA pore requires protein unfolding and subsequent refolding, thus limiting this approach to antigen-binding proteins that are readily folded within the reductive environment of the cytosol. In this sense, most of the aforementioned examples for the cellular delivery of nanobodies and antigen-binding proteins require endosomal escape mechanisms that constitute a major bottleneck for addressing intracellular antigens in living cells.

The use of cell-penetrating peptides (CPPs) might circumvent this limitation since they have been shown to enable the direct cellular uptake of functional full-length proteins.\textsuperscript{[117]} Therefore, the generation of cell-penetrating antigen-binding proteins by CPP fusion is the next logical step to fulfill the goal of immediate bioavailability and antigen binding. Herece and Schumacher et al. recently made use of this concept and ligated linear and cyclic HIV-derived TAT and deca-arginine (R$_{10}$) peptides to the C terminus of two different GFP-binding nanobodies by EPL (Figure 15a).\textsuperscript{[118]} Subsequent studies revealed that the conjugates produced transduction rates of up to 95% in cells from different cell lines when incubated with low $\mu$m concentrations (10 $\mu$m). Moreover, the uptake initiated by R$_{10}$ peptides is up to three times increased compared to that initiated by TAT, and cyclization of the peptides further increases uptake efficiency. Based on these findings, cyclic R10 peptides are most suitable for the generation of cell-permeable nanobodies. cR10 conjugates were then applied for the co-transport of GFP and GFP fusion proteins with a size of up to 83 kDa, including the therapeutically relevant MeCP2 protein.\textsuperscript{[119]} Moreover, the cell-permeable nanobodies have been used to visualize protein–protein interactions in living cells by slightly adopting the previously published three-hybrid assay.\textsuperscript{[40]} These experiments show that cell-permeable nanobodies are powerful tools for cell biology and the delivery of recombinant and

![Figure 15. Charge-induced membrane transduction of nanobodies initiated by cell penetrating peptides (CPPs).](Image)

Angew. Chem. Int. Ed. 2018, 57, 2314 – 2333 © 2018 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim www.angewandte.org 2329
therapeutically relevant proteins. Since arginine-rich CPPs localize to the nucleolus and bind to negatively charged RNA, addressing targets within the cytosol requires the rapid cleavage of the CPP after cellular uptake. Therefore, Herce and Schumacher et al. site-specifically conjugated a fluorophore via EPL to the C terminus of the nanobody and used the cysteine created at the EPL junction for linkage of the CPP via a disulfide (Figure 15b). Uptake studies and fluorescence microscopy revealed efficient internalization of the Cy5-labelled nanobody without nucleolar enrichment, thus indicating reductive CPP cleavage within cytosol (Figure 15c). This conjugate was used for the visualization of antigens in living cells, and constitutes a very promising tool for intracellular immunostaining and immunomanipulation.

7. Conclusion

Ever since their discovery, nanobodies have emerged as powerful antigen binders that, together with conventional antibodies and other classes of antigen-binding proteins, form a versatile toolbox for biochemistry, cell biology, and beyond. Nanobodies are characterized by their small size, increased solubility and stability compared to other antigen-binding proteins, and enlarged CDR loops that open the door towards previously inaccessible antigens. The fusion of nanobodies to fluorescent proteins initiated the generation of highly sophisticated, functional binders that have been further advanced through conjugation to organic fluorophores, tracers, and drugs, as well as the immobilization of nanobodies to solid supports. While such conjugates were originally synthesized using NHS chemistry, resulting in heterogeneous mixtures, strategies for the site-specific functionalization of nanobodies have been developed, leading to products with improved biophysical properties. New approaches for the cellular delivery of functional antigen-binding proteins allows their use within the cellular environment, which constitutes a major step in live-cell immunolabelling and antigen manipulation. Taken together, the versatility of antigen-binding proteins, methods for their functionalization, and strategies for their cellular delivery forms a powerful basis for the generation of next-generation diagnostics and therapeutic tools with striking properties.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin (Leibniz-Humboldt Professorship) and the Boehringer-Ingeheim Foundation (Plus 3a ward) to C.P.R.H., the Fonds der Chemischen Industrie (FCI) to C.P.R.H. and to D.S. (Kekulheim Foundation (Plus 3a ward) to C.P.R.H., the Leibniz-Humboldt Professorship) and the Boehringer-Ingeheim Foundation (Plus 3a ward) to C.P.R.H., the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungsgemeinschaft (SPP1623) to C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-2), the Einstein Foundation Berlin, the Deutsche Forschungs...
