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Review

Affibody molecules: Engineered proteins for therapeutic, diagnostic and biotechnological applications

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ABSTRACT

Affibody molecules are a class of engineered affinity proteins with proven potential for therapeutic, diagnostic and biotechnological applications. Affibody molecules are small (6.5 kDa) single domain proteins that can be isolated for high affinity and specificity to any given protein target. Fifteen years after its discovery, the Affibody technology is gaining use in many groups as a tool for creating molecular specificity wherever a small, engineering compatible tool is warranted. Here we summarize recent results using this technology, propose an Affibody nomenclature and give an overview of different HER2-specific Affibody molecules. Cumulative evidence suggests that the three helical scaffold domain used as basis for these molecules is highly suited to create a molecular affinity handle for vastly different applications.

1. Introduction

Affinity proteins are invaluable tools in the development of next generation therapeutics. Their ability to selectively and with high affinity bind a given molecular structure is an essential key feature also for in vitro and in vivo diagnostics, for basic research and for many biotechnological applications. High affinity and binding specificity are however not the only requirements. Depending on the therapeutic application, a drug protein must have rapid or slow in vivo kinetic properties. In imaging, small size is key, as well as ability to withstand the elevated temperature and harsh chemical conditions often involved in labeling of the targeting probe. For in vitro diagnostic purposes, minimal background interference and compatibility with a number of detection methods are important. As capturing agents in affinity chromatography, the molecules should withstand several tough regeneration procedures. In addition, for all applications, manufacturing cost, shelf life and intellectual property restrictions may eventually determine the usefulness of the affinity protein.

Currently, antibodies are the most successful and widespread affinity proteins for life science applications. Key to this success is their capability to be isolated to virtually any given target with high affinity and specificity and that the technology is readily available. There are however also historical reasons; until about 20 years ago, the immune system was the only source of affinity proteins from which binders to a desired target could be generated at will. Although antibodies often demonstrate strong binding and can be selected for high selectivity, they have some intrinsic limitations related to their molecular properties. The most used antibody type, the IgG molecule, is a large, bivalent, multidomain protein, dependent on disulphide bonds and with complex glycosylation pattern. This leads to relatively poor heat stability and a comparatively difficult and expensive manufacturing process. In addition, antibodies use only a minor part of the molecule for antigen recognition. Large domains have structural function and there are other defined binding sites that are responsible for interaction with complement factors and various Fc-receptors. These interactions may be desired or even essential for the intended application but adds up on the complexity when evaluating the molecules. We have learned how to generate and handle antibodies and their derivatives throughout the years, but substantial efforts have also been directed at finding new alternative affinity proteins with improved properties. Today, a number of techniques exist for in vitro generation of large non-antibody molecular repertoires, from which binders with high affinity and specificity can be selected. These so called alternative scaffold proteins often combine the favorable molecular recognition properties of antibodies with

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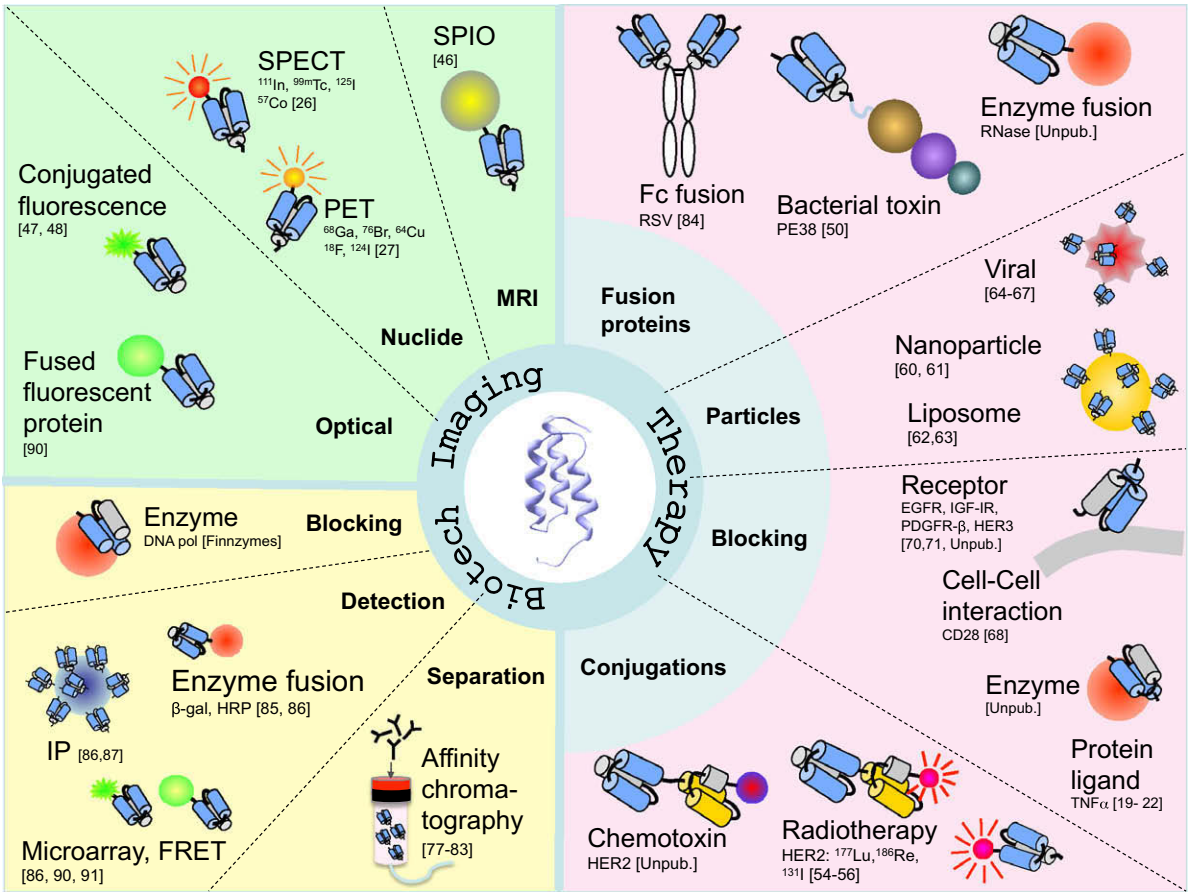

Fig. 1. Overview of selected applications of Affibody molecules. Applications are divided into three research areas: therapy, imaging and biotechnology. Please note that some constructs may be used in several application areas (e.g. enzymatic fusions in biotechnology as well as in therapy).

improved characteristics such as small size, high stability, absence of cysteines, high yield bacterial expression and the option for using multispecific constructs. Several of these scaffolds have made substantial scientific progress recently [1,2], and some are already in clinical trials.

In this work we will review Affibody molecules as a viable and sometimes superior alternative to antibodies and focus on recent results in therapeutic, in vivo imaging and biotechnological applications (Fig. 1). The amount of reports on Affibody molecules have increased dramatically over the last few years, as have the number of research groups working with Affibody molecules. Therefore, we here propose an Affibody molecule nomenclature in an attempt to facilitate comparison of results from different research groups.

2. Affibody technology

Representing a new class of affinity ligands, Affibody molecules were originally derived from the B-domain in the immunoglobulin-binding region of staphylococcal protein A [3]. The B-domain is a relatively short cysteine-free peptide of 58 amino acids that is folded into a three-helical bundle structure and the kinetics of the folding reaction is one of the fastest that has been reported [4]. The folding properties in combination with high solubility and a relatively high thermal stability has contributed to a high scientific interest. The B-domain was early mutated at key positions mainly for enhanced chemical stability and the resulting engineered variant was denoted the Z-domain [5]. The engineered Z-domain retained its affinity for the Fc part of the antibody while the weaker affinity for the Fab region was almost completely lost.

Exploiting their robust nature and capacity of specifically capturing a variety of different immunoglobulin species, protein A and derivatives of its immunoglobulin-binding domains have been widely used in biotechnology, and their suitability in a diverse set of applications is today well-established (discussed in more detail under Section 6). However, it was relatively early envisioned that the favorable properties of domain Z should be advantageous in an even broader variety of applications, resulting in the concept of engineering new so-called Affibody molecules based on the Z-domain scaffold with specific binding for theoretically any given target [6]. In contrast to monoclonal antibodies that may be generated by immunization of laboratory animals combined with hybridoma technology, isolation of new affinity proteins based on non-immunoglobulin scaffolds is performed using synthetic combinatorial libraries and in vitro selection systems (e.g. phage display technology). Affibody molecule libraries are generally constructed by combinatorial randomization of 13 amino acid positions in helices one and two that comprise the original Fc-binding surface of the Z-domain [6,7]. The libraries have typically been displayed on phages, followed by biopanning against desired targets. Using this strategy, Affibody molecules showing specific binding to a variety of different proteins (e.g. insulin, fibrinogen, transferrin, tumor necrosis factor-α, IL-8, gp120, CD28, human serum albumin, IgA, IgE, IgM, HER2 and EGFR) have been generated, demonstrating affinities (K_D) in the μM to pM range [8]. Should the affinity of the primary leads not suffice, affinity maturation generally results in improved binders and may be achieved by either helix shuffling or sequence alignment combined with directed combinatorial mutagenesis [9,10]. The newly identified molecules with their altered binding surface generally keep

the original helical structure as well as the high stability, although unique exceptions with interesting properties have been reported [11]. Importantly, due to their small size and rapid folding properties, Affibody molecules can be produced by chemical peptide synthesis [12]. This alternative to recombinant bacterial production enables site-specific incorporation of various chemical moieties, such as fluorescent probes or chelating groups for binding radioactive metal atoms in a single chemical process. Furthermore, the small size of the scaffold provides means for modular approaches, where different Affibody molecules are combined into fusion proteins with multiple properties while still retaining an overall size much smaller compared to a full-length antibody [13].

Phage display technology has traditionally been the preferred format for selection of new or improved Affibody molecules. However, today there are several more or less established alternative technologies available. Although these technologies are different in many aspects, some characteristics are generally important in order to achieve a successful outcome. Selection pressure should be focused on correct properties (e.g. high affinity for the target molecule) avoiding substantial bias, such as from expression level or amplification rate. The isolated clones should be easily amplified between rounds and identified after the selection process. Other preferred properties are functional display of the combinatorial protein library, evolution possibilities during selection, and means for monitoring the process in order to facilitate optimization. One important aspect in combinatorial protein engineering is the library complexity. A larger library results in a more complete coverage of the theoretical sequence space and hence a higher probability of containing high-affinity variants. Since proteins are difficult to sequence and amplification techniques not involving DNA are lacking, all successful selection systems are based on a physical coupling of phenotype to genotype. As mentioned above, the selection system should ideally be devoid of any undesired bias. Nevertheless, in practice all technologies suffer from some degree of bias, ultimately resulting in isolation of different sets of affinity proteins with different selection systems [14]. Consequently, a broad panel of available and compatible methods for generating the affinity protein of choice should be an advantage. In addition to phage display, several alternative selection technologies have been developed and evaluated for the Affibody scaffold.

Microbial display-based systems, including filamentous phage display, rely on an initial transformation of DNA encoding the library members to host cells. This procedure often become a limiting step when large and complex libraries are desired, despite that protocols for obtaining highly transformation competent cells and efficient delivery of the DNA by electroporation are available. To circumvent this particular restriction, cell-free methods not requiring transformation for construction of the molecular library have been investigated. One such technology is the microbead display system, which has been evaluated for selection of specific Affibody molecules [15]. Using spiked model libraries, Affibody molecules anchored onto small polystyrene beads could be efficiently selected using flow-cytometric sorting. Correspondingly, a variant of the more established ribosome display technology has been evaluated for similar purposes, enabling the construction of the largest Affibody molecule libraries to date as well as isolation of novel specific Affibody molecules to different targets (S. Grimm, P.-Å. Nygren, personal communication). The bypassing of the transformation step significantly simplifies the library construction, and thus allows the construction of large libraries in parallel for investigating and optimizing different library designs. Moreover, in vitro evolution may be included in the process, by applying error-prone PCR for amplification of isolated variants after each selection cycle.

Another recent alternative for selection of affinity proteins from combinatorial libraries is the protein complementation assay (PCA), normally employed for studying natural protein–protein interactions. In contrast to other selection systems, the target is expressed inside each cell (also harboring the affinity protein variant), thus circumventing the need for target protein production and purification. In addition, since PCA might be based on survival of positive clones in selective media, isolating specific variants can be accomplished by simple cultivation. However, although elegant in theory, the method has proven challenging in practice and several groups have reported problems with insufficient enrichment from scFv libraries, speculating on reasons associated with unspecific binding [16,17]. As a consequence, such efforts have required a pre-enrichment step using either phage or ribosome display (for selection of specific DARPins) [17,18]. In contrast to problems reported with scFvs, Nygren and co-workers recently demonstrated successful generation of specific Affibody molecules for human TNFα from a naïve library using PCA [19]. The approach was similar to a reported system for scFv libraries, employing a split version of the β-lactamase enzyme and selection using ampicillin-containing medium. Since both methods are nearly identical, the success of the latter is likely linked to the generally higher stability and solubility of the Z scaffold compared to scFv antibody fragments. It has also been speculated that the PCA methods are not suitable for affinity maturation because of the lack of control over the concentration of both affinity protein and target protein inside the cell [16]. Nevertheless, Nygren and co-workers recently reported that the affinity of TNFα specific Affibody molecules could be further improved up to 10-fold by PCA selection from an affinity maturation library [20].

Affibody libraries have also been displayed on cells for directed evolution purposes [21]. Cell display is analogous to phage display, but the larger size of the cell enables quantitative flow cytometry for both sorting libraries and characterizing isolated variants after selection. Using the Gram-positive bacterium *Staphylococcus carnosus* and a vector system for recombinant display of proteins on the bacterial surface, high affinity Affibody molecules for TNFα could be efficiently isolated and later characterized using FACS [21]. Not surprisingly, unique sets of Affibody molecules were obtained from the staphylococcal-displayed library, from phage display [22] and from PCA [19,20], respectively, although several binders were homologous (but never identical) even across selection platforms.

3. Imaging

Imaging widens and improves diagnosis of many diseases from bone fractures and lung TBC to blood flow and cancer. In cancer diagnosis it can provide a global view of all metastatic lesions in the body, in contrast to biopsy, which is usually restricted to the primary tumor and a limited number of local lesions. Furthermore, image-guided biopsy allows obtaining tissue samples from metastasis, which are otherwise not amenable for biopsy. Thus, in today's routine clinical practice, anatomical imaging such as computed tomography (CT) or magnetic resonance imaging (MRI) are important for diagnosis, staging, restaging and monitoring of therapy response of most common cancers. Lesions can be further characterized by functional or molecular imaging measuring biological processes at the molecular level using single-photon emission spectroscopy (SPECT) or positron emission tomography (PET). The vast majority of molecular imaging investigations are performed using the glucose analogue 2-[^{18}F]fluoro-2-deoxy-D-glucose (^{18}F-FDG PET), which has proven its value for detection of metastatic and recurrent disease, as well as for detection of early treatment response [23–25]. However, ^{18}F-FDG PET generates a relatively non-specific readout, showing increased glucose metabolism without providing information on the presence of cancer-specific cell-surface receptors such as human epidermal growth

factor receptor 2 (HER2), and therefore not providing guidance for receptor-targeted therapy. An ideal imaging agent should have high specificity and affinity for its target. Furthermore, rapid biodistribution and tissue penetration leading to high local concentrations at the intended sites as well as rapid clearance of unbound tracer will allow for high contrast tumor imaging and reduced time between injection and examination [26]. Recent studies indicate that Affibody molecules are among the most promising tracers for HER2-specific molecular imaging, compared to antibodies, antibody fragments (reviewed by Tolmachev in [27]) as well as alternative scaffold proteins such as DARPins [28]. Among alternative scaffold proteins, it is also the most investigated class of binders in terms of imaging [29]. Studies in rats and mice have confirmed the efficient extravasation and rapid biodistribution expected from their small size. Thus, Affibody molecules can find and bind to their targets within the first hours after administration. This together with rapid blood clearance (first half-life about 10–20 min in rats) allowed imaging of tumors in mouse xenografts models as early as 30–60 min after injection. The rapid in vivo pharmacokinetics makes Affibody molecules compatible with both very short-lived positron emitting nuclides like ^{18}F ($t_{1/2}$ 110 min) or ^{68}Ga ($t_{1/2}$ 68 min) or gamma emitting nuclides like ^{99m}Tc ($t_{1/2}$ 6 h) or ^{111}In ($t_{1/2}$ 2.8 days) with longer half-lives. This is in contrast to antibodies and scFv or Fab fragments, where the size and slow biodistribution prevents the use of short-lived positron emitters, due to the mismatch of the half-live of the tracer and the radionuclide. The development of Affibody molecules for molecular imaging applications was primarily performed with different HER2-binding Affibody molecules. The knowledge obtained from this work was later applied also for development of EGFR- and PDGFRβ-binding Affibody molecules [30] (unpublished). Fig. 2 shows the primary structure of the original HER2-binding Affibody molecule $Z_{HER2:4}$ [31], two affinity matured descendants $Z_{HER2:477}$ and $Z_{HER2:342}$ [10], and two molecules that were modified from $Z_{HER2:342}$: $Z_{HER2:2395}$ [32] and $Z_{HER2:2891}$ [33]. For comparison, the Z-domain scaffold sequence is included [5].

In the first studies Affibody molecules were radiolabeled by non-site-specific iodination or conjugation of chelators to the ε-NH_2-group of lysines using amine chemistry and the results have been summarized in several reviews [26,27,34].

Site-specific labeling of tracers is of utmost importance to allow the production of well defined and homogenous products for clinical use. Labeling was obtained either by site-specific coupling of a chelator during peptide synthesis or by incorporation of a unique cysteine and using thiol-directed chemistry. Appreciable effort has been applied to develop and optimize methods for ^{99m}Tc labeling of Affibody molecules. More than 20 different constructs having amino acid-based chelators placed either at the N- or C-terminus of $Z_{HER2:342}$ have been investigated. One of the lessons learned from these studies was that increasing the hydrophilicity of the chelator lead to a switch from predominantly hepatobiliary excretion to renal excretion. The absence of radiotracer uptake into the gastrointestinal tract dramatically improved image contrast (Fig. 3). The results of these studies have been summarized in detail in a recent review [35].

The first site-specific radiolabeling of Affibody molecules was reported by Orlova et al. [36]. In this study the Affibody molecule $Z_{HER2:342}$ was produced by peptide synthesis in a single chemical process with DOTA coupled to the N-terminal amine in the last synthesis step, yielding DOTA-$Z_{HER2:342\text{-}pep2}$ (ABY-002). ABY-002 was efficiently and stably labeled with ^{111}In within 30 min at temperatures in the range of 60–90 °C. Biodistribution of ^{111}In-ABY-002 in SKOV-3 tumor bearing mice revealed efficient tumor uptake with 23% ID/g already 1 h after injection. The tumor to blood ratio (T/B) rose from 8 after 1 h and 12 after 4 h to over 120 after 72 h. At all time points the tumor uptake was higher than the uptake in all other organs, except for the kidneys. Fast reduction of blood radioactivity and accumulation in the urine indicate rapid clearance, predominantly by renal filtration. Accumulation of radioactivity in the kidneys was expected for a small peptide labeled with the residualizing radiometal ^{111}In and a molecular weight below the exclusion limit for renal filtration. The rapid pharmacokinetics of ^{111}In-ABY-002 allowed visualization of HER2 overexpressing tumors as early as 1 h after injection. The gamma camera images showed high contrast and beside the tumor and the kidneys no other organ was visible. ABY-002 was also efficiently labeled with the positron emitter ^{68}Ga. Biodistribution and PET imaging studies performed in mice bearing SKOV-3 tumor xenografts showed specific tumor targeting [37]. The tumor uptake of ^{68}Ga-ABY-002 at 4 h post-injection (p.i.) was 12.4% ID/g and the T/B 31. Uptake in all other organs except the kidneys was low.

A dual-labeling experiment, i.e. co-injection of ^{68}Ga- and ^{111}In-ABY-002 in mouse xenografts allowed site-by-site comparison of the same Affibody molecule with two different radiometals, one gamma emitter and one positron emitter. The results revealed similar tumor uptake for both molecules. However, ^{68}Ga-ABY-002 had a significantly lower amount of radioactivity in blood, lung, spleen and the gastrointestinal tract as compared to ^{111}In-ABY-002, leading to higher T/B and tumor-to-organ ratios. Accordingly, the image contrast was slightly better on the ^{68}Ga-ABY-002 PET images then the ^{111}In-ABY-002 gamma camera images of the same mice obtained one day later after decay of the ^{68}Ga radioactivity.

The first clinical investigation of ^{68}Ga- and ^{111}In-labeld ABY-002 in three patients with metastatic breast cancer showed that HER2-specific Affibody molecules have the potential to visualize HER2-expressing metastatic lesions. High contrast SPECT or PET images were obtained already 2–3 h after injection. Overexpression of HER2 in two metastases was confirmed on biopsy tissue samples

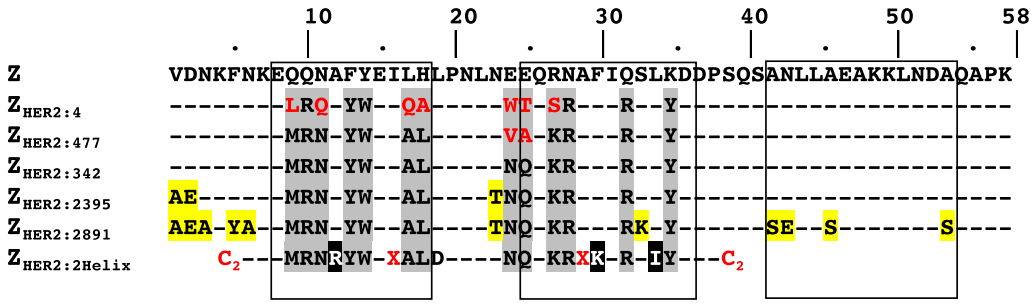

Fig. 2. Primary structure of HER2-binding Affibody molecules used for molecular imaging. The sequence of the Z-domain scaffold from which all Affibody molecules are derived is shown for comparison. The 13 amino acids in the binding site of Affibody molecules are marked grey for HER2-binding Affibody molecules. Amino acids in red show the differences in the binding site of HER2-binding Affibody molecules. Amino acids substituted in the improved scaffold are marked yellow. The two-helix variant of $Z_{HER2:342}$, also called Mut-DS is modified by: C_2 = homocystein, X = α-aminoisobutyric acid and amino acids marked black were originally selected by Braisted and Wells for the two-helix variant of the Z-domain. The three boxes indicate the position of helix 1, 2 and 3.

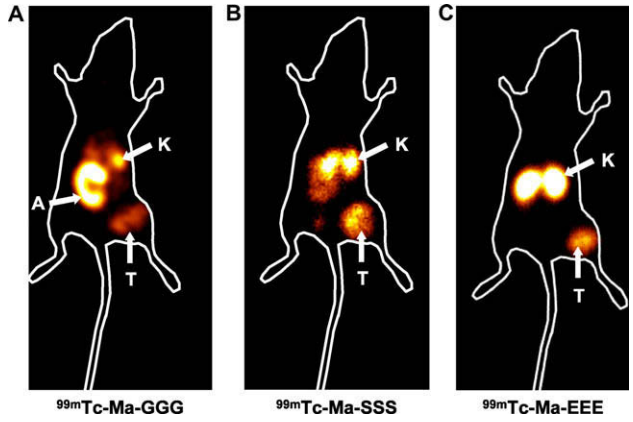

Fig. 3. Comparison of gamma camera images obtained 4 h p.i. with HER2-binding Affibody molecules with different N-terminal amino acid-based ^{99m}Tc chelators. (A) ^{99m}Tc-maGGG-$Z_{HER2:342}$, mice bearing a LS174T xenografts, (B) ^{99m}Tc-maSSS-$Z_{HER2:342}$, mice bearing a SKOV-3 xenograft, (C) ^{99m}Tc-maEEE-$Z_{HER2:342}$, mice bearing a SKOV-3 xenograft. ma, Mercaptoacetyl-; T, tumor; K, kidney; A, appendice. White contours surrounding the animals are superimposed. Note the shift from primarily hepatobiliary excretion to renal excretion with increasing hydrophilicity of the chelator. Published with permission of Bentham Science Publishers Ltd.

using the HercepTest™ indicating that ABY-002 specifically targets HER2 also in humans [38]. Further clinical studies are warranted to assess the sensitivity and specificity of radiolabeled HER2-targeting Affibody molecules.

A two-helix variant of the Affibody molecule $Z_{HER2:342}$ was recently produced by removal of helix 3, situated on the opposite site of the binding site and therefore not supposed to be involved in target binding [39]. The two alpha helices were stabilized by a disulfide bridge formed between two introduced homocysteines. However, the binding affinity of the resulting molecule $Z_{HER2:342}$-MUT-DS for HER2 was approximately 200-fold lower than the three-helical $Z_{HER2:342}$. Later, this molecule was further modified by conjugation of DOTA to the N-terminus [40]. SKOV-3 tumor uptake of ^{68}Ga-DOTA-$Z_{HER2:342}$-MUT-DS was 4.1% ID/g and the T/B ratio 6.9 at 2 h p.i. Tumor uptake exceeded the uptake in all other organs and all time points except the kidneys. However, at 2 h p.i. the two-helix variant had a 3-fold lower tumor uptake and a more than 4-fold lower T/B ratio compared to ^{68}Ga-ABY-002. Thus, the original three-helical Affibody molecule showed an overall better performance than the two-helix variant, indicating that helix 3 is not only important for structural stability but also for the overall performance of Affibody molecules in tumor targeting and imaging.

The three-helix Affibody molecule $Z_{HER2:342}$ was recently further improved by substitution of 11 amino acids in the scaffold. These substitutions resulted in higher hydrophilicity, higher thermal stability, diminished background interactions with immunoglobulins, full production flexibility as well as fully retained in vitro and in vivo functionality [33]. The resulting novel HER2 Affibody molecule was designated $Z_{HER2:2891}$. Further coupling of maleimido-DOTA to the C-terminal cysteine led to the new lead candidate drug for molecular imaging [MMA-DOTA-Cys^{61}]-$Z_{HER2:2891}$-Cys (ABY-025). Pre-clinical characterization of ABY-025 revealed no toxicity in rats and cynomolgus monkeys and no induction of antibody formation upon repeated administration in rats [41]. Approval for clinical trials has been obtained in Germany and Sweden.

Recently, ^{57}Co, a long-lived gamma emitting surrogate for positron emitting ^{55}Co was used for labeling of $Z_{HER2:2395}$-Cys [42] and showed favorable targeting of LS174T and SKOV-3 xenograft tumors detected using gamma camera.

^{18}F is currently the most commonly used positron emitter for PET studies. However, ^{18}F-labeling of small proteins and peptides was not very well-established. Thus, two new methods, both using different linker molecules containing a thiol-reactive maleimide group were developed for site-specific ^{18}F-labeling of Affibody molecules. The first published method used *N*-[2-(4-[^{18}F]fluorobenzamido)ethyl] maleimide (^{18}F-FBEM) for site-specific conjugation to the C-terminal cysteine of HER2-binding Affibody molecules to yield ^{18}F-FBEM-$Z_{HER2:342}$ or ^{18}F-FBEM-$Z_{HER2:2395}$ [43,44]. The second method used a bifunctional linker having a thiol-reactive maleimide group on one end and an aldehyde-reactive aminoxy group on the other end to obtain $Z_{HER2:477}$-ONH_2 and $(Z_{HER2:477})_2$-ONH_2 [45]. The aminoxy-functionalized Affibody molecules are labeled by conjugation of 4-[^{18}F]fluorobenzaldehyde (4-^{18}F-FBA) via formation of a stable oxime bond to obtain *N*-(4-[^{18}F]fluorobenzylidene)oxime ((^{18}F-FBO)-$Z_{HER2:477}$). Efficient tumor targeting was obtained with Affibody molecules ^{18}F-labeled by either method with a tumor uptake of 9.7% ID/g at 1 h p.i. for ^{18}F-FBEM-$Z_{HER2:342}$ and 4.8% ID/g 1 h p.i. for ^{18}F-FBO-$Z_{HER2:477}$. The T/B ratio was 7.5 and 3.2 for ^{18}F-FBEM-$Z_{HER2:342}$ and ^{18}F-FBO-$Z_{HER2:477}$, respectively. The amount of radioactivity in liver and kidney as well as the radioactive amount in the intestine was considerable higher for ^{18}F-FBO-labeled Affibody molecules, which lead to PET images with strong signals in the gastrointestinal tract.

Magnetic resonance imaging (MRI) is a powerful tool for in vivo anatomical and molecular imaging. Superparamagnetic iron oxide (SPIO) particles have been investigated as label for targeting vehicles to increase imaging contrast. Recently, a HER2-targeting Affibody was coupled to SPIO particles and shown to target tumors. Tumor imaging contrast was seen only in animals treated with targeted SPIO particles, demonstrating imaging utility [46].

Taken together, Affibody molecules can be labeled with a large variety of radionuclides. Thus development of Affibody based tracers both for SPECT and PET is possible. The first clinical use of Affibody molecules showed that high quality SPECT and PET images could be obtained within a few hours after injection. Thus Affibody molecules are well adapted for practical clinical use, where image acquisition on the day of administration is preferred over the imaging modalities one or several days after administration as demonstrated for antibodies or fragments thereof.

4. Optical imaging

In vivo optical imaging is rapidly being developed into a preferred analysis method in many labs for studying global receptor expression in live laboratory animals. One of the reasons is that the method facilitates study of the same labeled probe both in vitro in microscopy and flow cytometry, as well as in vivo, using mouse-imaging equipment. Importantly, optical labels provide an alternative to radioactive methods, which may be difficult to work with for many labs. Work with radioactivity for imaging need dedicated facilities, as well as expensive equipment like micro-PET or micro-SPECT devises. A disadvantage with optical imaging is that the technique is at best semi-quantitative and that the sensitivity is lower. The lower sensitivity may be an explanation for a somewhat surprising finding by Capala and co-workers [47]. They compared a monomeric HER2-specific Affibody molecule, alone or as fused to an albumin binding domain (ABD), with the antibody trastuzumab, all labeled with Alexa Fluor 750 (a near infrared probe). In contrast to many imaging studies using radionuclide-conjugated Affibody molecules, the best contrast was seen with the ABD-fused Affibody molecule, not the monomeric one. A clear signal could be detected with the monomeric Affibody molecule at about 6–8 h after injection, but it was washed out from the tumor relatively rapidly while the ABD-fused construct continued to accumulate, providing a better contrast than both the monomer and the Alexa-labeled antibody at 24 and 48 h after injection. Presumably, a high total dose on the tumor (as is

the case with the ABD-fused Affibody molecule) may be more important for optical imaging than for radionuclide based imaging where high contrast is pivotal.

Recently, however, successful in vivo optical imaging with monomeric NIR-labeled Affibody molecules specific for EGFR have been reported [48]. The data suggest imaging usefulness of the monomeric format in tumors as small as 11 mg, and with detection of the tumor from as early as 1 h after injection up to 3 days. The Affibody molecule was compared with the natural receptor ligand, EGF, labeled with the same NIR probe. Up to 1 h, binding and uptake of both agents increased over time. After 1 h, binding and uptake of labeled EGF decreased, whereas the signal of the labeled Affibody molecule increased, to reach its highest level at 4–6 h. Although the signal was higher for the natural ligand EGF, EGF also induced receptor mediated signaling, which is an unwanted property for an in vivo imaging reagent. In contrast, at high concentrations, the EGFR imaging Affibody molecule compromised the stimulatory effect of EGF on EGFR, thus having a weak therapeutic effect if any.

One advantage of optical imaging is the possibility to use multiple colors for simultaneous differentiation of several receptors within e.g. a tumor. This principle has been shown using an HER2-specific and an EGFR-specific Affibody labeled with two different fluorophores, respectively [48]. In mice transplanted with both a HER2-expressing breast cancer and an EGFR-expressing epithelial cancer, the two Affibody molecules could distinctively discriminate between the two tumors in the same animal. NIR-labeled Affibody molecules thus seem to have the potential to become useful in image guided diagnosis and even surgery of either superficial tumors or ones accessible to an endoscopic camera.

5. Towards therapeutic applications

For therapeutic applications, the most advanced results are with Affibody molecules targeting a payload. This is a natural consequence following the detailed characterization of the molecule as an imaging agent. In imaging, the "payload" is already present as a radionuclide or fluorescent molecule allowing for detailed characterization of the organ distribution and fate of the protein. This knowledge is essential for therapeutic applications, as a challenge in targeted therapy is the balance between achieving a sufficient therapeutic effect on the target while minimizing undesired toxic effects in normal tissues. With a large targeting molecule like a protein, either the payload has to be very potent, or large quantities have to be delivered, for instance by using nanoparticles or liposomes, or by amplification using viral delivery. A number of strategies are being developed using Affibody molecules to achieve this goal.

5.1. Targeting payloads

An attractive and highly potent class of payloads is immunotoxins; hybrid proteins composed of an affinity recognition moiety (often an antibody fragment) and a toxic domain derived from plant or bacterial toxins [49]. Pseudomonas exotoxin A is a well characterized bacterial protein toxin with proven therapeutic efficacy in numerous pre-clinical models as well as in clinical trials. A fusion construct consisting of the HER2-targeting Affibody molecule $Z_{HER2:342}$ and a truncated version of the toxin, PE38, was produced and demonstrated to bind to HER2-expressing cancer cell lines, but not to HER2-negative ones [50]. Furthermore, immunotoxin induced protein expression inhibition was analyzed in cell lines having different levels of HER2 on the cell surface, and the therapeutic efficacy was correlated to the expression level. For cells having high HER2 expression, even 1 pM concentration of immunotoxin was sufficient to inhibit protein synthesis (ED_{50}) and affect cell viability [50]. In tumor bearing mice, 250 μg/kg HER2-targeted immunotoxin (5 μg/mouse) injected three or six times was sufficient to eradicate large established xenografts of breast cancer (Jacek Capala, NCI/NIH; F.F., personal communication). This suggests a therapeutic utility of the fusion protein and indicates that Affibody molecules should be useful targeting molecules also for other immunofusion proteins.

Another highly potent class of payloads is radionuclides. Targeted radionuclide therapy can in fact be a closely related extension of imaging efforts. The imaging information providing exact distribution data of a targeting vehicle can be used to estimate the delivered therapeutic dose to a disease target, and toxic side effects for normal organs. One example is the use of the HER2-specific Affibody $Z_{HER2:342}$ together with radiometals attached via a chelate. The molecule was studied for tumor imaging using ^{111}In as a gamma emitting diagnostic radionuclide and found to have excellent imaging properties [51]. In biodistribution studies it was however clear that the calculated dose to the kidney would be too high to allow for a therapeutic effort. Therefore, vesicular instillation in urinary bladder cancer was suggested as a new locoregional therapeutic regimen. Here, the small size of the Affibody molecule is believed to be instrumental to allow penetration of the bladder wall into the tumors. DOTA-chelated $Z_{HER2:342}$ was successfully labeled with the radionuclides ^{90}Y or ^{177}Lu, both used therapeutically in the clinic, and shown to have retained tumor cell binding specificity and a favorable biodistribution profile in non tumor bearing mice [52]. Further studies should now be undertaken to prove the therapeutic utility in relevant animal models.

One approach for systemic therapy using Affibody molecules is based on slow internalization of Affibody molecules into malignant cells [53]. Then residualizing properties of a radionuclide are not critical for good radioactivity retention in tumors, and non-residualizing radionuclides reduce renal radioactivity retention. Selection of an optimal radiohalogen labeling chemistry enabled the design of an ^{131}I-HPEM-$Z_{HER2:342}$-C Affibody molecule providing low uptake in kidneys [54] and demonstrating dose to tumor 2.6-fold higher compared to dose to kidneys. Another potential Affibody conjugate for systemic therapy is the ^{186}Re-labeled HER2-specific maGSG-$Z_{HER2:342}$. By utilizing the ability to chemically produce the Affibody molecule by peptide synthesis, and based on previous Technetium-studies, Orlova et al. could label the molecule with ^{186}Re and show a favorable biodistribution profile with low kidney uptake [55]. Preliminary dosimetry estimation suggests that ^{186}Re-maGSG-$Z_{HER2:342}$ should provide a fivefold higher dose to the tumor compared to the dose to kidneys and more than 300-fold higher compared to the dose to blood, which is very promising for therapeutic applications.

The most advanced example of use of Affibody molecules for radioimmunotherapy to date is using the same HER2-specific Affibody molecule as described above, but fused to a small albumin binding domain (ABD). The resulting fusion protein associates with the serum albumin of the host and therefore adopts a much slower kinetic profile, a dramatically decreased kidney uptake, and a fivefold increase of the delivered dose to the tumor as compared to the non ABD-fused Affibody molecule [56]. Treatment of mice bearing HER2-expressing SKOV-3 ovary cancer microxenografts with ^{177}Lu-Affibody-ABD fusion protein completely prevented formation of tumors, in contrast to mice treated with saline or a radiolabeled but non-targeted mock Affibody-ABD fusion protein. In an experiment with tumors with a medium level of receptor expression, there was a lower, although significant therapeutic effect, stressing the fact that the payload has to be very potent when the target is not highly overexpressed. Antibodies have been extensively investigated for therapeutic targeting of radionuclides [57]. Recently, Carlsson and co-workers used the HER2-binding antibody pertuzumab, also labeled with ^{177}Lu and using the same

SKOV-3 tumor model and therapeutic protocol as used for the Affibody-ABD fusion protein described above [58]. Very encouraging results were obtained, but in contrast to the Affibody experiment, with no curative effect. This could be due to a too low dose in general, but it is also known that antibodies often cause a high radioactive dose to the bone marrow due to their long circulation half-life, which limits the total dose that can be given. To reduce the dose to blood, antibody fragments with shorter half-lifes have been investigated. A therapy experiment with an yttrium-90 labeled HER2-specific antibody fragment in a 54 kDa diabody format (^{90}Y-CHX-A″-C6.5K-A diabody), resulted in a rather modest therapeutic benefit in SKOV-3 xenografted mice, possibly due to the radioresistant nature of this tumor model [59]. As could be expected for a small antibody fragment, onset of kidney toxicity was seen at long term follow up, which would make it difficult to increase the therapeutic dose of the diabody, thus limiting its therapeutic potential. As an alternative to fragments of antibodies, or albumin association for small biomolecules, PEG has been used to increase the half-life of biomolecules. Zahnd et al. used PEG to modify the kinetic properties of a high affinity HER2-specific DARPin molecule (14.5 kDa) [28]. Whereas the unmodified molecule might be used as an imaging candidate, high kidney values would prevent use for radioimmunotherapy. However, by modifying the DARPin with PEG60, a distribution profile was obtained that warrants further investigation for therapy. Taken together, radiolabeled Affibody molecules are noteworthy candidates as targeting carriers for therapeutic radionuclides.

5.2. Redirecting particles

To increase the impact of a targeted drug, one can assemble many drug molecules to be activated per targeting event, for example by using particles. There are different strategies to develop particles, including solid particles composed of polymers, vesicular particles like immunoliposomes, or viral particles. Affibody molecules are well suited to provide small particles with affinity-mediated recognition for cellular targets. Such recognition is believed to constitute an important step in increasing the specificity and delivered amount of payload to e.g. unwanted cancer cells. Alexis et al. used commercially available HER2-specific Affibody molecules in conjunction with chemical co-polymers to create nanoparticle bioconjugates for tumor cell targeting [60]. The investigators found that the uptake and cell killing activity was significantly increased for the HER2-targeted particles carrying the cancer drug paclitaxel, as compared to non-targeted ones in in vitro cell studies. In another study, Canine et al. used biopolymers to develop nanoparticles that mimic viral vectors [61]. Here, genetic engineering techniques were used to develop highly controlled, monodisperse amino acid-based biomimetic vectors. The biopolymers consisted of a DNA condensing motif, a fusogenic peptide for endosomal disruption, a nuclear localization signal (NLS) and a C-terminal HER2 targeting Affibody molecule for cell specific uptake. HER2-targeting nanoparticles, with a diameter of 80 nm, were made by mixing plasmid DNA encoding enhanced green fluorescent protein (EGFP) and the biopolymer at fixed ratios. The nanoparticles were stable in plasma and could selectively transfect HER2-expressing SKOV-3 cells but not PC-3 cells with low HER2 expression, as measured in flow cytometry analyzing expressed EGFP. The Affibody molecule thus mediated tumor cell specific uptake of genes in vitro.

5.3. Liposomes

Another type of particles for delivery of payloads are liposomes; small vesicular carrier systems into which drugs can be encapsulated. Thereby the pharmacokinetics of the drug is altered and it is protected from elimination and degradation, resulting in improved efficacy and safety. It was recently demonstrated that EGFR-specific Affibody molecules could serve as affinity determinants for stabilized liposomes when conjugated to a PEG-moiety suitable for insertion in the lipid bilayer of the liposome [62]. Compared to non-targeted liposomes, targeting led to internalization in EGFR-expressing cell lines and a selective increase in cytotoxicity towards EGFR-expressing tumor cells when using mitoxantrone-loaded liposomes. The increased specificity is interesting, as liposomes per se are large molecules and not necessarily efficiently targeted by the affinity ligand only, but also subject to many other parameters influencing targeting due to their large size. Another group working with stabilized Affibody-conjugated liposomes has addressed the challenge of opening the liposome when at the target site as a measure to increase specificity. The authors could show temperature-induced opening of the liposomes at 41 °C, which can be achieved in vivo using focused ultrasound. Here, the liposomes were conjugated to a HER2-specific Affibody molecule that mediated binding and internalization into HER2-expressing cells but not HER2-negative ones [63]. The notion that further specificity by a secondary controlled opening step may be needed in addition to tumor cell targeting is important. In fact, for many nanoparticle approaches, it remains to be seen if therapeutically relevant doses can be delivered to the intended site in vivo, while sparing normal tissue.

5.4. Viral particles

Human adenoviruses have been used as cancer gene therapy vectors, both in clinical trials and in pre-clinical experiments. To make them useful in a therapeutic setting, their native binding to normal tissues must be muted and new specificity must be engineered. This can be done by genetic fusion of a suitable ligand with viral capsid proteins. Affibody molecules have been explored as retargeting ligands in this setting as they, in contrast to most antibody fragments, conveniently fold correctly in the reducing environment of the cell cytoplasm. There are several reports on fully functional and re-targeted viruses using different Affibody molecules. Recently, an adenovirus with dual binding specificity by tandem incorporation of two different Affibody molecules into the virus fiber was reported [64]. The virus particles were demonstrated to specifically infect two different cell lines, expressing either of the targets and could potentially be useful for increased target specificity. Anti-virus antibodies pose a significant problem in virus therapy as they neutralize the infectivity of the virus. Interestingly, most pre-formed antibodies are directed against the knob structure and Affibody reengineered virus particles were reported to escape recognition of virus-directed antibodies [65,66]. Most reports on re-targeted virus particles using Affibody molecules have used a fluorescent reporter gene as read out for successful transfection. Recently however, therapeutic activity was demonstrated in vitro by Belousova et al., using virus particles re-targeted by HER2-specific Affibody molecules containing a thymidine kinase gene [67]. Subsequently, ganciclovir, which is activated by thymidine kinase, was added to the cells and a much higher therapeutic activity was seen with cells incubated with re-targeted virus than with particles having a wild type infection machinery. This suggests that adenoviral particles redirected by using Affibody molecules warrants further investigations towards use as therapeutic molecules.

5.5. Blocking applications

One advantage with a blocking targeting agent is that the effector mechanism is mediated directly in the binding action and no effector function needs to be engineered, which reduces the risk of toxic side effects to normal tissues. A major part of antibodies approved for clinical use today block protein–protein interactions.

In fact, to date only three antibodies are approved for payload therapy (Gemtuzumab ozogamicin, Mylotarg; Ibritumomab tiuxetan, Zevalin; and Tositumomab-I131, Bexxar), disregarding that antibodies have an intrinsic payload function in the form of Fc-mediated effector mechanisms. A number of different Affibody molecules have been isolated and characterized that hold the potential for blocking a therapeutically relevant protein interaction. The costimulatory signaling interaction of CD28 and CD80 between two cell lines was for example blocked by a CD28 specific ABD-fused Affibody molecule, showing that proteins even of this small size (12 kDa) efficiently can block cell–cell interactions [68]. Other Affibody molecules that bind cell-surface receptors and compete with the natural ligand include molecules specific for: CD25, competing with IL2 [69], IGF1R, competing with IGF-1 [70], EGFR, competing with EGF [71], PDGFR-β, competing with PDGF-BB (unpublished) and HER3, competing with Heregulin (unpublished).

Affibody molecules can also block soluble protein ligands as exemplified with a TNFα specific Affibody molecule that could block binding of the TNF ligand to the TNF-receptor [21,22] in in vitro studies. An interesting case is an Affibody molecule specific for the amyloid-β (Aβ) peptide involved in Alzheimer's disease [72]. According to the amyloid hypothesis, the pathogenesis of Alzheimer's disease is associated with the oligomerization and aggregation of the Aβ peptide into protein plaques. The structure of the Affibody molecule in complex with Aβ was solved by NMR, revealing that two Affibody molecules dimerize upon binding the peptide [11]. Interestingly, both Affibody molecules and the Aβ peptide undergo structure reorganization when binding, resulting in a barrel formation of the Affibody molecules, which in turn encapsulate the Aβ peptide. By burying the hydrophobic parts of the aggregation prone Aβ peptide, the Affibody molecule inhibits amyloid fibrillation [11]. In a recent study, the same Aβ-specific Affibody molecule, expressed in the brain of a *Drosophila melanogaster* model of Alzheimer's disease, eliminated neurotoxicity by increasing the clearance of Aβ from the brain [73].

Affibody molecules can bind and retain proteins in the intracellular milieu indicating a potential as a therapeutic tool for targeted gene therapy. When expressed intracellularly, Affibody molecules specific for HER2 and EGFR have been shown to bind their respective receptors in the ER and thus prevent them from being transported to the cell surface [74,75]. For a more general cell-blocking tool, perhaps an Affibody molecule specific for the transcription factor c-jun could be useful [76].

In summary, Affibody molecules can block a range of therapeutically relevant target proteins, both targets that antibodies can access and targets that are difficult to address with antibodies such as intracellular proteins.

6. Biotechnology

Due to its natural affinity for several antibody species combined with a robust structure, staphylococcal protein A has been used extensively for bioseparation of antibodies, e.g. in affinity chromatography, immunoprecipitation and other bead-based assays. In addition to the work on natural protein A, Affibody AB together with GE Healthcare have engineered improved variants of this protein, such as an alkali stabile version (denoted MabSelect SuRe) for increased tolerance to alkali treatment during regeneration in affinity chromatography.

Based on the same concept, Affibody molecules with new specificities have been evaluated as capturing agents on affinity chromatography columns for protein purification purposes, targeting a variety of different proteins including apolipoprotein A-1M [77], Taq polymerase [77], the G protein of human respiratory syncytial virus [78] and recombinant human factor VIII [79]. In even more challenging studies, specific Affibody molecules have been successfully employed for depletion of human proteins (e.g. transferrin [80], amyloid-β peptide [72] and human IgA [81]) from complex body samples, such as human serum, plasma and cerebrospinal fluid. Moreover, engineered Affibody-like molecules based on the Z-domain with introduced positive and negative net charge, respectively, have been fused as tags to various proteins for purification by ion exchange chromatography [82,83].

Even though affinity bioseparation has been the most extensively evaluated application, the small size and favorable biophysical properties of Affibody molecules have also brought them into other biotechnology areas traditionally dominated by antibodies. Various proteins have been constructed by genetic fusions with targeting Affibody molecules to enable facile detection of targeting in assays such as Western blotting. Exploiting the wide array of commercially available Fc-specific secondary detection reagents, Affibody molecules have been fused to the Fc part from IgG, resulting in straightforward detection of the targeted protein in Western blot experiments as well as improved apparent affinity due to the introduced bivalency [84]. Similarly, functional fusions of Affibody molecules to different enzymes (e.g. β-galactosidase and horseradish peroxidase) have been reported [85,86], providing colorimetric read-out using appropriate substrates as successfully demonstrated in ELISA, dot blot assays and immunohistochemistry. Taking the approach further, specific Affibody molecules, targeting human IgA and IgE, have been fused to proteins on the surface of bacteria, resulting in whole-cell analytical tools that could be used in for example agglutination assays or in whole-cell immunoprecipitations [87].

Related to the ELISA setup, Affibody molecules have also been investigated as affinity probes in protein micro-array formats. For example, in one such study dimeric Affibody molecules with affinity for IgA, IgE, IgG, TNFα, Insulin and Taq polymerase, respectively, were immobilized on thiol dextran microarray slides followed by incubation with fluorescently labeled analyte, revealing specific binding of respective target protein with no observable cross-reactivity and a limit of detection as low as 70 fM for the best performing Affibody molecule [88]. Affibody molecules were also evaluated as capture agents in a sandwich array format with unlabeled target protein and monoclonal antibodies for detection, demonstrating specificity in a complex serum or plasma sample [88]. Employing Affibody molecules in such sandwich assays for analysis of human samples might also be advantageous in a cross-reactivity perspective, since human anti animal antibodies are occasionally present which might lead to cross-linking of capture and detection antibodies, resulting in elevated background signals, hence avoided using Affibody molecules [89].

Fluorescence-based assays using Affibody molecules coupled to fluorescent molecules (either chemically or by genetic fusions) have been investigated. Gräslund and co-workers reported on specific conjugation of an Oregon Green 488 dye to a C-terminal cysteine of a HER2-specific Affibody followed by quantitative analysis of HER2 expression levels on cancer cell lines as well as cryosections of SKOV-3 xenograft tumors [86]. Analysis was performed using both flow cytometry (for cell lines) and immunofluorescent confocal microscopy (for both cell lines and cryosections of tumors). Similarly, Capala and co-workers recently published a study in which genetic fusions of fluorescent proteins to both an HER2 and EGFR-specific Affibody were used for quantitative measurements of corresponding receptor levels on cell lines and in tissue using both flow cytometry and confocal imaging [90].

Many tumor cells express various levels of the EGFR- and HER2-receptors. In an attempt to evaluate if Affibody molecules can recognize and with high specificity distinguish tumor cells with a special composition of EGFR and HER2 expression, bispecific Affibody molecules were produced that recognized HER2 and EGFR [13]. Simultaneous binding to two cell lines expressing either of

the receptor was shown both in microarray format and in real time cell–cell interaction analysis. The next step will be to show binding discrimination between cell lines having various level of receptor co-expression.

Affibody molecules have also been investigated as biosensors in FRET-based detection of analytes in solution. In one such study [12], two different Affibody molecules with affinity for human IgA and IgG, respectively, were produced by solid phase peptide synthesis, enabling site-specific conjugation of EDANS and NBDX fluorochromes at opposite ends of the Affibody molecules. Adding target protein to the double-labeled Affibody molecules resulted in a concentration dependent shift in fluorescence ratio, induced by binding of target protein and reduction in FRET between the acceptor and donor fluorophore. In a similar study by the same group [91], two different anti-idiotypic Affibody pairs, consisting of an idiotypic anti-target Affibody molecule and an anti-idiotypic Affibody molecule competing for the same binding site as the target protein, were used for detection of unlabeled target protein in solution. The Affibody molecules were conjugated to a FRET donor and acceptor dye, respectively, enabling intramolecular FRET when interacting. Following titration of targeted protein to the solution, the anti-idiotypic interaction between the Affibody molecules was blocked by competition, resulting in decrease in FRET signal.

Finally, demonstrating the versatility of this particular class of molecules, Affibody molecules with specific affinity for various DNA polymerases are today used in products for hot start PCR, marketed by Finnzymes OY. At room temperature, the Affibody molecule binds the polymerase, thereby acting as a polymerase inhibitor, ensuring reduced levels of non-specific PCR products. At polymerization temperature, the Affibody molecule is dissociated, resulting in regained activity of the polymerase and amplification of the intended DNA sequence. Here, the rapid refolding capability of Affibody molecules after heating procedures is of importance.

7. Nomenclature

Based on a series of publications by researchers at the Royal Institute of Technology (KTH), Uppsala University and Affibody AB, Affibody molecules have become a tool used by many research groups. The number of reports on Affibody technology is increasing rapidly, involving a large variety of similar, yet different Affibody molecules. A common nomenclature denoting these reagents is highly warranted. Affibody proteins were first described as binding proteins based on the Z-domain derived from domain B of protein A. The letter Z has ever since remained a "denominator", even though the second generation Affibody molecule scaffold of today differ significantly from Z [33]. We suggest keeping Z as description for an Affibody molecule in general. Next, we propose to include the name of the target against which the Affibody was originally selected, followed by a number. In early work, the number referred to the clone plate/sequence number, e.g. $Z_{HER2:4}$ indicating clone 4 of a HER2-binding Affibody molecule [31]. Today, numbers refer to a serial number used at the company Affibody AB and collaborators. Here, in addition to newly selected molecules, also a change in the amino acid composition within the basic 58 aa sequence will result in a new number. Published variants of the affinity matured HER2-binding Affibody molecule derived from the original clone $Z_{HER2:4}$ are for example $Z_{HER2:342}$, $Z_{HER2:477}$, $Z_{HER2:2395}$ and $Z_{HER2:2891}$ (Fig. 2).

Sometimes multimeric constructs are desired, especially Affibody dimers. To name an oligomer, we propose that the name of the underlying molecule is put in brackets, and a number indicating the oligomeric state is used outside of the bracket. Thus, a dimer of $Z_{HER2:342}$ would be denoted $(Z_{HER2:342})_2$.

Finally, Affibody molecules are readily used as fusion partners with other molecules, such as enzymes, Fc-portion of IgG, albumin binding domains, bacterial toxins among others. We propose to simply add the name of the fusion partner N- or C-terminally of the Affibody name depending on its position in the molecule. For example, Tolmachev et al. [56] reported on the therapeutic application of a protein construct where an albumin binding domain (ABD) was fused N-terminally to a dimeric HER2-binding Affibody molecule, and thus the name would be: ABD-$(Z_{HER2:342})_2$. In a recent work, ABY027 is composed of an N-terminal monomeric second generation HER2-binding molecule fused to an affinity matured ABD and with a c-terminally cys-conjugated DOTA, hence named: $Z_{HER2:2891}$-ABD_{035}-DOTA conjugated from $Z_{HER2:2891}$-ABD_{035}-Cys (F.F., V.T., manuscript in preparation).

We propose this nomenclature to be used throughout the paper, especially if the study involves more than one Affibody molecule, and otherwise at least in the materials and methods section. In the materials and methods section, we propose that also purification tags and similar extensions are indicated,

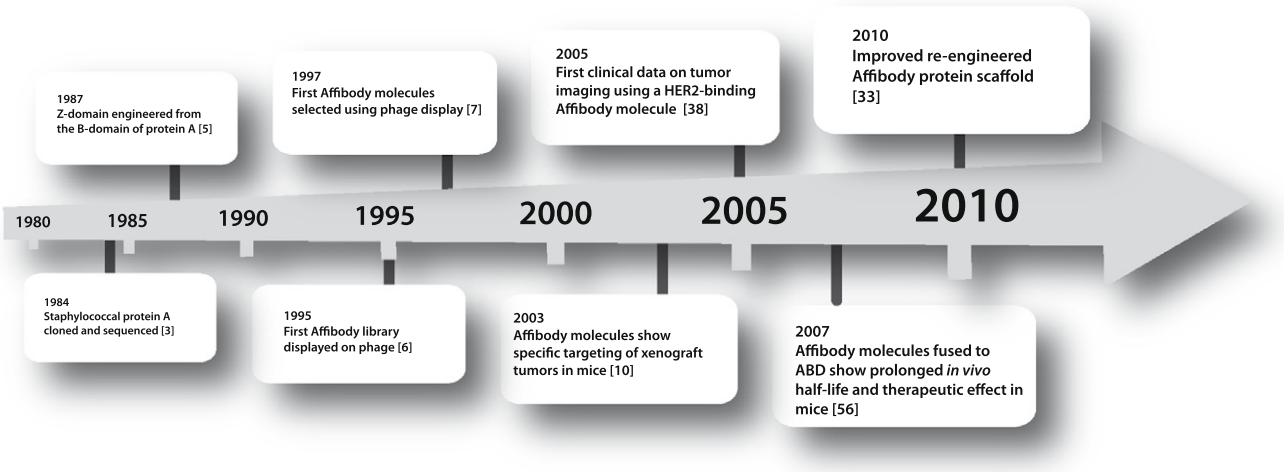

Fig. 4. History of Affibody molecules. Please note that the years in the text boxes for each event are for simplicity reasons the years of publication, except for the first clinical data as well as for the first in vivo targeting in mice where the years correspond to the years that the actual experiments were carried out.

although they may be omitted in the rest of the text for readability.

8. Conclusion and outlook

In conclusion, Affibody molecules represent a class of useful affinity proteins that have been investigated for a multitude of applications over the last 15 years, including use in therapy, in diagnostic imaging, and in biotechnology (Fig. 4). They have been successfully tested for targeted diagnostic utility in cancer patients with HER2-expressing metastases, and they are used as affinity ligand in an IgG affinity purification column with annual sales of tens of million dollars. While many different binding members have been explored for biotechnological use, several Affibody molecules with different specificities have been used for in vivo purposes. Most early work on in vivo targeting has been done with HER2 and later EGFR-targeting Affibody molecules. Interesting features with Affibody molecules are their intrinsic small size, fast folding and simple but robust non-cysteine containing structure. As only 13 amino acid positions differ between binding members specific for different receptors and proteins, much of the knowledge and techniques on modulation and labeling of one Affibody molecule can be applied to another. Thus, labeling and characterization procedures for imaging purposes can be standardized, facilitating the development of imaging ligands. The same holds true for targeted therapeutic payload approaches that in addition could take advantage of target validation and preexisting knowledge obtained from molecular imaging studies.

Taken together, Affibody molecules have favorable properties for development of novel therapeutic, diagnostic imaging and biotechnological applications.

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