Affibody- and DARPin-Conjugated Nanomaterials in Cancer Therapy

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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.

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.
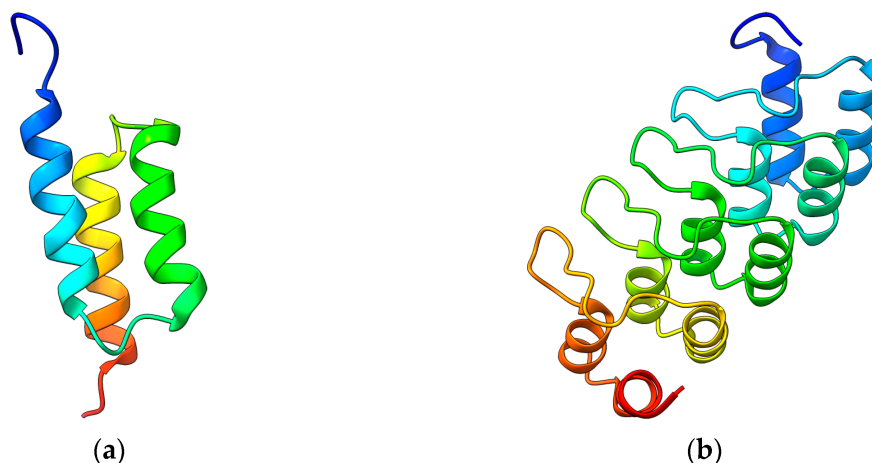

(**a**)   (**b**)

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 EDAC/NHS	[41]
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

Inorganic 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 dipeptide and PABC spacer	[48]
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]

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]

Inorganic Nanomaterials

Material	Synthesis	Shape	Size [1]	Bioconjugation Strategy	Reference
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]

[1] Average size.

References

1. Jin, S.; Sun, Y.; Liang, X.; Gu, X.; Ning, J.; Xu, Y.; Chen, S.; Pan, L. Emerging new therapeutic antibody derivatives for cancer treatment. Sig. Transduct. Target Ther. 2022, 7, 39.

2. Gasser, M.; Waaga-Gasser, A.M. Therapeutic Antibodies in Cancer Therapy. Adv. Exp. Med. Biol. 2016, 917, 95–120.

3. Ståhl, S.; Gräslund, T.; Eriksson Karlström, A.; Frejd, F.Y.; Nygren, P.Å.; Löfblom, J. Affibody Molecules in Biotechnological and Medical Applications. Trends Biotechnol. 2017, 35, 691–712.

4. Farahavar, G.; Abolmaali, S.S.; Gholijani, N.; Nejatollahi, F. Antibody-guided nanomedicines as novel breakthrough therapeutic, diagnostic and theranostic tools. Biomater. Sci. 2019, 7, 4000–4016.

5. Simeon, R.; Chen, Z. In vitro-engineered non-antibody protein therapeutics. Protein Cell 2018, 9, 3–14.

6. Shilova, O.N.; Deyev, S.M. DARPins: Promising Scaffolds for Theranostics. ActaNaturae 2019, 11, 42–53.

7. Shipunova, V.O.; Deyev, S.M. Artificial Scaffold Polypeptides As an Efficient Tool for the Targeted Delivery of Nanostructures In Vitro and In Vivo. ActaNaturae 2022, 14, 54–72.

8. Löfblom, J.; Feldwisch, J.; Tolmachev, V.; Carlsson, J.; Ståhl, S.; Frejd, F.Y. Affibody molecules: Engineered proteins for therapeutic, diagnostic and biotechnological applications. FEBS Lett. 2010, 584, 2670–2680.

9. De, A.; Kuppusamy, G.; Karri, V.V.S.R. Affibody molecules for molecular imaging and targeted drug delivery in the management of breast cancer. Int. J. Biol. Macromol. 2018, 107, 906–919.

10. Jia, D.; Liu, H.; Zheng, S.; Yuan, D.; Sun, R.; Wang, F.; Li, Y.; Li, H.; Yuan, F.; Fan, Q.; et al. ICG-Dimeric Her2-Specific Affibody Conjugates for Tumor Imaging and Photothermal Therapy for Her2-Positive Tumors. Mol. Pharm. 2023, 20, 427–437.

11. Oroujeni, M.; Bezverkhniaia, E.A.; Xu, T.; Liu, Y.; Plotnikov, E.V.; Karlberg, I.; Ryer, E.; Orlova, A.; Tolmachev, V.; Frejd, F.Y. Evaluation of an Affibody-Based Binder for Imaging of Immune Check-Point Molecule B7-H3. Pharmaceutics 2022, 14, 1780.

12. Karsten, L.; Janson, N.; Le Joncour, V.; Alam, S.; Müller, B.; Tanjore Ramanathan, J.; Laakkonen, P.; Sewald, N.; Müller, K.M. Bivalent EGFR-Targeting DARPin-MMAE Conjugates. Int. J. Mol. Sci. 2022, 23, 2468.

13. Vorobyeva, A.; Schulga, A.; Rinne, S.S.; Günther, T.; Orlova, A.; Deyev, S.; Tolmachev, V. Indirect Radioiodination of DARPin G3 Using N-succinimidyl-Para-Iodobenzoate Improves the Contrast of HER2 Molecular Imaging. Int. J. Mol. Sci. 2019, 20, 3047.

14. Qi, S.; Hoppmann, S.; Xu, Y.; Cheng, Z. PET Imaging of HER2-Positive Tumors with Cu-64-Labeled Affibody Molecules. Mol. Imaging Biol. 2019, 21, 907–916.

15. Shipunova, V.O.; Kolesnikova, O.A.; Kotelnikova, P.A.; Soloviev, V.D.; Popov, A.A.; Proshkina, G.M.; Nikitin, M.P.; Deyev, S.M. Comparative Evaluation of Engineered Polypeptide Scaffolds in HER2-Targeting Magnetic Nanocarrier Delivery. ACS Omega 2021, 10, 16000–16008.

16. Uhlén, M.; Guss, B.; Nilsson, B.; Gatenbeck, S.; Philipson, L.; Lindberg, M. Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. J. Biol. Chem. 1984, 259, 1695–1702.

17. Moks, T.; Abrahmsén, L.; Nilsson, B.; Hellman, U.; Sjöquist, J.; Uhlén, M. Staphylococcal protein A consists of five IgG-binding domains. Eur. J. Biochem. 1986, 156, 637–643.

18. Ibrahim, S. Immunoglobulin Binding Specificities of the Homology Regions (Domains) of Protein A. Scand. J. Immunol. 1993, 38, 368–374.

19. Jansson, B.; Uhlén, M.; Nygren, P.-Å. All individual domains of staphylococcal protein A show Fab binding. FEMS Immunol. Med. Microbiol. 1998, 20, 69–78.

20. Gouda, H.; Torigoe, H.; Saito, A.; Sato, M.; Arata, Y.; Shimada, I. Three-dimensional solution structure of the B domain of staphylococcal protein A: Comparisons of the solution and crystal structures. Biochemistry 1992, 31, 9665–9672.

21. Starovasnik, M.A.; Skelton, N.J.; O'Connell, M.P.; Kelley, R.F.; Reilly, D.; Fairbrother, W.J. Solution Structure of the E-Domain of Staphylococcal Protein A. Biochemistry 1996, 35, 15558–15569.

22. Nilsson, B.; Moks, T.; Jansson, B.; Abrahmsén, L.; Elmblad, A.; Holmgren, E.; Henrichson, C.; Jones, T.A.; Uhlén, M. A synthetic IgG-binding domain based on staphylococcal protein A. Protein Eng. Des. Sel. 1987, 1, 107–113.

23. Tashiro, M.; Tejero, R.; Zimmerman, D.E.; Celda, B.; Nilsson, B.; Montelione, G.T. High-resolution solution NMR structure of the Z domain of staphylococcal protein A. J. Mol. Biol. 1997, 272, 573–590.

24. Zheng, D.; Aramini, J.M.; Montelione, G.T. Validation of helical tilt angles in the solution NMR structure of the Z domain of Staphylococcal protein A by combined analysis of residual dipolar coupling and NOE data. Protein Sci. 2004, 13, 549–554.

25. Nord, K.; Nilsson, J.; Nilsson, B.; Uhlén, M.; Nygren, P.A. A combinatorial library of an alpha-helical bacterial receptor domain. Protein Eng. Des. Sel. 1995, 8, 601–608.

26. Nord, K.; Gunneriusson, E.; Ringdahl, J.; Ståhl, S.; Uhlén, M.; Nygren, P. Binding proteins selected from combinatorial libraries of an α-helical bacterial receptor domain. Nat. Biotechnol. 1997, 15, 772–777.

27. Feldwisch, J.; Tolmachev, V.; Lendel, C.; Herne, N.; Sjöberg, A.; Larsson, B.; Rosik, D.; Lindqvist, E.; Fant, G.; Höidén-Guthenberg, I.; et al. Design of an optimized scaffold for affibody molecules. J. Mol. Biol. 2010, 398, 232–247.

28. Votsmeier, C.; Plittersdorf, H.; Hesse, O.; Scheidig, A.; Strerath, M.; Gritzan, U.; Pellengahr, K.; Scholz, P.; Eicker, A.; Myszka, D.; et al. Femtomolar Fab binding affinities to a protein target by alternative CDR residue co-optimization strategies without phage or cell surface display. mAbs 2012, 4, 341–348.

29. Arora, P.; Oas, T.G.; Myers, J.K. Fast and faster: A designed variant of the B-domain of protein A folds in 3 microsec. Protein Sci. 2004, 13, 847–853.

30. Eigenbrot, C.; Ultsch, M.; Dubnovitsky, A.; Abrahmsén, L.; Härd, T. Structural basis for high-affinity HER2 receptor binding by an engineered protein. Proc. Natl. Acad. Sci. USA 2010, 107, 15039–15044.

31. Merz, T.; Wetzel, S.K.; Firbank, S.; Plückthun, A.; Grütter, M.G.; Mittl, P.R. Stabilizing ionic interactions in a full-consensus ankyrin repeat protein. J. Mol. Biol. 2008, 376, 232–240.

32. Breeden, L.; Nasmyth, K. Similarity between cell-cycle genes of budding yeast and fission yeast and the Notch gene of Drosophila. Nature 1987, 329, 651–654.

33. Kane, E.I.; Spratt, D.E. Structural Insights into Ankyrin Repeat-Containing Proteins and Their Influence in Ubiquitylation. Int. J. Mol. Sci. 2021, 22, 609.

34. Binz, H.K.; Stumpp, M.T.; 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.

35. Steiner, D.; Forrer, P.; Plückthun, A. Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display. J. Mol. Biol. 2008, 382, 1211–1227.

36. Schilling, J.; Schoeppe, J.; Plückthun, A. From DARPins to LoopDARPins: Novel LoopDARPin design allows the selection of low picomolar binders in a single round of ribosome display. J. Mol. Biol. 2014, 426, 691–721.

37. Schilling, J.; Jost, C.; Ilie, I.M.; Schnabl, J.; Buechi, O.; Eapen, R.S.; Truffer, R.; Caflisch, A.; Forrer, P. Thermostable designed ankyrin repeat proteins (DARPins) as building blocks for innovative drugs. J. Biol. Chem. 2022, 298, 101403.

38. Plückthun, A. Designed ankyrin repeat proteins (DARPins): Binding proteins for research, diagnostics, and therapy. Annu. Rev. Pharmacol. Toxicol. 2015, 55, 489–511.

39. Shipunova, V.O.; Belova, M.M.; Kotelnikova, P.A.; Shilova, O.N.; Mirkasymov, A.B.; Danilova, N.V.; Komedchikova, E.N.; Popovtzer, R.; Deyev, S.M.; Nikitin, M.P. Photothermal Therapy with HER2-Targeted Silver Nanoparticles Leading to Cancer Remission. Pharmaceutics 2022, 14, 1013.

40. Proshkina, G.M.; Shramova, E.I.; Shilova, M.V.; Zelepukin, I.V.; Shipunova, V.O.; Ryabova, A.V.; Deyev, S.M.; Kotlyar, A.B. DARPin_9-29-Targeted Gold Nanorods Selectively Suppress HER2-Positive Tumor Growth in Mice. Cancers 2021, 13, 5235.

41. Pourshohod, A.; Zeinali, M.; Ghaffari, M.A.; Kheirollah, A.; Jamalan, M. Improvement of specific aiming of X-ray radiotherapy on HER2-overexpressing cancerous cell lines by targeted delivery of silver nanoparticle. J. Drug Delivery Sci. Technol. 2022, 76, 103746.

42. Zhang, C.; Zhang, F.; Han, M.; Wang, X.; Du, J.; Zhang, H.; Li, W. Co-delivery of 5-fluorodeoxyuridine and doxorubicin via gold nanoparticle equipped with affibody-DNA hybrid strands for targeted synergistic chemotherapy of HER2 overexpressing breast cancer. Sci. Rep. 2020, 10, 22015.

43. Roy, S.; Curry, S.D.; Corbella Bagot, C.; Mueller, E.N.; Mansouri, A.M.; Park, W.; Cha, J.N.; Goodwin, A.P. Enzyme Prodrug Therapy with Photo-Cross-Linkable Anti-EGFR Affibodies Conjugated to Upconverting Nanoparticles. ACS Nano 2022, 16, 15873–15883.

44. Al-Ani, A.W.; Zamberlan, F.; Ferreira, L.; Bradshaw, T.D.; Thomas, N.R.; Turyanska, L. Near-infrared PbS quantum dots functionalized with affibodies and ZnPP for targeted imaging and therapeutic applications. Nano Express 2021, 2, 040005.

45. Ma, S.; Gu, C.; Xu, J.; He, J.; Li, S.; Zheng, H.; Pang, B.; Wen, Y.; Fang, Q.; Liu, W.; et al. Strategy for Avoiding Protein Corona Inhibition of Targeted Drug Delivery by Linking Recombinant Affibody Scaffold to Magnetosomes. Int. J. Nanomed. 2022, 17, 665–680.

46. Shipunova, V.O.; Sogomonyan, A.S.; Zelepukin, I.V.; Nikitin, M.P.; Deyev, S.M. PLGA Nanoparticles Decorated with Anti-HER2 Affibody for Targeted Delivery and Photoinduced Cell Death. Molecules 2021, 26, 3955.

47. Zhang, F.; Yin, J.; Zhang, C.; Han, M.; Wang, X.; Fu, S.; Du, J.; Zhang, H.; Li, W. Affibody-Conjugated RALA Polymers Delivering Oligomeric 5-Fluorodeoxyuridine for Targeted Therapy of HER2 Overexpressing Gastric Cancer. Macromol. Biosci. 2020, 20, e2000083.

48. Xia, X.; Yang, X.; Huang, W.; Xia, X.; Yan, D. Self-Assembled Nanomicelles of Affibody-Drug Conjugate with Excellent Therapeutic Property to Cure Ovary and Breast Cancers. Nano-Micro Lett. 2022, 14, 33.

49. Yang, X.; Xia, X.; Huang, W.; Xia, X.; Yan, D. Highly efficient tumor-targeted nanomedicine assembled from affibody-drug conjugate for colorectal cancer therapy. Nano Res. 2023, 16, 5256–5264.

50. Komedchikova, E.N.; Kolesnikova, O.A.; Tereshina, E.D.; Kotelnikova, P.A.; Sogomonyan, A.S.; Stepanov, A.V.; Deyev, S.M.; Nikitin, M.P.; Shipunova, V.O. Two-Step Targeted Drug Delivery via Proteinaceous Barnase-Barstar Interface and Doxorubicin-Loaded Nano-PLGA Outperforms One-Step Strategy for Targeted Delivery to HER2-Overexpressing Cells. Pharmaceutics 2023, 15, 52.

51. Shipunova, V.O.; Komedchikova, E.N.; Kotelnikova, P.A.; Zelepukin, I.V.; Schulga, A.A.; Proshkina, G.M.; Shramova, E.I.; Kutscher, H.L.; Telegin, G.B.; Kabashin, A.V.; et al. Dual Regioselective Targeting the Same Receptor in Nanoparticle-Mediated Combination Immuno/Chemotherapy for Enhanced Image-Guided Cancer Treatment. ACS Nano 2020, 27, 12781–12795.

52. Wang, H.; Jia, D.; Yuan, D.; Yin, X.; Yuan, F.; Wang, F.; Shi, W.; Li, H.; Zhu, L.-M.; Fan, Q. Dimeric Her2-specific affibody mediated cisplatin-loaded nanoparticles for tumor enhanced chemo-radiotherapy. J. Nanobiotechnol. 2021, 19, 138.

53. Novoselova, M.V.; Shramova, E.I.; Sergeeva, O.V.; Shcherbinina, E.Y.; Perevoschikov, S.V.; Melnikov, P.; Griaznova, O.Y.; Sergeev, I.S.; Konovalova, E.V.; Schulga, A.A.; et al. Polymer/magnetite carriers functionalized by HER2-DARPin: Avoiding lysosomes during internalization and controlled toxicity of doxorubicin by focused ultrasound induced release. Nanomedicine 2023, 47, 102612.

54. Jia, D.; Yang, Y.; Yuan, F.; Fan, Q.; Wang, F.; Huang, Y.; Song, H.; Hu, P.; Wang, R.; Li, G.; et al. Increasing the antitumor efficacy of doxorubicin liposomes with coupling an anti-EGFR affibody in EGFR-expressing tumor models. Int. J. Pharm. 2020, 586, 119541.

55. Jia, D.; Wang, F.; Yang, Y.; Hu, P.; Song, H.; Lu, Y.; Wang, R.; Li, G.; Liu, R.; Li, J.; et al. Coupling EGFR-Antagonistic Affibody Enhanced Therapeutic Effects of Cisplatin Liposomes in EGFR-expressing Tumor Models. J. Pharm. Sci. 2022, 111, 450–457.

56. Shipunova, V.O.; Shramova, E.I.; Schulga, A.A.; Shilova, M.V.; Deyev, S.M.; Proshkina, G.M. Delivery of Barnase to Cells in Liposomes Functionalized by Her2-Specific DARPin Module. Russ. J. Bioorg. Chem. 2020, 46, 1156–1161.

57. Jun, H.; Jang, E.; Kim, H.; Yeo, M.; Park, S.G.; Lee, J.; Shin, K.J.; Chae, Y.C.; Kang, S.; Kim, E. TRAIL & EGFR affibody dual-display on a protein nanoparticle synergistically suppresses tumor growth. J. Control Release 2022, 349, 367–378.

58. Zhang, C.; Han, M.; Zhang, F.; Yang, X.; Du, J.; Zhang, H.; Li, W.; Chen, S. Enhancing Antitumor Efficacy of Nucleoside Analog 5-Fluorodeoxyuridine on HER2-Overexpressing Breast Cancer by Affibody-Engineered DNA Nanoparticle. Int. J. Nanomed. 2020, 15, 885–900.

59. Zhang, C.; Fu, S.; Zhang, F.; Han, M.; Wang, X.; Du, J.; Zhang, H.; Li, W. Affibody Modified G-quadruplex DNA Micelles Incorporating Polymeric 5-Fluorodeoxyuridine for Targeted Delivery of Curcumin to Enhance Synergetic Therapy of HER2 Positive Gastric Cancer. Nanomaterials 2022, 12, 696.