# Affibody- and DARPin-Conjugated Nanomaterials in Cancer Therapy

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Contributor: Federica Gabriele , Marta Palerma , Rodolfo Ippoliti , Francesco Angelucci , Giuseppina Pitari , Matteo Ardini

Affibodies and designed ankyrin repeat proteins (DARPins) are synthetic proteins originally derived from the *Staphylococcus aureus* virulence factor protein A and the human ankyrin repeat proteins, respectively. The use of these molecules in healthcare has been recently proposed as they are endowed with biochemical and biophysical features heavily demanded to target and fight diseases, as they have a strong binding affinity, solubility, small size, multiple functionalization sites, biocompatibility, and are easy to produce; furthermore, impressive chemical and thermal stability can be achieved, especially when using affibodies. In this sense, several examples reporting on affibodies and DARPins conjugated to nanomaterials have been published, demonstrating their suitability and feasibility in nanomedicine for cancer therapy.

affibodies DARPins cancer therapy targeting delivery nanoparticles liposomes proteins DNA

## 1. Introduction

Therapies based on antibodies (Abs) are undoubtedly pivotal in several fields, including cancer treatments. Abs, especially monoclonals, have entered the mainstream for their use in the targeted delivery of chemotherapeutic agents and to manipulate anticancer immune responses. It is not surprising, therefore, that the number of approved Abs-based therapies is growing worldwide, propelling their clinical relevance [1][2][3]. In parallel, advances in nanotechnology to produce several types of nanomaterials have revolutionized nanomedicine for their small size, customizable surfaces, solubility, and biocompatibility, which make them able to interact with biological surfaces. For this purpose, biological macromolecules, particularly Abs, have been used as ligands to create advanced, smart hybrid nanomaterials addressed to therapeutic approaches [4].

However, although Abs exhibit strong binding and high selectivity toward the target epitopes and endless engineering possibilities, they also come with undesired drawbacks for applied purposes. Namely, Abs are large, bivalent, and multidomain proteins showing intramolecular oxidized cysteines forming disulfide bonds and often a glycosylation pattern. These features lead to relatively poor thermal and chemical stability. In addition, Abs only use the small complementarity-determining regions (CDRs) to interact with the antigen, and in some cases, the high cost of manufacturing at a large scale has been identified due to the complexity in producing the full Ab. This

problem and the potential difficulty in penetrating solid tissues for cancer therapy justified the need for engineered derivatives with reduced size and composition [5].

In this context, other biological molecules with affinity properties toward ligands have been identified as valid alternatives to Abs. Nonantibody-binding proteins with low molecular weight have been identified and proposed as valuable tools and are currently being designed with improved properties. These antibody-mimicking molecules are grouped into two categories according to the location of the amino acid residues that mediate the binding to the ligands: those where the binding occurs via exposed, unstructured loops, and those where the interactions involve secondary structures, usually α-helices [5]. Among all, the so-called “affibodies” and “designed ankyrin repeat proteins (DARPins)”, both belonging to the second category, are the most representative of therapeutic means [1][3][6][7]. These affinity proteins have become invaluable tools in the development of next-generation therapeutics in vitro and in vivo for their unique biophysical and biochemical properties (see next paragraph), and their suitability in several applications is now well-established. For instance, affibodies can be easily designed with combined protein engineering approaches resulting in small and robust protein scaffolds showing favorable folding and stability. Moreover, affibodies encompass only 13 amino acid positions that differ between binding members and therefore much of the knowledge to manipulate and functionalize these proteins is known [8][9]. A few examples can be recalled that highlight the importance of affibodies and DARPins in several biomedical applications that include both therapeutics [10][11][12] and imaging agents for tumors [13][14].

Considering such high versatility and biological activity, it is not surprising that nanomaterials have also been functionalized with both affibodies and DARPins to create hybrid and advanced structures for targeted delivery in vitro and in vivo [15]. Note, in some cases, they gave even more efficient results than immunoglobulins (Igs)-based approaches [15].

# 2. Structural and Biochemical Features of Affibodies and DARPins

## 2.1. Affibodies

In 1984, the amino acid sequence of the virulence factor from *Staphylococcus aureus* called protein A (SpA) was published, unveiling five highly homologous domains A−E that encompassed 58 amino acid residues each [16]. These domains lacked cysteines and have been found to bind to Igs with high affinity [17][18][19]. The structural characterization provided by nuclear magnetic resonance revealed a simple bundle of three α-helices [20][21].

The SpA protein represents the precursor of affibodies, a new class of small, high-affinity Igs-binding proteins. The first affibody, named Z-domain, has been realized by mutating key amino acids of the SpA B-domain resulting in enhanced chemical stability and preserving the binding affinity. Furthermore, it showed enhanced resistance against low pH [22] and the typical native three-helix bundle [23][24] (**Figure 1**a). The Z-domain is a 58 amino acid molecule with an approximately 6.5 kDa molecular weight. It has been used to generate all known affibody libraries by combined mutations able to interact with various molecular targets. Examples of targets are Taq DNA

polymerase, human insulin, and human apolipoprotein, showing $K_D$ affinities in the μM range [25][26]. Moreover, affibodies targeting the epidermal growth factor receptor 2 (HER2), tumor necrosis factor α (TNFα), insulin and the platelet-derived growth factor receptor (PDGFR), and showing very high melting temperatures and $K_D$ down to pM and fM were also realized [27][28]. Furthermore, other biochemical and biophysical aspects, such as the folding kinetics of the three-helix bundle, have been improved [29], thus contributing to enhance their properties.

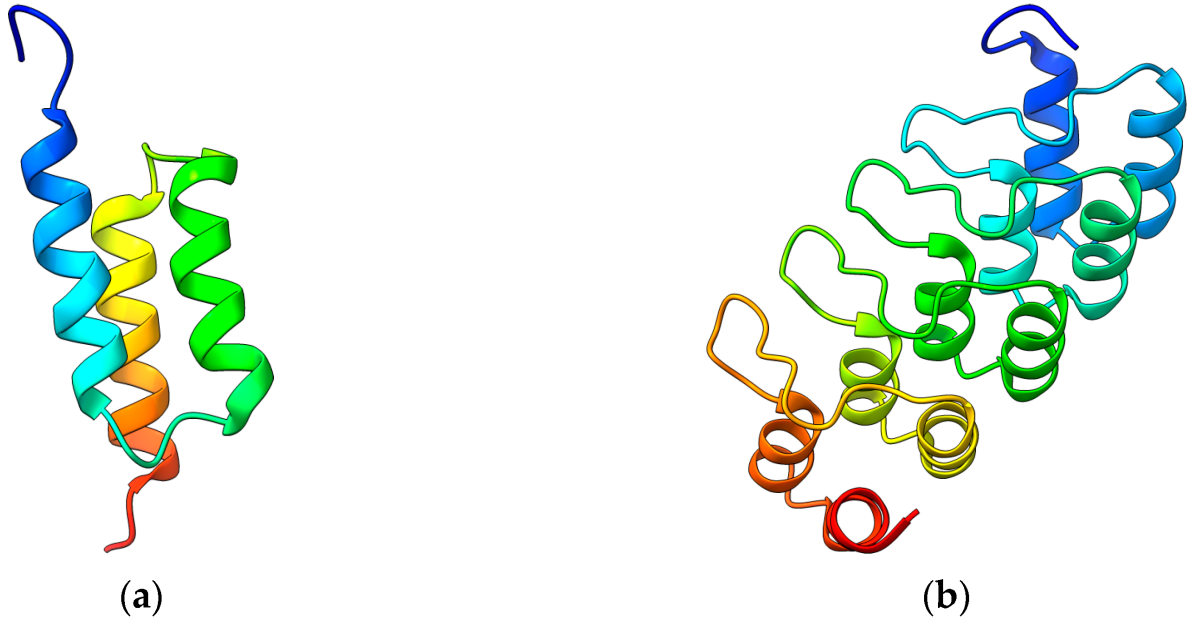


**Figure 1.** (**a**) Nuclear magnetic resonance structure of a Z affibody (PDB 2KZJ) [30]. (**b**) Crystal structure of a DARPin (2QYJ) [31]. Images obtained with ChimeraX v1.4.

As a result, modern Z affibodies are small 58 amino acid polypeptides lacking cysteines and capable of rapid folding, which show high affinity for several molecular partners. Moreover, they can be easily engineered and expressed as soluble and proteolytically stable molecules in various host cells on their own or fused with other partners. These properties contributed to increasing the interest in affibodies for practical purposes, making them more appealing than Abs.

## 2.2. DARPins

Ankirin repeats (ARs) were discovered in the cell cycle regulators Swi6 from *Saccharomyces cerevisiae* and the cell division control protein Cdc10 and Notch from *Drosophila melanogaster* [32]. Since this discovery, ARs have been found in many eukaryotic proteins, becoming one of the most abundant repeat domains in the eukaryotic proteome alongside other repeats, i.e., leucine-rich repeats (LRR), armadillo repeats (ARM), and tetratricopeptide repeats (TPR). It is not surprising that more than 367,000 predicted AR domains have been found. Proteins with AR repeats show tightly packed tandem sequences of 4 to 6 repeats, which usually encompass 33 amino acids each. The repeats form a structural unit consisting of a β-turn followed by two antiparallel α-helices resulting in a typical helix−loop−helix−β-hairpin/loop structure. Short interdomain interactions stabilize a particular right-handed solenoid-like fold rather than a globular shape [33].

Similar to the original B-domain used to produce affibodies, the AR scaffold has been exploited to identify and randomize amino acids to manipulate the recognition properties, thus obtaining libraries of DARPins with an incredibly high yield of production (200 mg per liter of bacterial culture) and thermal stability [34]. Next-generation DARPins have been then produced by introducing a continuous convex paratope similar to the long CDR-H3 loop found in Igs without altering the biophysical properties of the original scaffold (**Figure 1**b). The resulting DARPins showed extended epitope-binding properties with affinity down to the pM range toward several targets, including human Igs, TNFα, the epidermal growth factor receptor (EGFR), and HER2 [35][36]. Further studies revealed that single point mutations strongly increased the thermal stability of these proteins up to melting temperatures of 90 °C [37].

Modern DARPins can recognize targets with specificities and affinities equal to or greater than Abs, disclosing a multitude of practical applications, including cancer therapies [38]. Furthermore, they can be produced with high yield through common bacterial expression systems, reaching high concentrations without aggregating, and show length-dependent stability against boiling and chemical denaturation.

## 3. Affibody- and DARPin-Conjugated Nanomaterials in Cancer Therapy

A brief description is recalled herein, and a comprehensive overview is provided in **Table 1**, describing main properties in terms of constitutive matter, shape, and conjugation strategy.

**Table 1.** Main features of the affibody- and DARPin-conjugated nanomaterials cited here.

| Inorganic Nanomaterials | | | | | |
|---|---|---|---|---|---|
| **Material** | **Synthesis** | **Shape** | **Size** [1] | **Bioconjugation Strategy** | **Reference** |
| Ag | Biological synthesis | Particle | 35 nm | Crosslinking with EDC/NHS | [39] |
| Au | Chemical synthesis | Rod | 50 × 8 nm | Crosslinking with 2-iminothiolane hydrochloride and sulfo-EMCS | [40] |
| Ag | Chemical synthesis | Particle | 120 nm | Crosslinking with sulfo-SMCC or | [41] |

| Inorganic Nanomaterials | | | | | |
|---|---|---|---|---|---|
| **Material** | **Synthesis** | **Shape** | **Size** [1] | **Bioconjugation Strategy** | **Reference** |
| | | | | EDAC/NHS | |
| Au | Chemical synthesis | Particle | 31–39 nm | Crosslinking with sulfo-EMCS | [42] |
| Nd, Yb and Tm | Chemical synthesis | Particle | 18 nm | Crosslinking with NHS-PEG-azide | [43] |
| Pb, S | Chemical synthesis | Dot | 5 nm | Crosslinking with EDC/NHS | [44] |
| $Fe_3O_4$, $Fe_3S_4$ | Biological synthesis | Particle | 30–120 nm | Crosslinking with SPDP | [45] |
| **Organic Nanomaterials** | | | | | |
| **Material** | **Synthesis** | **Shape** | **Size** [1] | **Bioconjugation Strategy** | **Reference** |
| PLGA | Chemical synthesis | Particle | 120 nm | Crosslinking with EDC/NHS | [46] |
| RALA | Biological synthesis | Particle | 104.5 nm | Fusion synthesis | [47] |
| MMAE | Chemical synthesis | Micelle | 153 nm | Crosslinking with valine-citrulline | [48] |

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| Inorganic Nanomaterials | | | | | |
|---|---|---|---|---|---|
| **Material** | **Synthesis** | **Shape** | **Size** [1] | **Bioconjugation Strategy** | **Reference** |
| | | | | dipeptide and PABC spacer | |
| MMAE | Chemical synthesis | Micelle | 130 nm | Crosslinking with valine-citrulline dipeptide and PABC spacer | [49] |
| PLGA | Chemical synthesis | Particle | 218 nm | Fusion synthesis; protein-protein high affinity interaction | [50] |
| PLGA | Chemical synthesis | Particle | 140 nm | Fusion synthesis; crosslinking with EDC/NHS | [51] |
| **Hybrid Nanomaterials** | | | | | |
| **Material** | **Synthesis** | **Shape** | **Size** [1] | **Bioconjugation Strategy** | **Reference** |
| PDA, $MnO_2$ | Chemical synthesis | Particle | 163 nm | Fusion synthesis; crosslinking with Michael addition/Schiff base reaction | [52] |
| $CaCO_3$, $Fe_3O_4$, polyarginine, dextran sulfate | Chemical synthesis | Particle | 400 nm | Crosslinking with EDC/NHS | [53] |

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| Inorganic Nanomaterials | | | | | |
|---|---|---|---|---|---|
| **Material** | **Synthesis** | **Shape** | **Size** [1] | **Bioconjugation Strategy** | **Reference** |
| **Biological Nanomaterials** | | | | | |
| **Material** | **Synthesis** | **Shape** | **Size** [1] | **Bioconjugation Strategy** | **Reference** |
| Hydrogenated soybean phosphatidylcholine, cholesterol and mPEG 2000-DSPE | Chemical synthesis | Micelle | 110–137 nm | Crosslinking with maleimide-PEG DSPE | [54] |
| Hydrogenated soybean phosphatidylcholine, cholesterol, and mPEG 2000-DSPE | Chemical synthesis | Micelle | 140 nm | Crosslinking with maleimide-PEG DSPE | [55] |
| L-α-phosphatidylcholine and phosphatidylethanolamine | Chemical synthesis | Micelle | 117 nm | Crosslinking with 2-iminothiolane hydrochloride and sulfo-EMCS | [56] |
| *Aa*LS protein | Biological synthesis | Particle | 40 nm | Crosslinking through spontaneous ST-SC isopeptide covalent bond | [57] |
| DNA | Chemical synthesis | Tetrahedron | 23 nm | Crosslinking with EMCS | [58] |
| DNA | Chemical synthesis | Micelle | 132 nm | Crosslinking with EMCS | [59] |

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