Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Structural Biology

Chaperone-assisted structure elucidation with DARPins

Peer RE Mittl, Patrick Ernst and Andreas Plückthun

Designed ankyrin repeat proteins (DARPins) are artificial binding proteins that have found many uses in therapy, diagnostics and biochemical research. They substantially extend the scope of antibody-derived binders. Their high affinity and specificity, rigidity, extended paratope, and facile bacterial production make them attractive for structural biology. Complexes with simple DARPins have been crystallized for a long time, but particularly the rigid helix fusion strategy has opened new opportunities. Rigid DARPin fusions expand crystallization space, enable recruitment of targets in a host lattice and reduce the size limit for cryo-EM. Besides applications in structural biology, rigid DARPin fusions also serve as molecular probes in cells to investigate spatial restraints in targets.

Address
Department of Biochemistry, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Corresponding author: Plückthun, Andreas (plueckthun@bioc.uzh.ch)

Current Opinion in Structural Biology 2020, **60**:93–100

This review comes from a themed issue on **Proteins**

Edited by **Jan Steyaert** and **Todd Yeates**

https://doi.org/10.1016/j.sbi.2019.12.009

Introduction

For the understanding of almost any molecular process in life science the knowledge of the three-dimensional structure of biological macromolecules is indispensable. Diffraction methods, particularly X-ray crystallography and electron microscopy, are the prevailing techniques to obtain high resolution structural information. Both methods possess strengths and weaknesses. X-ray crystallography requires well-ordered crystals to amplify the diffracted waves, while electron microscopy requires a minimal particle size to identify molecular particles in electron micrographs. Both methods are largely complementary, because large macromolecules are typically more difficult to crystallize and small macromolecules are difficult to spot on images, favoring electron microscopy and X-ray crystallography for large and small macromolecules, respectively (reviewed in Ref. [1]).

One strategy to overcome both limitations would be to bind or fuse specific chaperone proteins to the target molecule. In X-ray crystallography, the thereby extended molecular surface offers additional opportunities to form crystal contacts, which improves the likelihood for obtaining crystals. Early crystallization chaperones that bind the target by non-covalent interactions were derived from natural binding proteins, such as antibody Fab fragments (reviewed in Refs. [2–4]). Initially, these were derived by immunizing lab animals, but *in vitro* selection methods from synthetic libraries have also been used in recent years. The antibody fragments from advanced synthetic libraries typically express much better as recombinant proteins than those from natural sources [5,6] — an important factor considering the protein amounts required for typical structural biology projects.

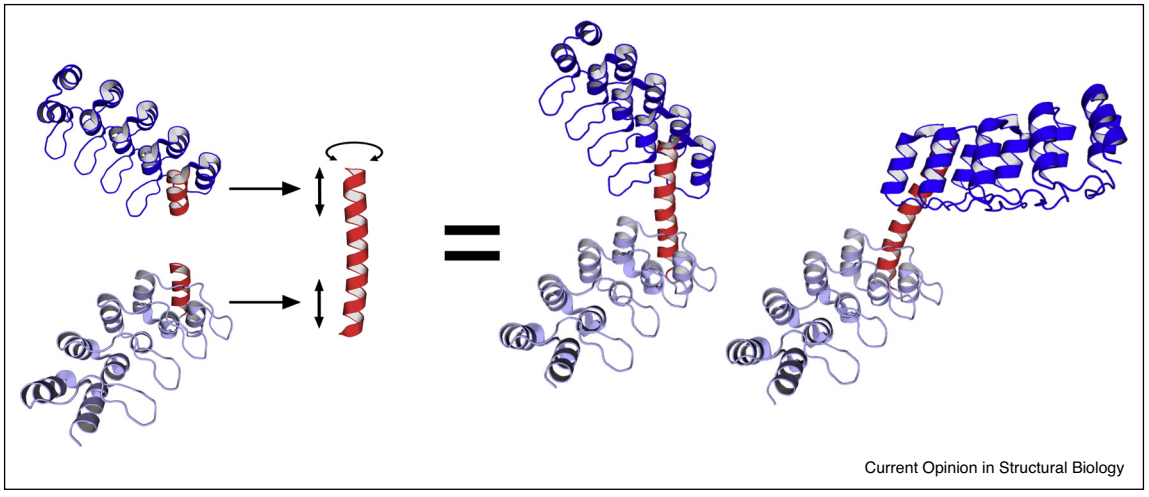
After the establishment of selection technologies from synthetic antibody libraries, the antibody molecule itself became dispensable. Thus, binders from alternative scaffolds, such as camelid antibody VHH domains ('nanobodies') [7,8], fibronectin type III domains ('monobodies') [9], lipocalins ('anticalins') [10], SH3 domains ('fynomers') [11], *Staphylococcus aureus* IgG-binding protein A domain Z ('affibodies') [12], and designed ankyrin repeat proteins (DARPins) were also used, the latter being the focus of this review [13,14].

What are DARPins?

Natural ankyrin repeat proteins interact with their targets on the concave surface, typically using a subset out of four to six individual ankyrin repeats. Considering this principle, highly stable consensus repeats were designed with self-complementary molecular surfaces that can be stacked in any combination, and are randomized in the potential interaction residues [15,16]. Each repeat folds into two antiparallel α-helices followed by a short β-turn (Figure 1). The stacked repeats form a superhelical solenoid protein, where the extended hydrophobic core is shielded by specific capping repeats on either side that provide a hydrophilic surface [17,18]. DARPins possess several beneficial traits to serve as crystallization chaperones and EM scaffolds: They can be robustly selected *in vitro* against virtually any folded protein target [14], and DARPins have also been made against particular DNA structures [19], peptides [20•] and small molecules [21]. DARPins are extremely stable against heat and chemical denaturants [22,23], they do not contain cysteine residues and they can be expressed at very high yield from bacterial expression cultures (reviewed in Ref. [14]).

Each internal ankyrin repeat comprises 33 residues, of which 27 framework residues are kept constant to ensure

Figure 1



Concept of rigid DARPin fusions with DARPin-DARPin fusions as an example.
Two DARPins (blue and light blue) can be connected rigidly by overlapping or fusing the terminal helices to a rigid, designed α-helical linker of variable length. This changes the relative orientation of the two DARPins with respect to each other. Examples shown are rigid fusions 5LE8 (left) and 5LEL (right) with a 15 or 10 amino acid long shared helix, respectively.

structural integrity and six residues that are potentially forming part of the paratope can be randomized. Two or three stacked internal repeats typically form the intearction surface to recognize a variety of target proteins [13]. Binders against almost any target can be selected from synthetic DARPin libraries by ribosome display in a high-throughput manner, selecting against 96 targets in parallel, typically resulting in a series of picomolar or low nanomolar binders, all with different sequences [24]. Ribosome display allows the panning of libraries with up to 10^{12} molecules, but other methods, such as phage display [25], yeast surface display [21], and SNAP display [26] have also been applied successfully.

DARPins and their applications in general have been previously reviewed [14,27], and specialized reviews of their applications in developmental biology [28], gene delivery [29], and as protein therapeutics [14,27] have been published. Several DARPins are in clinical development [30]. Here, we focus on the application of DARPins as structural chaperones, particularly in electron microscopy and X-ray crystallography. For NMR, working with DARPins is less straightforward, because the repetitive architecture makes the assignment of overlapping resonances difficult, but NMR has given unprecedented insight into the structural determinants of the extreme protein stability, and was thus instrumental in the further engineering of the DARPin platform [17,23,31].

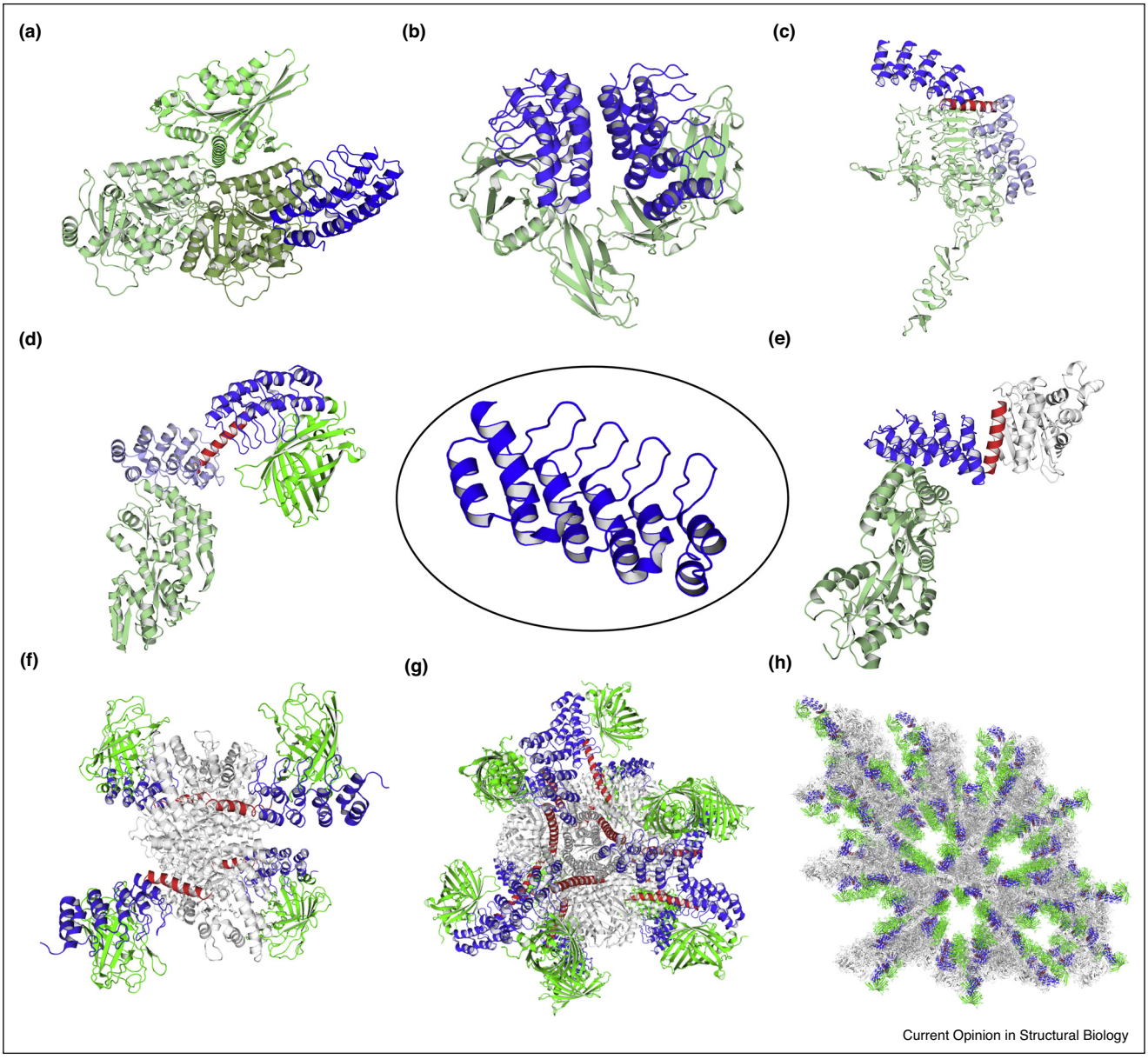
The early application of unmodified DARPins to help in the structure elucidation by X-ray crystallography has been previously reviewed [32], but subsequent engineering has increased the options considerably, and this will be one focus of this review (Figure 2). DARPin-target complexes have not only been made to enhance crystallization, or to stabilize a particular structural state for structural studies, but also to structurally understand biological effects. Since it is impossible to separate the impact of DARPins on crystallization from the biological effect, we review many of recent crystal structures of DARPins, regardless of whether the DARPin was used to enhance crystallization or to study the biological effect (or both). To identify these complexes, we searched the PDB database with the sequence of the full-consensus DARPin N3C [22] and looked at all entries from the past four years. It should be noted that an earlier summary of DARPin complexes has been published [33], and a few older ones will be mentioned for context.

Crystal structures of single DARPin complexes

Tubulin is a nice example of how DARPins enable the structural analysis of oligomerization-prone proteins (Figure 2a). The assembly of tubulin monomers into filaments is a process that competes with crystallization. DARPin D1, as well several other DARPins, prevents oligomerization [34]. Using the D1 co-crystallization strategy, several structures of ternary tubulin complexes have been determined that could explain the molecular details of nucleation, growth and disassembly of microtubules, even allowing data collection at room temperature by serial crystallography [35–40,41•].

The RAS protein is crucial for signal transduction and an attractive target to combat roughly 30% of all human cancers. Crystal structures of $KRAS^{G12V}$ in complex with DARPins K27/K55 and K13/K19 have been determined

Figure 2



DARPins as structural chaperones.
DARPins are in blue, rigid fusion partners in light blue (other DARPins) or white (non-DARPin fusions), bound targets in green (GFP) or light green/pale green (non-GFP targets) **(a)** DARPin binding tubulin (PDB-ID:4LNU). **(b)** Back-to-back DARPin dimer binding erythropoietin receptor (EpoR) (PDB-ID:6MOH). **(c)** DARPin-DARPin rigid fusion serving as a sterically sensitive structural probe for binding to the receptor Her2. **(d)** DARPin-DARPin rigid fusion binding MBP and GFP (PDB-ID:6LEM). **(e)** DARPin-β-lactamase fusion binding MBP (PDB-ID:5AQ9). **(f)** Aldolase-DARPin fusion as an EM-scaffold binding GFP (PDB-ID:6MWQ). **(g)** DARPin-cage fusion as an EM-scaffold binding GFP (PDB-ID: 6NHV and 6NHT). **(h)** EngBF-DARPin fusion as a crystal lattice host, binding GFP as the guest (PDB-ID 6SHA).

at high resolution. While the DARPins K27/K55 bind RAS in its inactive, GDP-bound or active, GTP-bound conformation, respectively, the DARPins K13 and K19 exclusively recognize the isoform KRAS, independently of the nucleotide bound. The specificity for just one isoform is mediated by the interaction with the allosteric lobe of KRAS, which exhibits most of the isoform-differentiating point mutations within the RAS G-domain. Interestingly, these different DARPins are thought to execute their cytotoxic effects by discrete modes of action, including the inhibition of GEF-mediated nucleotide exchange, downstream effector recruitment and RAS nanoclustering [42,43•]. Taking into account the amount of surface that is covered when the small RAS protein is bound by one of the

DARPins, it remains questionable whether there is really a single, discrete mode of action. Furthermore, another DARPin has been developed, which recognizes all RAS isoforms with similar affinity and shows only a slight preference for the active conformation (Kapp *et al.*, manuscript in preparation). In this case, the authors propose a combination of multiple modes of action.

One of the strategies to block signaling by a soluble protein is to generate a high-affinity binding protein that targets the same surface that interacts with the cognate receptor. This is typically the surface most prone for protein-protein interactions, by being more rigid and somewhat more hydrophobic. DARPins binding to interleukins (IL) 4 and 13 were generated that could impair IL signaling. The IL-13Rα1/IL-4Rα receptor pair binds IL-4 as well as IL-13. The crystal structures of IL-4:DARPin_44 and IL-13:DAPRin_6G9 complexes revealed that the DARPins recognize distinct epitopes: DARPin_44 binds IL-4 with picomolar affinity and mimics the interactions of the IL-4Rα receptor [44], whereas DARPin 6G9 prevents binding of IL-13Rα1, the second half of the receptor pair, to IL-13 [45]. Induced-fit movements were observed in both complexes, although all molecules were initially considered structurally stable.

DARPins have also been used to modulate the activity of enzymes, such as recently Cathepsin B [46], and in earlier work, various kinases [47–50] and other proteases [51,52]. Because DARPins can be expressed in the cytosol and fold there, they have become valuable reagents to study and influence enzymatic reactions in living cells.

Recently, a standardized procedure was developed to model DARPin:target complexes that can be used to predict the structures of unknown complexes, requiring only the sequence of a DARPin and a structure of the unbound target [53•]. For a set of diverse DARPin:target complexes tested, it generated a single model of the complex that well approximates the native state of the complex, as found in the crystal structure of the complexes.

DARPins targeting membrane receptors

Besides soluble proteins and polymerizing structural proteins, also membrane proteins have been targeted by DARPins to enhance crystallization. One target particularly well studied is the bacterial multidrug efflux pump AcrB (reviewed in Ref. [32]). Overexpression of this efflux pump is often observed in multi-drug resistance, making the AcrABZ-TolC complex an attractive target for the development of novel antibiotics. DARPins in complex with full-length AcrB and an AcrB fragment comprising the periplasmic domain have been used to crystallize the efflux pump in complex with fusidic acid and pyranopyridine-based inhibitors, respectively [54,55]. The combined study of AcrABZ-TolC by cryo-EM at 5.9 Å and AcrBZ:puromycin:DARPin by X-ray crystallography at 3.2 Å resolution gave rise to a better understanding of the deviation of AcrB from the three-fold symmetry upon ligand binding and cargo transport. DARPins bind AcrB with a 2:3 stoichiometry, exploiting the asymmetry of the transporting AcrB trimer, which is also seen by cryo-EM in the absence of DARPins [56].

Membrane-anchored receptors have been targeted in different ways: Steric interference of monomeric DARPins with dimerizing receptors can inhibit signaling, as shown, for example, for the vascular endothelial growth factor [57]. In contrast, in the case of hepatocyte growth factor receptor (c-MET), different bi-paratopic DARPins crosslink the receptors in an inactive state, thereby inhibiting MET kinase activity and downstream signaling [58]. The latter application follows a strategy previously developed for HER2 [59–61] that is now being translated to the clinic in a similar form.

In the case of the Frizzled family of Wnt receptors the combination of *in silico* design followed by experimental selection with yeast display was instrumental for deriving subtype-specific DARPin binders [62••]. In a very elegant study, several DARPin complex structures were used to guide the topological tuning of the erythropoietin receptor (EpoR). The anti-EpoR DARPin E2 was dimerized and E2 dimers were subsequently used to impose different angles and distances between EpoR extracellular domains. Crystal structures of EpoR:E2 dimer complexes allowed a correlation of the spatial orientation of EpoR domains with receptor activity in human erythroid cells [63••] (Figure 2b).

DARPin fusions expand the structural repertoire

DARPins have a unique architecture that makes the creation of versatile rigid fusion proteins possible. The N-termini and C-termini of DARPins form α-helices, permitting rigid fusions of DARPins to any other helical scaffold by extending (and/or ovelapping) these helices (the 'shared helix approach') (Figure 1). The 'shared helix approach' was initially developed for the design of self-assembling protein oligomers [64]. This design strategy was later improved to engineer the helix to contact both domains simultaneously and thus to minimize any bending motion, and it was used for the design of monomeric binding proteins extended by a well-crystallizing domain in different orientations [65]. In DARPins, the termini are lateral to the binding site (on either side), permitting a variety of architectures that are fully compatible with target binding. Rigidity is one key advantage of this concept, of essence in structural biology, but it can also be exploited in functional studies. In addition, the 'shared helix approach' allows to control the directionality of the fused scaffolds, for example, the orientation of epitopes relative to each other. When designed properly, the helix at least partially contacts, at the same time, both protein domains to be joined, a feature essential for rigidity, and this requires the helix sequence to be adjusted to allow this.

A long 'flagpole' helix, where the two helices are simply consecutively joined, leads to a non-rigid fusion. These considerations are explained in the first paper describing this strategy [65].

Crystallization chaperones were the first DARPin-based fusion proteins where this concept was applied, as the versatility of the concept allows not only to fuse the binding DARPin to different well-crystallizing fusion proteins, but each one of them also in many orientations, thereby creating a very large number of new crystallization interfaces. This has greatly extended the utility of DARPins as crystallization chaperones. Furthermore, the same strategy has been highly useful in developing rigidly fused DARPins as functional tools in other applications (see below).

The first example for such a rigid fusion was the extension of the molecular surface of the DARPin named 'off 7', which binds to maltose binding protein (MBP), by fusing its C-terminus to the N-terminal alpha-helix of TEM-1 β-lactamase as a robust, well folding and well crystallizing monomeric protein [65] (Figure 2e). Six different constructs with different linking geometries were generated by inserting additional residues in the shared helix, which has to be adjusted in sequence, as it now contacts both proteins with part of its surface. Some of them were crystallized in complex with maltose-binding protein, the target of the DARPin [65], and the designed structures were largely confirmed. Subequently, a similar strategy was applied to fuse the C-terminus of DARPin off7 to the N-terminus of protein A [66,67].

For use as a crystallization chaperone, it was particularly advantageous to fuse the DARPin D12 to the target-binding DARPin, as D12 was found to have unusual properties [68•]. The DARPin D12 paratope frequently makes versatile crystal contacts. The interactions of DARPin D12 are weak enough for constructs containing this DARPin to remain monomeric during expression and purification. Upon crystallization, the paratope-paratope interactions of this DARPin are strong enough to form prominent crystal contacts under a wide variety of conditions and in different geometries. This strategy was used to determine the structures of designed repeat proteins recalcitrant to yielding well diffracting crystals, such as in the case of BRIC2 and an Armadillo repeat protein [69,70], by exploiting the possibility of creating different fusion geometries. Several more examples are currently being prepared for publication, including G protein-coupled receptors (Deluigi *et al.*, in preparation). DARPin D12 may thus be added to the portfolio of well crystallizing proteins such as, for example, T4 lysozyme, apo-cytochrome B(562)RIL and several others, yet with the additional advantage of being able to be fused to other proteins in a rigid manner and thus be part of binding complexes. The ability to adjust the directionality of DARPin epitopes adds another dimension in crystallization screens.

Furthermore, the rigid fusion approach can be utilized to build crystallization scaffolds that serve defined purposes, like shielding a binding surface from influencing crystal contacts. This strategy was applied to determine the structure of designed Armadillo repeat proteins, to keep their peptide-binding site undisturbed by crystal contacts [70].

Since the rigid-fusion approach with a shared helix is a facile and versatile way to connect two proteins in a defined way, DARPins were also used to determine the structures of other biomolecules in a novel host:guest approach. For this purpose, the DARPin was inserted in an existing crystal lattice. Normally, crystallization chaperones do not eliminate the need to search for crystallization conditions — they merely increase the chance of finding suitable ones. To solve this last problem, a DARPin fusion protein was developed that crystallizes under predictable conditions. The bacterial protein EngBF builds a porous but well diffracting crystal lattice. The EngBF lattice permits rigid fusions to a specific DARPin and the so created EngBF-DARPin host lattice allows site-specific recruitment of a guest molecule [20•] (Figure 2g). This method, designated host-lattice display, was used to determine the structures of short peptides. In principle, the EngBF-DARPin host lattice has been shown to be capable of accommodating targets up to 40 kDa [20•], but currently those targets still show high B-factors and rather weak electron density, having motivated ongoing further redesign to rigidify the positioning of the guest.

DARPin-DARPin rigid fusions

To extend this strategy further, target-binding DARPins themselves that are helical on either side can be rigidly connected with adjustable geometries to other DARPins (D12 or other binders) by the shared helix concept (Figure 1). When fused to a binding DARPin with nine different connector molecules, eight of them could be crystallized and the molecular design could thus be confirmed [68•]. One recent example where this strategy of crystallizing with DARPin-DARPin rigid fusions (of a binding DARPin with DARPin D12) was applied is the c-Jun N-terminal kinase 1 [50], for which a simple monomeric DARPin never resulted in crystals of the complex.

Furthermore, the development of multivalent DARPin chains further extends the initial concept of DARPin-fusion chaperones, because each DARPin domain can not only be a well-crystallizing entity like DARPin D12, but additionally be directed against a separate crystallization enhancer like MBP or GFP. Both tags are frequently used in protein biochemistry; MBP to improve solubility and GFP to visualize the target, and both typically crystallize well. Tight-binding DARPins against MBP and GFP derivatives and homologs, such as sfGFP and teal fluorescent protein 1, have been developed and co-crystallized with the targets [71,72]. Human dual-specificity phosphatase 1 that failed to crystallize as an MBP fusion protein only crystallized readily in complex with DARPin

off7, binding to the fused MBP [73]. DARPins off7 and 3G124 have been inserted in DARPin-DARPin rigid fusions, and crystal structures in complex with MBP and GFP were determined [68•] (Figure 2d).

Polyvalent DARPin-DARPin rigid fusions therefore serve as molecular probes with precisely defined geometries, and are thus useful not only to improve crystallization, but also to investigate the spatial restraints for receptor signaling, for example, HER2 [74]. Here, a HER2-binding DARPin was fused to a non-binding DARPin in two different geometries, and with the differential binding of the two fusions it could be shown that HER2 in the cell membrane does not assume a tethered conformation to any detectable amount — unlike all other EGFR family members [74] (Figure 2c).

DARPin fusions for cryo-EM

The necessity to amplify the signal of the diffracted waves by a crystalline arrangement of molecules limits the application of protein crystallography. Single particle cryo-EM doesn't have this limitation, but it is currently restricted to molecules larger than 40–60 kDa (mass of the particle, that is, monomer or oligomeric assembly). DARPins were also instrumental to shift this limit further down. Using the rigid helix fusion strategy described above, DARPins were linked to a large oligomeric, symmetric assembling unit. An artificial cage protein with tetrahedral symmetry and $A_{12}B_{12}$ stoichiometry was selected as the assembling unit and the DARPin was fused to subunit A, which generated 12 identical target binding sites. 3D image analysis and symmetry averaging yielded a 3.1 Å resolution structure of this EM chaperone [75]. To show that this DARPin-cage fusion protein is useful for determining structures of small proteins, the structure of the 26 kDa protein GFP was determined with a resolution overall and for the target of 3.5 Å and 3.8 Å, respectively [76•] (Figure 2g). In parallel, the cryo-EM structure of GFP was determined by an alternative DARPin-based design. In this systematic approach, six different assembling units were tested [77•]. Finally, the DARPin was fused to the N-terminus of aldolase, a protein that shows D2 tetrameric symmetry (Figure 2f). This EM chaperone was also used to determine the structure of GFP with resolutions of 3 Å and 4–8 Å for the aldolase and GFP:DARPin domains, respectively.

In the cage-based assembling unit there are three DARPin/target complexes located around the vertices, which provides more data for structure averaging, albeit at the expense of steric hindrance between them. In the aldolase-based assembling unit there is more space for the target but fewer copies for averaging, which probably causes the lower resolution of the target. Currently, a number of other oligomeric designs are under development.

Conclusions

Since their development, artificial binding molecules from the DARPin architecture have now matured into very useful tools for protein biochemistry, including structural biology — besides their use in the clinic. Several crystal structures of DARPin:target complexes show that even naked monomeric DARPins are useful for solving issues in crystallization, but fusing the DARPin via a rigid helix to other robust domains significantly expands their functionality in a very versatile manner. This strategy allows researchers already to tackle some fundamental challenges in structural biology, such as the crystallization problem in X-ray diffraction and the size problem in cryo-EM, and further design approaches are on the way to further build on the versatile DARPin platform.

Conflict of interest statement

Nothing declared.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Muench SP, Antonyuk SV, Hasnain SS: **The expanding toolkit for structural biology: synchrotrons, X-ray lasers and cryoEM**. *IUCrJ* 2019, **6**:167-177.
2. Prongay AJ, Smith TJ, Rossmann MG, Ehrlich LS, Carter CA, McClure J: **Preparation and crystallization of a human immunodeficiency virus p24-Fab complex**. *Proc Natl Acad Sci U S A* 1990, **87**:9980-9984.
3. Kovari LC, Momany C, Rossmann MG: **The use of antibody fragments for crystallization and structure determinations**. *Structure* 1995, **3**:1291-1293.
4. Hunte C, Michel H: **Crystallisation of membrane proteins mediated by antibody fragments**. *Curr Opin Struct Biol* 2002, **12**:503-508.
5. Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellnhofer G, Hoess A, Wolle J, Plückthun A, Virnekäs B: **Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides**. *J Mol Biol* 2000, **296**:57-86.
6. Ewert S, Huber T, Honegger A, Plückthun A: **Biophysical properties of human antibody variable domains**. *J Mol Biol* 2003, **325**:531-553.
7. Yan J, Li G, Hu Y, Ou W, Wan Y: **Construction of a synthetic phage-displayed Nanobody library with CDR3 regions randomized by trinucleotide cassettes for diagnostic applications**. *J Transl Med* 2014, **12**:343.
8. Yu X, Yang YP, Dikici E, Deo SK, Daunert S: **Beyond antibodies as binding partners: the role of antibody mimetics in bioanalysis**. *Annu Rev Anal Chem* 2017, **10**:293-320.
9. Koide A, Abbatiello S, Rothgery L, Koide S: **Probing protein conformational changes in living cells by using designer binding proteins: application to the estrogen receptor**. *Proc Natl Acad Sci U S A* 2002, **99**:1253-1258.
10. Schonfeld D, Matschiner G, Chatwell L, Trentmann S, Gille H, Hulsmeyer M, Brown N, Kaye PM, Schlehuber S, Hohlbaum AM *et al.*: **An engineered lipocalin specific for CTLA-4 reveals a combining site with structural and conformational features similar to antibodies**. *Proc Natl Acad Sci U S A* 2009, **106**:8198-8203.

11. Grabulovski D, Kaspar M, Neri D: **A novel, non-immunogenic Fyn SH3-derived binding protein with tumor vascular targeting properties**. *J Biol Chem* 2007, **282**:3196-3204.

12. Nygren PA: **Alternative binding proteins: affibody binding proteins developed from a small three-helix bundle scaffold**. *FEBS J* 2008, **275**:2668-2676.

13. Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, Forrer P, Grütter MG, Plückthun A: **High-affinity binders selected from designed ankyrin repeat protein libraries**. *Nat Biotechnol* 2004, **22**:575-582.

14. Plückthun A: **Designed ankyrin repeat proteins (DARPins): binding proteins for research, diagnostics, and therapy**. *Annu Rev Pharmacol Toxicol* 2015, **55**:489-511.

15. Binz HK, Stumpp MT, Forrer P, Amstutz P, Plückthun A: **Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins**. *J Mol Biol* 2003, **332**:489-503.

16. Forrer P, Stumpp MT, Binz HK, Plückthun A: **A novel strategy to design binding molecules harnessing the modular nature of repeat proteins**. *FEBS Lett* 2003, **539**:2-6.

17. Interlandi G, Wetzel SK, Settanni G, Plückthun A, Caflisch A: **Characterization and further stabilization of designed ankyrin repeat proteins by combining molecular dynamics simulations and experiments**. *J Mol Biol* 2008, **375**:837-854.

18. Kramer MA, Wetzel SK, Plückthun A, Mittl PRE, Grütter MG: **Structural determinants for improved stability of designed ankyrin repeat proteins with a redesigned C-capping module**. *J Mol Biol* 2010, **404**:381-391.

19. Scholz O, Hansen S, Plückthun A: **G-quadruplexes are specifically recognized and distinguished by selected designed ankyrin repeat proteins**. *Nucleic Acids Res* 2014, **42**:9182-9194.

20. • Ernst P, Plückthun A, Mittl PRE: **Predictable protein crystallisation by host/guest crystal lattice engineering**. *Sci Rep* 2019, **9**:15199
DARPins were inserted in different orientations into the EngBF crystal lattice. The EngBF-DARPin fusion protein crystallizes under predictable conditions and independently of the target (guest) molecule

21. Schütz M, Batyuk A, Klenk C, Kummer L, de Picciotto S, Gulbakan B, Wu Y, Newby GA, Zosel F, Schöppe J *et al.*: **Generation of fluorogen-activating designed ankyrin repeat proteins (FADAs) as versatile sensor tools**. *J Mol Biol* 2016, **428**:1272-1289.

22. Merz T, Wetzel SK, Firbank S, Plückthun A, Grütter MG, Mittl PRE: **Stabilizing ionic interactions in a full-consensus ankyrin repeat protein**. *J Mol Biol* 2008, **376**:232-240.

23. Wetzel SK, Settanni G, Kenig M, Binz HK, Plückthun A: **Folding and unfolding mechanism of highly stable full-consensus ankyrin repeat proteins**. *J Mol Biol* 2008, **376**:241-257.

24. Hanes J, Plückthun A: **In vitro selection and evolution of functional proteins by using ribosome display**. *Proc Natl Acad Sci U S A* 1997, **94**:4937-4942.

25. Steiner D, Forrer P, Stumpp MT, Plückthun A: **Signal sequences directing cotranslational translocation expand the range of proteins amenable to phage display**. *Nat Biotechnol* 2006, **24**:823-831.

26. Houlihan G, Gatti-Lafranconi P, Kaltenbach M, Lowe D, Hollfelder F: **An experimental framework for improved selection of binding proteins using SNAP display**. *J Immunol Methods* 2014, **405**:47-56.

27. Boersma YL: **Advances in the application of designed ankyrin repeat proteins (DARPins) as research tools and protein therapeutics**. *Methods Mol Biol* 2018, **1798**:307-327.

28. Harmansa S, Affolter M: **Protein binders and their applications in developmental biology**. *Development* 2018, **145**:dev148874.

29. Buchholz CJ, Friedel T, Büning H: **Surface-engineered viral vectors for selective and cell type-specific gene delivery**. *Trends Biotechnol* 2015, **33**:777-790.

30. Rao L, De Veirman K, Giannico D, Saltarella I, Desantis V, Frassanito MA, Solimando AG, Ribatti D, Prete M, Harstrick A *et al.*: **Targeting angiogenesis in multiple myeloma by the VEGF and HGF blocking DARPin® protein MP0250: a preclinical study**. *Oncotarget* 2018, **9**:13366-13381.

31. Wetzel SK, Ewald C, Settanni G, Jurt S, Plückthun A, Zerbe O: **Residue-resolved stability of full-consensus ankyrin repeat proteins probed by NMR**. *J Mol Biol* 2010, **402**:241-258.

32. Bukowska MA, Grütter MG: **New concepts and aids to facilitate crystallization**. *Curr Opin Struct Biol* 2013, **23**:409-416.

33. Jost C, Plückthun A: **Engineered proteins with desired specificity: DARPins, other alternative scaffolds and bispecific IgGs**. *Curr Opin Struct Biol* 2014, **27**:102-112.

34. Cao L, Wang W, Jiang Q, Wang C, Knossow M, Gigant B: **The structure of apo-kinesin bound to tubulin links the nucleotide cycle to movement**. *Nat Commun* 2014, **5**:5364.

35. Sharma A, Aher A, Dynes NJ, Frey D, Katrukha EA, Jaussi R, Grigoriev I, Croisier M, Kammerer RA, Akhmanova A *et al.*: **Centriolar CPAP/SAS-4 imparts slow processive microtubule growth**. *Dev Cell* 2016, **37**:362-376.

36. Weinert T, Olieric N, Cheng R, Brunle S, James D, Ozerov D, Gashi D, Vera L, Marsh M, Jaeger K *et al.*: **Serial millisecond crystallography for routine room-temperature structure determination at synchrotrons**. *Nat Commun* 2017, **8**:542.

37. Zheng X, Ramani A, Soni K, Gottardo M, Zheng S, Ming Gooi L, Li W, Feng S, Mariappan A, Wason A *et al.*: **Molecular basis for CPAP-tubulin interaction in controlling centriolar and ciliary length**. *Nat Commun* 2016, **7**:11874.

38. Wang W, Cantos-Fernandes S, Lv Y, Kuerban H, Ahmad S, Wang C, Gigant B: **Insight into microtubule disassembly by kinesin-13s from the structure of Kif2C bound to tubulin**. *Nat Commun* 2017, **8**:70.

39. Trofimova D, Paydar M, Zara A, Talje L, Kwok BH, Allingham JS: **Ternary complex of Kif2A-bound tandem tubulin heterodimers represents a kinesin-13-mediated microtubule depolymerization reaction intermediate**. *Nat Commun* 2018, **9**:2628.

40. Nithianantham S, Cook BD, Beans M, Guo F, Chang F, Al-Bassam J: **Structural basis of tubulin recruitment and assembly by microtubule polymerases with tumor overexpressed gene (TOG) domain arrays**. *eLife* 2018, **7**: e38922.

41. • Campanacci V, Urvoas A, Cantos-Fernandes S, Aumont-Nicaise M, Arteni AA, Velours C, Valerio-Lepiniec M, Dreier B, Plückthun A, Pilon A *et al.*: **Insight into microtubule nucleation from tubulin-capping proteins**. *Proc Natl Acad Sci U S A* 2019, **116**:9859-9864
The kinetically unfavorable process of nucleation is controlled by inhibiting proteins, and has been structurally and kinetically studied with natural and synthetic binding proteins, such as DARPins, that interact with protofilaments at either end. Interference is shown to occur at the (+) end.

42. Guillard S, Kolasinska-Zwierz P, Debreczeni J, Breed J, Zhang J, Bery N, Marwood R, Tart J, Overman R, Stocki P *et al.*: **Structural and functional characterization of a DARPin which inhibits Ras nucleotide exchange**. *Nat Commun* 2017, **8**:16111.

43. • Bery N, Legg S, Debreczeni J, Breed J, Embrey K, Stubbs C, Kolasinska-Zwierz P, Barrett N, Marwood R, Watson J *et al.*: **KRAS-specific inhibition using a DARPin binding to a site in the allosteric lobe**. *Nat Commun* 2019, **10**:2607
Some DARPins specifically inhibit KRAS/effector interactions and block the downstream signalling pathways in cancer cells.

44. Obmolova G, Teplyakov A, Malia TJ, Keough E, Luo J, Sweet R, Jacobs SA, Yi F, Hippensteel R, O'Neil KT *et al.*: **Induced conformational change in human IL-4 upon binding of a signal-neutralizing DARPin**. *Proteins* 2015, **83**:1191-1197.

45. Teplyakov A, Malia TJ, Obmolova G, Jacobs SA, O'Neil KT, Gilliland GL: **Conformational flexibility of an anti-IL-13 DARPin**. *Protein Eng Des Sel* 2017, **30**:31-37.

46. Kramer L, Renko M, Zavrsnik J, Turk D, Seeger MA, Vasiljeva O, Grütter MG, Turk V, Turk B: **Non-invasive in vivo imaging of**

tumour-associated cathepsin B by a highly selective inhibitory DARPin. *Theranostics* 2017, **7**:2806-2821.

47. Kummer L, Parizek P, Rube P, Millgramm B, Prinz A, Mittl PRE, Kaufholz M, Zimmermann B, Herberg FW, Plückthun A: **Structural and functional analysis of phosphorylation-specific binders of the kinase ERK from designed ankyrin repeat protein libraries**. *Proc Natl Acad Sci U S A* 2012, **109**:2248-2257.

48. Parizek P, Kummer L, Rube P, Prinz A, Herberg FW, Plückthun A: **Designed ankyrin repeat proteins (DARPins) as novel isoform-specific intracellular inhibitors of c-Jun N-terminal kinases**. *ACS Chem Biol* 2012, **7**:1356-1366.

49. Kummer L, Hsu CW, Dagliyan O, MacNevin C, Kaufholz M, Zimmermann B, Dokholyan NV, Hahn KM, Plückthun A: **Knowledge-based design of a biosensor to quantify localized ERK activation in living cells**. *Chem Biol* 2013, **20**:847-856.

50. Wu YF, Honegger A, Batyuk A, Mittl PRE, Plückthun A: **Structural basis for the selective inhibition of c-Jun N-terminal kinase 1 determined by rigid DARPin-DARPin fusions**. *J Mol Biol* 2018, **430**:2128-2138.

51. Kawe M, Forrer P, Amstutz P, Plückthun A: **Isolation of intracellular proteinase inhibitors derived from designed ankyrin repeat proteins by genetic screening**. *J Biol Chem* 2006, **281**:40252-40263.

52. Schweizer A, Roschitzki-Voser H, Amstutz P, Briand C, Gulotti-Georgieva M, Prenosil E, Binz HK, Capitani G, Baici A, Plückthun A *et al.*: **Inhibition of caspase-2 by a designed ankyrin repeat protein: specificity, structure, and inhibition mechanism**. *Structure* 2007, **15**:625-636.

53. • Radom F, Paci E, Plückthun A: **Computational modeling of designed ankyrin repeat protein complexes with their targets**. *J Mol Biol* 2019, **431**:2852-2868

A docking procedure has been developed which allowed seven out of seven DARPin:target complexes to result in near-native models as the top-scoring model, exploiting the rigid paratope of the DARPin.

54. Oswald C, Tam HK, Pos KM: **Transport of lipophilic carboxylates is mediated by transmembrane helix 2 in multidrug transporter AcrB**. *Nat Commun* 2016, **7**:13819.

55. Sjuts H, Vargiu AV, Kwasny SM, Nguyen ST, Kim HS, Ding X, Ornik AR, Ruggerone P, Bowlin TL, Nikaido H *et al.*: **Molecular basis for inhibition of AcrB multidrug efflux pump by novel and powerful pyranopyridine derivatives**. *Proc Natl Acad Sci U S A* 2016, **113**:3509-3514.

56. Wang Z, Fan G, Hryc CF, Blaza JN, Serysheva II, Schmid MF, Chiu W, Luisi BF, Du D: **An allosteric transport mechanism for the AcrAB-TolC multidrug efflux pump**. *eLife* 2017, **6**:e24905.

57. Thieltges KM, Avramovic D, Piscitelli CL, Markovic-Mueller S, Binz HK, Ballmer-Hofer K: **Characterization of a drug-targetable allosteric site regulating vascular endothelial growth factor signaling**. *Angiogenesis* 2018, **21**:533-543.

58. Andres F, Iamele L, Meyer T, Stüber JC, Kast F, Gherardi E, Niemann HH, Plückthun A: **Inhibition of the MET kinase activity and cell growth in MET-addicted cancer cells by bi-paratopic linking**. *J Mol Biol* 2019, **431**:2020-2039.

59. Jost C, Schilling J, Tamaskovic R, Schwill M, Honegger A, Plückthun A: **Structural basis for eliciting a cytotoxic effect in HER2-overexpressing cancer cells via binding to the extracellular domain of HER2**. *Structure* 2013, **21**:1979-1991.

60. Tamaskovic R, Schwill M, Nagy-Davidescu G, Jost C, Schaefer DC, Verdurmen WP, Schaefer JV, Honegger A, Plückthun A: **Intermolecular biparatopic trapping of ErbB2 prevents compensatory activation of PI3K/AKT via RAS-p110 crosstalk**. *Nat Commun* 2016, **7**:11672.

61. Schwill M, Tamaskovic R, Gajadhar AS, Kast F, White FM, Plückthun A: **Systemic analysis of tyrosine kinase signaling reveals a common adaptive response program in a HER2-positive breast cancer**. *Sci Signal* 2019, **12**:eaau2875.

62. •• Dang LT, Miao Y, Ha A, Yuki K, Park K, Janda CY, Jude KM, Mohan K, Ha N, Vallon M *et al.*: **Receptor subtype discrimination using extensive shape complementary designed interfaces**. *Nat Struct Mol Biol* 2019, **26**:407-414

DARPins were computationally designed and refined by directed evolution to distinguish surface differences between Frizzled (Fz) receptor subtype. The selectivity was also observed *in vivo*.

63. •• Mohan K, Ueda G, Kim AR, Jude KM, Fallas JA, Guo Y, Hafer M, Miao Y, Saxton RA, Piehler J *et al.*: **Topological control of cytokine receptor signaling induces differential effects in hematopoiesis**. *Science* 2019, **364**:eaav7532

DARPins have been used to control EpoR dimerization by creating rigid homodimers in different angles, corroborated by several ligand-EpoR complex crystal structures. This has been used to modulate cytokine receptor signaling output.

64. Padilla JE, Colovos C, Yeates TO: **Nanohedra: using symmetry to design self assembling protein cages, layers, crystals, and filaments**. *Proc Natl Acad Sci U S A* 2001, **98**:2217-2221.

65. Batyuk A, Wu Y, Honegger A, Heberling MM, Plückthun A: **DARPin-based crystallization chaperones exploit molecular geometry as a screening dimension in protein crystallography**. *J Mol Biol* 2016, **428**:1574-1588.

66. Jeong WH, Lee H, Song DH, Eom JH, Kim SC, Lee HS, Lee H, Lee JO: **Connecting two proteins using a fusion alpha helix stabilized by a chemical cross linker**. *Nat Commun* 2016, **7**:11031.

67. Youn SJ, Kwon NY, Lee JH, Kim JH, Choi J, Lee H, Lee JO: **Construction of novel repeat proteins with rigid and predictable structures using a shared helix method**. *Sci Rep* 2017, **7**:2595.

68. • Wu Y, Batyuk A, Honegger A, Brandl F, Mittl PRE, Plückthun A: **Rigidly connected multispecific artificial binders with adjustable geometries**. *Sci Rep* 2017, **7**:11217

General strategy to rigidly fuse DARPins to each other in different orientations, verified by crystal structures. This paper also reports DARPin D12, which has favorable properties that facilitate crystal contacts in many orientations.

69. ElGamacy M, Coles M, Ernst P, Zhu HB, Hartmann MD, Plückthun A, Lupas AN: **An interface-driven design strategy yields a novel, corrugated protein architecture**. *ACS Synth Biol* 2018, **7**:2226-2235.

70. Ernst P, Honegger A, van der Valk F, Ewald C, Mittl PRE, Plückthun A: **Rigid fusions of DARPins and dArmRP efficiently protect a binding surface crystal contacts**. *Sci Rep* 2019, **9**:16162.

71. Hansen S, Stüber JC, Ernst P, Koch A, Bojar D, Batyuk A, Plückthun A: **Design and applications of a clamp for Green Fluorescent Protein with picomolar affinity**. *Sci Rep* 2017, **7**:16292.

72. Vigano MA, Bieli D, Schaefer JV, Jakob RP, Matsuda S, Maier T, Plückthun A, Affolter M: **DARPins recognizing mTFP1 as novel reagents for in vitro and in vivo protein manipulations**. *Biol Open* 2018, **7**:bio036749.

73. Gumpena R, Lountos GT, Waugh DS: **MBP-binding DARPins facilitate the crystallization of an MBP fusion protein**. *Acta Crystallogr F Struct Biol Commun* 2018, **74**:549-557.

74. Jost C, Stüber JC, Honegger A, Wu Y, Batyuk A, Plückthun A: **Rigidity of the extracellular part of HER2: evidence from engineering subdomain interfaces and shared-helix DARPin-DARPin fusions**. *Protein Sci* 2017, **26**:1796-1806.

75. Liu Y, Gonen S, Gonen T, Yeates TO: **Near-atomic cryo-EM imaging of a small protein displayed on a designed scaffolding system**. *Proc Natl Acad Sci U S A* 2018, **115**:3362-3367.

76. • Liu Y, Huynh DT, Yeates TO: **A 3.8 Å resolution cryo-EM structure of a small protein bound to an imaging scaffold**. *Nat Commun* 2019, **10**:1864

GFP is bound to an anti-GFP DARPin which is symmetrically oligomerized by fusion to a synthetic cage complex, facilitating cryo-EM structure determination of GFP.

77. • Yao Q, Weaver SJ, Mock JY, Jensen GJ: **Fusion of DARPin to aldolase enables visualization of small protein by cryo-EM**. *Structure* 2019, **27**:1148-1155

GFP is bound to an anti-GFP DARPin which is symmetrically oligomerized by fusion to aldolase, facilitating cryo-EM structure determination of GFP.