Engineering of Affibody molecules targeting the Alzheimer’s-related amyloid β peptide

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Till mina fina på Åsen
Abstract

In the field of biotherapeutics and diagnostics, small binding-proteins are emerging as interesting drug candidates. A promising class of molecules for such applications is the small non-immunoglobulin derived Affibody molecule. Protein engineering is a powerful tool to modify and improve certain properties of such binding-agents. In this thesis, strategies for protein engineering have been employed to generate Affibody molecules binding to various conformations of the aggregation-prone and neurotoxic Alzheimer’s-related amyloid beta (Aβ) peptide, for use in research and potentially therapeutic as well as diagnostic settings.

The first three studies of the thesis are based on a previously generated Affibody molecule (denoted Z_{Aβ3}) that targets non-aggregated forms of Aβ. It has been demonstrated that Z_{Aβ3} adopts a complex disulfide-linked dimeric structure upon binding to the Aβ peptide. In order to perform successful engineering of the binder, the first study successfully investigated a novel staphylococcal cell surface display technology for both library and rational design purposes. In the second study, affinity maturation libraries were created, based on a new truncated and dimeric single chain format of the Affibody molecule, and displayed on staphylococci for screening of high-affinity binders using FACS. An Affibody molecule (denoted Z_{SYM73}) with a 50-fold improved affinity (∼300 pM) was generated. As fused to an albumin-binding domain it demonstrated efficient capture of Aβ_{1−42} peptides at physiologically relevant concentrations from a complex mixture of biomolecules while simultaneously binding to albumin, a highly relevant function in a potential therapeutic setting. In the third study, the high-affinity Z_{SYM73} molecule was investigated in a preclinical study that aimed to prevent development of Aβ-related pathology in an APP/PS1 transgenic AD mouse model. The mice received three weekly intraperitoneal injections of 100 µg/dose of the Aβ-binding Affibody molecule for 13 weeks, starting at the onset of pathology development at 3-4 months of age. Behavioral assessment and histological evaluation demonstrated that the treatment significantly improved the working memory and cognitive functions of the mice, and that this amelioration was coupled with substantial reductions in amyloid brain burden. These promising results motivate additional investigations of the Affibody molecule as a therapeutic candidate.

In the fourth study, combinatorial protein engineering and phage display technology was used to generate a new set of Affibody molecules that bind a stable engineered mimic of wild type Aβ_{1−42} protofibrils. Several low nanomolar binders were isolated, which also recognized wild-type protofibril assemblies. Dimeric head-to-tail fusion proteins were created, with subnanomolar binding affinities and very slow dissociation rates. Mapping of the chemical properties of selected side-chains onto the Affibody scaffold surface indicated that the majority of the binders reflected the same binding surface, which presumably matched an established binding epitope on the protofibrils. These binders have potential to be used in several different future applications to target aggregated Aβ.

In the last study, a method for screening of new protein-based aggregation-inhibitors was developed. The platform was based on co-expression of a reporter and an inhibitor in E. coli. The reporter comprised the aggregation-prone Aβ_{1−42} peptide in fusion to green fluorescent protein (GFP). Expression of the reporter resulted in a low fluorescent signal. However, when co-expressed with a control Affibody-based inhibitor, the reporter was rescued from aggregation, resulting in an increased fluorescent signal. In a model FACS-screen, the Affibody-based aggregation-inhibitor could be successfully enriched from a large background of a non-inhibiting Affibody molecule. These results demonstrate the potential of the system to be extended to library applications for isolation of aggregation-inhibitors to different disease-related aggregation-prone proteins and peptides.
Taken together, the work in this thesis describes the development of Affibody molecules targeting both non-aggregated and aggregated forms of the Alzheimer’s-associated \( \text{A}\beta \) peptide, with promising results for use in both research as well as therapeutic and diagnostic applications.

**Keywords:** Affibody molecules, Alzheimer’s disease, AD, amyloid beta, \( \text{A}\beta \), combinatorial protein engineering, staphylococcal surface display.
Populärvetenskaplig sammanfattning


I de tre första studierna, har dessa tekniker använts för att förbättra en redan existerande Affibodymolekyl som binder till Aβ peptiden i monomerform och på så sätt förhindrar att den aggregerar till toxiska aggregat. Vi förbättrade Affibodymolekylen med avseende

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## Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABD</td>
<td>Albumin-binding domain</td>
</tr>
<tr>
<td>ABP</td>
<td>Albumin-binding protein</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid β</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen-binding</td>
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<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug administration</td>
</tr>
<tr>
<td>Fv</td>
<td>Fragment variable</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized metal ion affinity chromatography</td>
</tr>
<tr>
<td>KD</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>kf</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>kon</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>scFv</td>
<td>Single chain fragment variable</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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</table>
Structure of the thesis

This thesis is based on the work that is presented in the five appended publications and manuscripts. The thesis offers a perspective on the next-generation of biotherapeutic agents, and the technologies that enable generation of such protein-based tools. In this introductory section, the objectives of the thesis are presented, followed by an outline of the eight chapters and a list of the papers that the work is based upon.

Objectives

The general aim of this work has been to use protein-engineering strategies to develop Affibody molecules that target various conformations of the Alzheimer’s-related amyloid beta (Aβ) peptide. Towards these ends, methods for directed evolution and rational design have been employed to generate and modify such binding-molecules. In studies I-III, the aims were to engineer, and assess the effects of such engineering, of a preexisting Affibody molecule that targets non-aggregated forms of Aβ. In study IV, the aim was to generate Affibody molecules targeting aggregated protofibrillar forms of Aβ. In study V, a new method for generation of protein-based binding-molecules with capacity to inhibit aggregation of aggregation-prone peptides and proteins was developed. The specific aims of each study are presented in greater detail in chapter 7.

Thesis outline

The eight chapters of the thesis offer a general and theoretical overview of what next-generation biotherapeutics are and how these can be developed, with emphasis on the methodology used in this work.

In chapter one, proteins are introduced in the contexts of how they can be involved in severe diseases, and how they have emerged as attractive candidates for therapeutic treatment as
well as in medical imaging applications for in vivo diagnostics.

The second chapter presents the concept of protein engineering, which can be used to improve certain characteristics of a protein for various purposes. The chapter introduces two approaches, rational design and directed evolution, which have both been employed for modification of proteins in this work.

In chapter three, an overview of different microbial-based display technologies for protein engineering is provided. Such platforms have been used for screening of protein libraries to enable identification and isolation of affinity proteins with desired traits.

In the fourth chapter, the term affinity proteins is introduced. The chapter elaborates on the naturally evolved antibody class of proteins, being the archetype of affinity proteins in both research and medicine, and how it has paved the way for the development of engineered alternative scaffold proteins. Affibody molecules are one such class of alternative binding-molecules, which are central to the work of this thesis.

The fifth chapter provides an overview of one of the most devastating and fatal neurodegenerative disorders throughout history, Alzheimer’s disease. The underlying molecular mechanisms of the disorder are currently not fully understood, and partly therefore, treatments to cure or reverse the disease progression are still lacking. The most established theory for development and progression of disease pathology is known as the amyloid cascade hypothesis. Based on this theory, the chapter provides a brief description of the principles for different therapeutic strategies, and an overview of on-going interventions for treatment of the disorder.

The sixth chapter describes a preexisting Aβ-binding Affibody molecule (denoted Z_{Aβ3}) that binds non-aggregated forms of Aβ. The first three studies of this work are based on the Z_{Aβ3} Affibody molecule and improved versions thereof.

In chapter seven, the work of the appended publications and manuscripts is reviewed.

Lastly, in chapter eight, some final concluding remarks on the work is presented.
List of appended papers

The thesis is based on the work presented in the five articles and manuscripts listed below. Full versions of the papers are appended at the end of the thesis.


*These authors contributed equally to this work.

All articles are reproduced with permission from the copyright holders.
Other scientific contributions, not included in the thesis


*These authors contributed equally to this work.
Proteins in therapeutic applications

Proteins are known as one of the main building blocks of life, as they are involved in nearly every cellular process in living organisms. Proteins have a huge functional variability, which includes mediating signaling between cells, protection against pathogens, catalysis and regulation of biochemical reactions. Because of their importance, proteins are also central in the mechanism of many disorders. A small error in a single protein can be enough to cause a severe and fatal disease [1]. Over the last decades, proteins have also emerged as attractive candidates for treatment of different disorders, as they possess several attributes that can be favorable in therapeutic and diagnostic settings [2]. This first chapter of the thesis provides an overview of the protein domain and how it can be used in treatment of various diseases.

Proteins as disease markers

Within each nucleic cell in the human body, approximately 20,000 protein-encoding genes, within the deoxyribonucleic acid (DNA), carry all information that is required for protein production and composition [3, 4]. The genetic code is built by sequential arrangement of four bases [5, 6], of which triplets translate into specific amino acids that are strung together into a polypeptide chain that build up the encoded protein. There are 20 standard, natural amino acids that can build up a protein. Each of these building blocks have distinct chemical and physical properties [7]. The order of which amino acids are incorporated into the polypeptide chain is of great importance, as it dictates the structure, properties, and functions of the folded protein [8]. In most proteins, local regions of the polypeptide chain spontaneously fold into energetically favorable secondary structures, e.g. $\alpha$-helices or $\beta$-sheets [9–11]. Secondary structures of the polypeptide chain are further arranged in space, providing the protein with a global tertiary structure. The major driving force of this later process is to hide hydrophobic residues in the protein core in order to shield them from the surrounding water molecules, since exposure of non-polar groups to solvent is entropically costly. Proteins that consist of two or more polypeptide chains finally associate
Proteins in therapeutic applications

and arrange their subunits into a quaternary structure, providing the protein with its final function (Figure 1.1) [9–11]. A number of proteins also undergo additional modifications, such as glycosylation or phosphorylation, in order to attain the right functional properties. This flow of information is known as the Central dogma [3].

Figure 1.1: The different states of protein structure. (a) The amino acid sequence of a protein is called the primary structure. (b) The polypeptide chain of amino acids is typically organized into sections of \( \alpha \)-helices and \( \beta \)-sheets, making up the secondary structural elements of the protein. (c) Secondary structures are organized into a tertiary structure, and (d) several polypeptide chains with their tertiary folds can be combined into a quaternary structure.

While the function of proteins depend on their three-dimensional structure, their biological roles commonly depend upon different types of interactions. Protein-protein interactions have essential implications in almost all cellular signaling networks, where they operate to maintain cellular structure, regulate gene expression, transport molecules across biological membranes, etc. [12]. A malfunctioning protein may disrupt the regulation of this complex and delicate network, and hence result in various defects or diseases. Malfunctioning can arise from inherited or acquired gene variations, or abnormal amino acid modifications. Such changes may result in the loss of a critical function, structural alterations, functional dysregulation, inappropriate interactions, or affect the folding process of the protein [13,14], which can lead to diseases such as amyloidosis [15,16] or cancer [17]. Today, it is recognized
that the protein domain is the ubiquitously most affected molecular domain in human disorders.

**Protein therapeutics**

Since the early 1980s, therapeutic proteins have revolutionized the pharmaceutical market and emerged as attractive alternatives to small chemical compounds for treatment of various disorders [18]. Generally, proteins possess several physiochemical properties that make them suitable as therapeutic modalities. For example, the high specificity of protein-interactions usually lead to fewer unexpected side-effects as compared to small chemical compounds, including less potential for off-target adverse effects [19]. In addition, proteins can generally perform more complex functions and exhibit longer *in vivo* half-lives. The unprecedented success of protein-based therapeutics has in large part been endorsed by advances in molecular biology and recombinant DNA technologies that has allowed proteins to be easily engineered for improved performance [20].

**Advances**

Early biopharmaceuticals that were approved by the US Food and Drug administration (FDA) for clinical use were variants of human proteins, which aimed to replace their lacking natural counterparts in certain disorders [21]. In 1982, recombinant *insulin* was the first human protein therapeutic that was approved for clinical use [22]. Over the years, the field of protein therapeutics has seen a transition from the development of recombinant versions of naturally occurring proteins to the design of new agents for optimal target recognition, pharmacokinetics, therapeutic function, and biodistribution [23–25]. Based on their mode of action, therapeutic proteins generally aim to *i)* replace a protein that is lacking or abnormal, *ii)* interfere with an existing pathway, *iii)* provide a novel function or activity, *iv)* interfere with other molecules such as pathogens, or *v)* deliver a compound or another protein such as a radionuclide, effector protein or cytotoxic drug [20].

Although the majority of the currently approved drugs are small molecules (approximately 1600), the estimated clinical approval success rate is higher for protein therapeutics [26,27]. Presently, there are over 200 commercialized protein-based therapeutics, diagnostics, and vaccines on the market [24], and more than 40% of all the pharmaceutical industry research is devoted to biopharmaceuticals [28]. Antibodies were early discovered as naturally evolved mediators of the immune system to tackle infections, and currently represent the most widely studied class of proteins for therapeutic use. Despite the many valuable char-
acteristics of antibodies in biotherapeutic applications, there is a large quest to develop even better next-generation protein drugs. The rationale for developing improved biotherapeutics comes from the convergence of clinical, scientific, commercial and technological drivers that collectively have identified unmet needs with current therapeutics. To address these requests, strengths of present antibody therapeutics provide a foundation upon which to build, whereas their limitations pinpoint several significant challenges that need to be conquered, including enhanced efficacy, greater safety, higher quality, improved delivery, and reduced costs of production and treatment.

Challenges

A critical consideration for clinical approval of a drug is related to the pharmaceutical properties of the agent. Criteria that need to be fulfilled when developing therapeutics for clinical use include high safety and efficacy, good quality (including stability and solubility), low immunogenicity, and an appropriate pharmacokinetics profile of the drug [20,29,30].

Immunogenicity is a unique risk factor for protein-based therapeutics, and is related to their inherent potential to induce an unintended and undesired immune response to the administered therapeutic protein itself [31]. Such responses can neutralize or diminish the therapeutic effect of the therapeutic protein, and might also compromise the safety of the drug [20]. Predicting clinical immunogenicity is challenging, as it can be influenced by both intrinsic factors (including the amino acid sequence, tertiary structure and post-translational modifications of the protein) and extrinsic factors (including the route of administration, formulation, dose and even the human individual). Therefore, much effort is put into strategies for predicting and reducing immunogenicity of candidate therapeutics, using e.g. in silico algorithms for predicting T cell epitope content [31].

The pharmacokinetic (PK) profile is also a critical factor, which differ protein drugs from small molecular drugs [32]. The PK profile refers to the relationship between absorption, distribution, metabolism and excretion (ADME) of a drug in a patient. Delivery of a protein drug to a patient is commonly performed by intravenous (i.v.), intramuscular (i.m.) or subcutaneous (s.c.) injections, whereas chemical drugs can be administered in more convenient ways, e.g. orally. Oral administration is typically not possible for proteins due to the high proteolytic activity and low pH in the stomach. Distribution of protein drugs in the body is usually limited to the vasculature and the extracellular compartments due to their large size, whereas small molecules generally distribute more easily by diffusion throughout tissues and into cells. The bioavailability of the drug (including distribution and half-life) can be a limiting factor for proteins as they are metabolized differently in the body [30,33,34]. The half-life commonly depends on the route of excretion. Proteins
that are smaller than $\sim 60$ kDa are generally cleared from the circulation through renal filtration (usually within minutes to hours), whereas larger proteins are mainly metabolized in the liver [35].

In addition to these critical factors, a candidate-drug can be faced with additional challenges that are related to the purpose of the drug. One such challenge, which is essential to the work of this thesis, is transfer across the blood-brain barrier (BBB) for treatment of neurodegenerative disorders (transfer over the BBB can also be an issue for treatment or diagnosis of tumors that originate or have metastasized to the brain.) Generally, proteins show a poor ability to permeate the specialized endothelial structure of the BBB, which can limit the efficacy of the drug [25,36–38]. It has been demonstrated that $\sim 0.1\%$ of a peripherally administered dose of antibodies can penetrate the BBB to reside in the brain [39].

All these different parameters are critically assessed in multiple in vivo preclinical and clinical investigations prior to potential approval of a drug. Notably, the majority of all candidate-drugs never reach the market as they fail to meet these essential criteria. Consequently, much effort is focused to improving their features using protein-engineering strategies [30].
Protein engineering

Over the last decades, the field of protein engineering has emerged as a tool to increase the functionality of proteins by reshaping or introducing changes to modify certain properties, such as molecular recognition, stability, and solubility. Due to the complexity of proteins, predicting proper sequence modifications to obtain a desired property can be extremely challenging. Different strategies for protein engineering have emerged that are commonly classified as either rational predictions or library approaches. These strategies typically include three fundamental steps: i) determining how a protein should be modified, ii) introducing such modifications, and iii) screening/selecting for variants with desired traits [40].

Protein engineering can often be considered a sub-discipline to the broader category of genetic engineering, as changes are typically introduced as mutations at specific positions in the underlying DNA sequence. This has been endorsed by the technological advancements in recombinant DNA technology, including enzymatic digestion and ligation of DNA as well as PCR to amplify DNA sequences. The corresponding proteins can thereafter be produced by exploiting the natural protein synthesis machinery of living organisms or extracts, using principles that result in a physical linking of the phenotype (protein) to its encoded genotype (gene). The effect of the introduced modification can subsequently be characterized and compared to the parental protein [40].

In the work of this thesis, combinations of both rational design and library strategies have been employed for engineering of Aβ-binding Affibody molecules. Consequently, this chapter provides an overview of both approaches.

Rational design

Rational protein engineering principles often rely on hypothesis-driven predictions of how changes in the amino acid sequence may affect the traits of the protein. Modifications can include a wide range of changes, including single amino acid replacements, insertions,
Protein engineering

deletions, and fusions to unrelated protein domains to add functions [41]. This approach is often effective when the three-dimensional structure and physiochemical properties of the protein are known, as such information offers valuable assistance to effectively locate key residues near active sites or in binding-interfaces [42–44]. Within this discipline, much progress has been made in defining three-dimensional structures of proteins by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, providing an increasing number of available structures that can be used for such purposes [42, 45–49]. In addition to sequence-based design strategies, computational simulations and algorithms for thermodynamic calculations of the most energetically favorable folding and interaction states have become valuable tools to effectively explore the impact of amino acid substitutions on protein structure and stability [2, 43, 50–56].

Nevertheless, these techniques for prediction cannot be universally applied. In cases when there is limited amount of information on the structure and mechanisms of the protein, alternative strategies can instead be exploited to help guide which parts of a protein to vary. Alanine scanning is one such method that elucidates the functional contributions of each amino acid by individually substituting them to a small non-charged alanine amino acid (which minimally disturbs the protein conformation) [57, 58]. Saturation mutagenesis is a similar but very laboursome approach, which samples all possible 20 amino acids at a particular position. The importance of each substituted amino acid is subsequently determined relative to the functionality of the parental protein [59, 60].

Rational design is a practical approach for design or redesign of proteins that have a relatively straightforward relationship between structure and function [56, 61]. The approach has extensively been used as an engineering tool for enzymes [42], and has been applied with reasonable success to manipulate the binding-interface in protein-protein interactions [62, 63], to improve pharmacokinetic profiles including increased stability, reduced immunogenicity, and reduced aggregation of biotherapeutic candidates [2]. However, despite major improvements in prediction-strategies and advanced computational algorithms, predicting proper substitutions for proteins that require more extensive engineering is still challenging. The challenge mainly reside in predicting how a large number of combined alterations will affect the folded state (which is affected by the sequence, backbone topology and amino acid side-chain conformation), the physiological function, and hence the desired improved function [43, 62, 64–66]. By any strategy for rational design, predictions and assessments are generally laborious and time consuming. The predicted mutants are typically produced and characterized one-by-one [43, 67], which often restricts the approach to a small number of variants.
Directed evolution

Library approaches for protein engineering has emerged as a powerful alternative to rational design. It dispenses with the requirement for structural and physiochemical knowledge about the protein to generate molecular diversity (even though such information is most useful), and allows a large number of mutants to be assayed simultaneously. The strategy is commonly termed directed evolution, as it relies on a process that resembles the natural evolutionary principle for proteins, wherein randomly occurring mutations result in the generation of new protein variants that become functionally tested by the environment to assess if the mutations have provided the protein with an advantage or not. In general, combinatorial strategies are based on two key elements: i) diversification of the parental domain to generate a large pool of up to billions of variants that differ in the amino acid composition (denoted library), and ii) iterative high-throughput assaying and isolation of candidates with desired traits using a robust screening/selection platform (described in the next chapter). Library approaches are not unique for protein engineering, but have been used for chemical libraries of small molecules in combination with different screening techniques, and are routinely used in early phases of drug discovery [42,68].

The genes encoding a protein library can be derived from natural sources or synthesized in the laboratory [69–71]. Natural library sources can for example be generated by isolating antibody encoding genes from either naïve non-immunized B-cell pools, or pools that have been pre-enriched by immunization with an antigen [69]. In contrast, synthetic libraries are generated in vitro by diversification of the protein-encoding template gene by different means, including error-prone PCR (epPCR), shuffling and codon-based diversification (these strategies are covered in the next section). Synthetic strategies provide a large flexibility and possibility to tailor the design of the library [72]. It is generally recognized that the probability to identify a protein variant with improved traits, such as improved affinity, increases with the complexity of the library [73]. Importantly, this requires high-throughput selection/screening methods in order to be able to cover the large library sizes. However, many selection platforms are limited by their transformation frequencies and/or screening capacities. Therefore, to increase the chances of identifying variants with improved features, combinatorial libraries can often be created with elements of rational design to direct the mutagenesis to specific positions or a certain part of the protein, e.g. a protein-protein interface [42,74]. Integration of the two approaches is often referred to as semi-rational or knowledge-based library design, and tailoring the library design in this fashion enables the possibility to choose which positions to include in the diversification process, including the type of amino acids to allow in the randomization, and what type of degenerate codons that should be allowed in these positions. Such a combined strategy usually reduces the final library size while increasing its functionality [72,74,75]. In studies
Protein engineering

I, II and IV of the thesis, semi-rational approaches are explored for protein engineering of Affibody molecules.

Methods for diversifying libraries

Generally, methods for creating genetic diversity can be divided into three categories: random mutagenesis, DNA recombination, and focused mutagenesis. Within each category, a large variety of techniques have been reported with different degrees of technical difficulty, cost effectiveness, and library quality [74]. The most commonly employed methods are described below, whereof site-directed methods have been employed in this work.

Error-prone polymerase chain reaction (epPCR) is the most common method to introduce non-targeted, and hence random, diversification. Point or multiple mutations are randomly incorporated into a template gene by mutagenic PCR strategies, using a polymerase with low proof-reading to enhance the error rate throughout the newly synthesized gene fragments [76,77]. Generation of libraries by random mutagenesis suffers some limitations, which can influence the final library diversity. Such limitations include that it is not possible to position the introduced mutation to a specific residue, but rather to a region, that the procedure might introduce stop codons, or unwanted amino acids, and biases due to natural codon degeneracy (certain amino acids are more likely to be incorporated as they are encoded by several codons, whereas other amino acids are only encoded by one codon) [78,79]. Another limitation with the method is that it most commonly only alters one base per codon, and consequently restricts the diversity per codon to a few amino acids. Nevertheless, important advantages of epPCR are that it is rapid and cost-efficient.

DNA recombination is another popular strategy to introduce random diversity into a protein. Such diversification is based on random fragmentation of genes that encode naturally homologous proteins, followed by reassembly of homologous sequences into full-length chimeric variants by PCR [80–82]. Shuffling is commonly applied to further diversify clones with desired traits that have been identified from epPCR mutagenesis strategies [71].

Site-directed diversification can be considered a semi-rational approach for generation of libraries, as it enables specific predetermined positions or regions to be altered. There are several different approaches for site-directed diversification. Library genes can be synthesized using different mixtures of the four nucleotides for incorporation at the three positions of the randomized codon [41]. Depending on the mixture that is used, i.e. if using codons that are either fully randomized or restricted to certain codon combinations, different diversities of the library can be generated. Fully randomized codons contain any of the four nucleotides at all three positions in the triplet, i.e NNN, where N = equal mix of A, T, C, and G. Such a redundant mixture covers all 64 codons of the genetic code, including the
three stop codons. Approaches for restricted diversities are often employed to reduce codon redundancy and the biases that can be observed with NNN codons. Using e.g. NNK (K = G or T), the redundancy can be reduced to 32 different codons, including one stop codon, which cover all 20 amino acids. Even though the redundancy is lowered with NNK, it is not optimal since codons for certain amino acids are still represented to a higher degree than others.

In recent years, the use of pre-made trinucleotide blocks (instead of single nucleotides) has become more predominant in the synthesis of library genes. It provides full codon control over the diversification since only desired codons are used [83]. In principle, a mixture of pre-made desired codons (according to the customized needs) is used in the library synthesis and added in one step for the particular randomized position. The step-wise added trinucleotide blocks are assembled into a gene on a solid-phase by sequential ligation [79, 84–86]. Hence, the use of trinucleotides provides full control of the diversification process as it avoids degenerate codons and allows the ratio of the amino acids introduced at any position to be pre-decided. The Slonomics® approach is one such novel method, which has been used for library synthesis in studies I, II and IV.
Selection systems

Library-based protein engineering resembles the natural evolutionary process of diversification, selection and amplification of variants with desired traits. As described in the previous chapter, there are several methods to create large and diverse protein-encoding DNA libraries. In order to generate and identify desired protein-candidates from the large pool, directed evolution relies on technologies that i) enables physical linkage of the phenotype (protein) to the encoded genotype (genetic information), and allows subsequent ii) screening and physical separation of proteins with desired traits based on certain binding or enzymatic-properties in an iterative enrichment process.

There is a plethora of available systems with distinct features that make them more or less suitable for different approaches of selection. The systems are generally classified as i) cell-dependent, ii) cell-independent, or iii) non-display systems. Cell-dependent platforms usually exploit the natural machinery of microbial organisms (including phage, bacteria, yeast and mammalian cells) for transcription, translation and display of the recombinant proteins. Depending on the host, libraries of $10^7-10^9$ protein variants can be generated. In studies I, II and IV of this thesis, staphylococcal cells and page particles were utilized for display of recombinant library proteins. In cell-independent systems, e.g. ribosome display, a key feature is that library genes are transcribed into mRNA and subsequently translated into proteins in a cell-free environment using cell extracts. Library sizes of $10^{12}$ variants are commonly generated using such platforms [87,88]. Non-display systems, such as protein fragment complementation assays (PCA), relies on co-expression of a library member and the target in the same cell, which are each genetically linked to one half of a so-called split-reporter-protein. The interaction of a library protein and the target brings the two halves of the reporter protein in close proximity, promoting the reconstitution of the reporter protein-activity that can be monitored by various means [89–91]. One such type of system was developed in study V.

Due to the major scope of this thesis, only cell-dependent systems are further elaborated on in this chapter, with emphasis on the technologies used in the studies.
Selection systems

Cell-dependent systems

There are several available cell-based methods for directed evolution, including e.g. phage, yeast, mammalian or bacterial cells. These methods all follow the same essential steps, including cloning of the library genes into a host-specific expression vector for subsequent transformation into the host (typically by electroporation), expression of the protein-library on the surface of the host, incubation of the displayed library with target molecules, screening or selection to enrich for interesting candidates, amplification of isolated clones between selection cycles, and lastly characterization of a set of candidates that have been isolated.

For optimal directed evolution performance, the method should ideally fulfill certain requirements, including i) functional display of the combinatorial protein library, ii) possibility to construct large libraries in order to increase the likelihood of finding the best candidates [69, 74], iii) possibility to apply a high selection pressure on the desired properties, such as affinity or function, iv) high-throughput, and v) no potential biases in expression levels or amplification rates. Additional characteristics of a selection system that are advantageous, although not essential, include the possibility to vi) perform additional diversification between selection cycles, vii) monitor the progress of the screening or selection process in order to facilitate potential optimization, viii) that the selection can be performed without need for special resources/instrumentation or requires complicated procedures, and that ix) the isolated variants can be easily amplified between selection rounds, as well as x) easily identified and characterized after the selection [92–96].

Phage display

Phage display technology was the groundbreaking selection system within the field of library engineering. The method was initially described three decades ago by Smith et al. for display of peptides on the surface of filamentous phage (Ff) particles [97]. Filamentous bacteriophages are a non-lytic family of bacterial viruses that infect Gram-negative bacteria bearing F-pili (mainly Ff bacteria such as M13, fd, and f1). The filamentous phage is rod-shaped, ∼ 900 nm long and 6-7 nm in diameter with a viral coat built by five coat proteins (pIII, pVI, pVII, pVIII and pIX) that encloses a circular single-stranded DNA molecule [98, 99]. The viral capsid is built primarily by ∼ 2700 copies of coat protein pVIII, and ∼ 5 copies of the four distinct proteins pIII, pVI, pVIII and pIX at the ends of the phage particle. The minor coat protein pIII is critical for infection of E. coli cells, since it interacts with the F-pili in order to inject the packed viral DNA into the host.
Selection systems

cell [98]. The principle of phage display is based on recombinant fusion of a protein to a capsid protein. In theory, this would be feasible for any of the capsid proteins present on the phage surface, enabling multivalent display of the recombinant protein on the phage surface. However, pIII has been the most frequent choice as fusion partner, followed by fusions to the major coat protein pVIII [69,100,101].

Protein-fusions to all five copies of pIII on an individual phage can potentially interfere with the ability to infect the *E. coli* host [100]. In addition, multivalent display might generate avidity effects during selection and thereby counteract the isolation of the strongest binders. To meet these limitations, the monovalent phagemid system was developed. In this method, the protein for display is fused to an extra copy of pIII that is encoded by an additional plasmid (phagemid) that lacks all the other phage genes, but contains a region necessary for packing into phage particles. Consequently, the system depends on helper phages that encode all the proteins that are essential for phage assembly and replication, to co-infect *E. coli* cells. Over the years, the phage and phagemid display systems have been subjected to constant improvements to enable functional display of full-length antibodies [102], various antibody fragments, including Fabs [103], and scFvs [104], as well as non-immunoglobulin scaffolds [105–107]. Libraries of naïve, immune and synthetic origins with library diversities of $10^{10}$ variants are routinely generated [100].

The most widespread strategy for selection by phage display is by a procedure known as biopanning. In this method, phage-displayed libraries enable selections against e.g. biotinylated target molecules through capture of target-binding clones on streptavidin (SA)-coated magnetic beads or SA-immobilized to a surface. The unbound variants are thereafter washed away to retrieve the bound population in a final elution step. The captured library genes in the eluted binders are amplified by infection of Gram-negative bacteria, such as *E. coli*, in order to provide an enriched population that can be reintroduced in the next selection cycle. The selection is repeated either by a default number of selection rounds or until an enrichment of specific variants is observed (Figure 3.1a) [96].

**Cell surface display**

Inspired by the robust and straightforward system for display on phage, alternative techniques have emerged during the last two decades, that aim to improve certain features of the screening/selection process. Cell surface display technologies have become attractive platforms, mainly because of the larger size of the cell and the multivalent display of library proteins on the cell surface, which enable high-throughput screening and sorting of target-binding cells by fluorescence-activated cell sorting (FACS) (Figure 3.1b).
Selection systems

Figure 3.1: Overview of the selection/screening process using a) phage display, and b) cell surface display.

In FACS, the cell-displayed library is screened for potential target-interacting variants, based on light-scattering properties and the amount of fluorescent markers on the surface of the cells. Fluorescently labeled targets that interact with cell-surface proteins are de-
Selection systems

tected by a laser, and the cells can be separated from non-binding cells by an electrical field. In principle, any property of interest that can be linked to laser light scattering or fluorescence can be measured quantitatively in real time by flow-cytometry (Figure 3.2) [108]. Compared to the phage display method, in which the selection is performed by a ‘capture and elution’ procedure, FACS screening and isolation permit selections to be conducted in a more controlled manner since it provides a real-time overview of the process [109].

**Fluorescence-activated cell sorting (FACS)**

![Fluorescence-activated cell sorting (FACS)](image)

**Figure 3.2:** Cell display in combination with fluorescence-activated cell sorting (FACS). FACS enables separation of cells that express target-binding library proteins on their surfaces from cells that express non-binding library proteins.

Furthermore, FACS offers the possibility to position the sorting gates and to use both negative and positive screens to control selection stringency, whereas the general biopanning strategies commonly lower the target concentration and intensifies the washing conditions between selection cycles for increased stringency. The multivalent display of library proteins on cells allows for a quantitative measurement of the affinity during a selection. Since the recombinant proteins are typically displayed in fusion to a reporter protein, the target-binding signal can be normalized against cell-to-cell expression level variations. Cell-
Selection systems

display methods also offer the possibility to perform on-cell characterization of the selected binders [93, 110].

Eukaryotic yeast cells provide the main platform for cell surface display systems. The eukaryotic system is considered beneficial for the display of proteins from higher organisms that require certain glycosylations or rely on disulfide bridges, as it promotes efficient oxidative protein folding and N-linked glycosylation. As first described by Boder and Wittrup in 1997, the recombinant protein is typically displayed in fusion to the Aga2p subunit of the agglutinin receptor, which attaches to the cell wall through disulfide bonds to the Aga1p subunit on the surface of Saccharomyces cerevisiae. A yeast cell typically displays $10^{4-5}$ copies of a library protein variant with an N-terminal hemaglutinin tag for normalization of expression levels in the flow cytometer, and a C-terminal c-myc tag for detection of correctly displayed full-length proteins or normalization purposes [94, 111, 112]. Due to limited transformation frequencies of library plasmids into yeast cells, typical yeast displayed libraries contain $\sim 10^5-7$ members [113, 114]. For these reasons, yeast display has been extensively used for affinity maturation efforts [93, 115–117] and epitope mapping [118]. However, libraries in the order of $10^{10}$ clones have been reported [113]. Several kinds of affinity protein libraries have been expressed on yeast, including scFvs from both immune and synthetic libraries [111], Fab-fragments [117, 119], and scaffold proteins [112].

Prokaryotic bacterial cell systems, mainly using Gram-negative E. coli as host strain, have also been well explored for display of libraries. E. coli cells have a rapid growth rate, high-transformation frequency, and there is a wide range of different strains and promoters that can be exploited. In E. coli surface display systems, the recombinant protein is fused to a natural surface protein that is capable of transporting the protein through the periplasmic space and across the outer membrane. Different carrier proteins have been employed for such display, including outer membrane proteins, flagella and autotransporters [108, 109]. However, the translocation of larger and more complex proteins through both the inner and the outer membrane of Gram-negative bacteria have been reported challenging [108]. Another attractive prokaryotic host for cell surface display is the Gram-positive Staphylococcus carnosus. This platform was exploited in the first two studies of this thesis and is therefore described separately in the next section.

Staphylococcal cell surface display

The use of Gram-positive bacteria for surface display of recombinant combinatorial protein libraries has been intensely investigated using Staphylococcus carnosus as host [120–123]. S. carnosus is a food-grade bacteria that is commonly found in fermented meat products, in which it is exploited as a component of the starter culture [125]. The bacterium
is GRAS (generally recognized as safe) classified, share little DNA homology with the pathogenic *Staphylococcus aureus*, and does not produce virulence factors such as protein A or enterotoxins [126]. Contrasting *S. aureus* and many other Gram-positives that grow in clusters, *S. carnosus* grows predominantly as single cells or in pairs [126]. This single cell growth pattern in combination with the size (0.5-1.5 \( \mu m \) in diameter), lack of secreted or displayed virulent factors, very low extracellular proteolytic activity, thick peptidoglycan cell wall that provides the cell with a robust surface, and single membrane that needs to be traversed for display are characteristics that make the bacterium a suitable host for surface display of heterologous proteins [109, 124, 126, 127]. Furthermore, these characteristics allow though selection conditions such as low pH, presence of added proteases, and high-speed FACS screening [108, 123, 128]. The first expression system for display of heterologous proteins on *S. carnosus* was first described two decades ago [129]. The system has been modified over the years to improve features including genetic stability [130], means to monitor cell surface expression levels [110], enhanced transformation frequencies through improved transformation protocols [131], and refinement of the expression vector through introduction of protease recognition sites flanking the recombinant protein both N-terminally [109] and C-terminally, along with a purification tag [132] to enable recombinant protein purification directly from the cell surface without the need for recloning.

*Figure 3.3:* Schematic illustration of the staphylococcal expression vector and display of recombinant proteins on the *S. carnosus* cell surface. 

**a)** The *S. carnosus* display vector.  
**b)** Covalent attachment of the displayed heterologous protein to the staphylococcal cell wall. Cells are labeled with fluorescent target and HSA to facilitate detection of target-binding candidates and normalization of the surface expression level in FACS.
production and purification of the protein [109, 120, 132].

The display vector (Figure 3.3a) exploits a i) constitutive promoter (P_lip) ii) signal sequence (S), and iii) propeptide (PP), that all originate from a Staphylococcus hyicus lipase gene, iv) the recombinant protein for cell surface display (Z), v) an albumin-binding protein (ABP) from streptococcal protein G to serve as a normalization tag, and vi) a conserved cell wall anchoring region (XM) that is derived from S. aureus protein A [129]. Upon translocation, the N-terminal of the fusion protein is secreted through the cell membrane whereas the C-terminus remains attached to the cell (Figure 3.3b). A specific peptide motif in the XM region is recognized by a cell wall anchored enzyme, which proteolytically cleaves the membrane-spanning C-terminal element and subsequently covalently attaches it to cross-linking peptides in the peptidoglycan layer [123,124]. After translocation to the cell surface, the signal sequence is cleaved off from the displayed fusion-protein, whereas the propeptide remains as a part of the displayed recombinant protein [133]. Moreover, the albumin-binding protein in the expression-cassette (consisting of three albumin-binding domains), introduces space between the displayed protein and the cell wall to improve accessibility, functions as an affinity handle for purification of cell-wall extracted fusion proteins, and provides means for flow-cytometric monitoring of the level of surface accessible fusion proteins, as well as normalization through its affinity to human serum albumin (HSA) [110, 123]. In addition to the expression cassette, the vector also contains an origin of replication derived from S. aureus and a chloramphenicol acetyl transferase gene for replication and selection in staphylococci. Moreover, in order to facilitate subcloning in E. coli, the shuttle-vector also contains an origin of replication for E. coli and a β-lactamase gene conferring ampicillin resistance.

Typically, $10^{4-5}$ copies of the recombinant protein are displayed per cell [132]. Fine affinity discrimination, through normalization against expression level [110], makes the S. carnosus display platform a suitable system for library applications. Successful display of particularly Affibody libraries has been reported [120,134,135]. Typically, libraries of $10^{6-7}$ variants are readily obtained, making the system specifically appropriate for affinity maturation purposes [120,122,134,135]. Recently, an affinity maturation effort of a Human Epidermal growth factor Receptor 3 (HER3)-binding Affibody molecule generated new binders in the low pM-range [120]. The largest library displayed on the surface of S. carnosus, containing $10^9$ different members, was recently generated through extensive transformation efforts (Jonsson et. al., unpublished data). Staphylococcal display has also been employed for epitope mapping of antibodies [136], engineering and library work of nanobodies [122], ADAPTs [121], and scFv fragments (Hu et. al., unpublished data). Taken together, the S. carnosus display system has several attractive features for protein engineering, which make it an interesting complement to the more established techniques described above.
Affinity proteins for molecular recognition

Many proteins participate in biological processes by recognizing and interacting with other biomolecules. The attractive forces that cause proteins to interact is a natural phenomenon known as \textit{affinity}. In order to both understand the function of various units in specific networks and to successfully design biotherapeutic agents with predefined properties, knowledge and understanding about this interaction is essential [14]. Antibodies have been the archetype of affinity proteins for more than a century. They can be raised to bind virtually any biomolecule with high selectivity and affinity. During the last decades, a trend towards development of smaller binding proteins with improved binding capacity, production, and reduced costs have emerged to meet practical limitations associated with the large format of the antibody molecule [137]. Even though such tailored binding proteins have raised great commercial expectations [138], antibodies still remain at the forefront of versatility and applicability in several areas of biotechnology, ranging from detection agents to biopharmaceuticals [100,139].

This chapter provides a description of advantages and challenges with the antibody class of molecules, which have paved the way for novel alternative affinity proteins. One such class of proteins, which the work of this thesis is based upon, are the small \textit{Affibody molecules} that are derived from staphylococcal protein A. Affibody molecules are described in greater detail in a separate section below. Moreover, strategies to arm the small alternative binding proteins with effector functions, in order to e.g. improve their pharmacokinetics profile for biotherapeutic efforts is discussed.
Antibodies

Antibodies, also denoted immunoglobulins (Ig), are a diverse set of affinity molecules that B cells naturally generate as a part of the humoral response of the immune system of vertebrates, as a defense towards a wide range of invading pathogens. Antibodies are bifunctional molecules that are able to interact with a target while simultaneously modulating several key immune responses [140]. The in vivo repertoire of antibodies is enormous, and principally, the immune system can generate antibodies to any molecule. Based on their exceptional properties of natural molecular recognition (which is applicable in many different areas of biological research) they have been investigated in medical diagnostics and therapeutics. There are five main antibody isotopes, which are classified based on the sequence of their constant region: IgG, IgM, IgA, IgE and IgD. IgG1 is the most abundant isotype in the circulation (approximately 75-85%) [32,141], and hence the most exploited class for research and engineering of therapeutic antibodies [32,142].

Antibodies are large and complex proteins, typically with a size of 150 kDa. They are composed of two identical heavy polypeptide chains (H) and two identical light polypeptide chains (L), which are linked and stabilized by 12 intra-domain and four inter-domain disulfide bonds (Figure 4.1) [143,144]. Each chain consists of a variable region (V) and a constant region (C). Molecular recognition is mediated by three hypervariable loops, denoted complementarity-determining regions (CDRs), at the amino-terminal part of each V_H and V_L chain. These regions are characterized by a high degree of amino acid sequence variation between different antibodies, and can adopt various conformations. The great diversity of the CDR regions is generated by random combinations of a number of variable gene segments in the germline of B-cells, followed by affinity maturation through a process called somatic hypermutation [145,146]. The affinity between the antibody and its antigen can be considered as the strength of the interaction between the CDR regions and the binding-site (epitope) of the antigen. The bivalent structure of antibodies allows them to bind two antigens at the same time, which may result in avidity effects and typically in a high apparent affinity, depending on the presentation of the antigen [147].

In addition to the specific antigen-binding properties, antibodies are responsible for recruiting several key immune effectors functions for elimination of an encountered antigen. Biological effector functions are mediated via interaction of the conserved Fc-domain with various immune effector pathways, including 1) binding to Fcγ receptors (FcγR) on the surface of immune effector cells that lead to for example antibody-dependent cellular cytotoxicity (ADCC), 2) phagocytosis [148–152], or 3) interaction with Cq1-component of the complement system, which through a cascade of events lead to complement dependent cytotoxicity (CDC) [148,149,151]. Another effect, due to the conserved Fc-domain, is the
Affinity proteins for molecular recognition

Figure 4.1: Schematic illustration of the domains of an antibody (IgG), a Fab fragment, a scFv, and a \( V_{H/L} / V_{H} \) single-domain antibody. The IgG molecule consists of two identical heavy chains (H) and two identical light chains (L), which are stabilized by several disulfide bonds. The heavy chain consists of three constant domains (\( C_{H 1-3} \)) and one variable domain (\( V_{H} \)), whereas the light chain consists of one constant domain (\( C_{L} \)) and one variable domain (\( V_{L} \)). The variable domains are responsible for antigen-binding and the Fc-domain for mediating effector functions by the immune system via the FcRn and Fc\( \gamma \)R binding sites.

Potential of antibodies to interact with the neonatal Fc receptor (FcRn) on endothelial cells and circulating monocytes, which influences IgG homeostasis resulting in prolonged serum half-life [32,153,154] and consequently prolonged therapeutic effect. Interaction with FcRn protects the IgG from degradation, and allows the molecule to remain within circulation from a few days up to four weeks in humans (depending on the isotype) [150,153]. The extraordinary long time in circulation is also attributed to the large size of the antibody, which exceeds the renal filtration cut-off of \( \sim 60 \) kDa [30]. These properties are advantageous and often central in the mechanism of action of therapeutic antibodies [150–152].

For very long, the immune system was the only available source for generation of antibodies of different kinds. Traditionally, antibodies have been produced through immunization of animals with an antigen, which produces a pool of polyclonal antibodies that can be
isolated from the sera. These antibodies generally recognize different epitopes on the antigen. In 1975, a method for generating monoclonal antibodies in vitro emerged through the hybridoma technology. In this technique, an antibody-expressing lymphocytic B-cell from an immunized animal is fused with an immortal myeloma cell to generate an immortal antibody-producing hybridoma cell [155]. Despite the fact that the hybridoma technology is broadly used for generation of antibodies, the method is relatively laborious. Furthermore, the hybridoma technology has been linked to some limitations for clinical use, including that the produced antibodies generally are of non-human origin and hence can induce an immune response in the human recipient. Strategies to potentially overcome such limitations have been developed by grafting the CDRs of antibodies produced by hybridoma technology into a human antibody framework [156]. As an alternative, today, monoclonal antibodies can be generated by the use of transgenic animals or through combinatorial protein engineering principles for selection from antibody libraries of either synthetic or natural origin (as previously described) [69,96]. Generation of antibodies by library engineering and selection technologies allow different types of engineering strategies and selection pressures to be applied, including selection for high stability and multiple specificities.

Limitations

Antibodies are excellent and powerful tools in many applications. However, the intact format of the antibody is linked to some restrictions and disadvantages in both production and utilization of the molecule. First, the antibody is a large multimeric protein with a complex structure that relies on several essential disulfide bonds and final glycosylation of the protein for function. In this context, production and manufacturing can be complicated and costly due to the need of mammalian expression systems [142,149,157]. Second, for certain applications, the large size might cause steric hindrance, which restricts tissue penetration (into solid tumors or poorly vascularized tissues). In addition, the generally planar binding interfaces make the binding to grooves and catalytic sites of enzymes difficult [158]. Third, only a fraction of the molecule is essential for interaction with the target, whereas the majority of the constant region of the antibody is responsible for functions that are only required in certain therapeutic applications (including for recruitment of effector functions and to maintain the pharmacokinetic profile) [96,148]. In other applications, e.g. for blocking of interactions, the constant region may not be necessary [148], and in fact, it can raise unpredictable and unwanted Fc-mediated effector functions that might lead to unwanted side-effects [159,160] (see chapter 5 for such examples that have been identified in clinical trials of Alzheimer’s disease). Moreover, the slow blood clearance may be disadvantageous in diagnostic molecular imaging applications, wherein it typically results in a decreased contrast (and undesired side-effects) [159,161,162]. Such limitations
have motivated the development of smaller domain-proteins that are based both on the antibody and other protein frameworks.

**Antibody derivatives**

The modular structure of antibodies has enabled genetic reformatting of the molecule into smaller and more stable units that are focused on the antigen-binding domain (Figure 4.1). These smaller fragments usually retain selectivity and affinity of the parental antibody but display different pharmacokinetic and biochemical characteristics relative to the larger format. In general, antibody fragments lack many of the limitations of conventional antibodies and can be produced more easily and economically in non-mammalian systems. Several antibody derived constructs have been developed and many are currently under evaluation for clinical use [163,164]. Antibody fragments were originally generated by enzymatic treatment of IgG, nevertheless, such methods have now been replaced by recombinant techniques.

The Fab fragment was one of the first reported antibody derivatives. The Fab fragment consists of the antibody V_H and V_L domains, linked to their flanking constant regions (C_H1 and C_L), generating a ~55 kDa protein [158]. The single-chain fragment variable (scFv) is a single polypeptide construct of ~28 kDa, in which the variable domains of the heavy (V_H) and light chain (V_L) are connected by a flexible polypeptide linker [158]. The Fab and scFv fragments are monovalent binders but can be engineered into multivalent binders to gain avidity effects [158,165]. Another type of antibody derivative is the single domain antibody scaffold. In nature, single domain antibodies have been discovered in distinct types of organisms, including camelids and sharks. These naturally evolved single domains are ~15 kDa in size and comprise a single heavy chain domain (V_HH and V_NAR, camelid and shark, respectively) to confer antigen-binding [158,166]. These domains commonly display long CDR 3 loops that are often larger than for conventional human antibodies, and are hence capable of more efficiently penetrating cavities such as enzyme active sites in target antigens [158].

**Alternative scaffold proteins**

Immunoglobulins are not the only class of proteins that can be employed for selective and tight binding of target molecules. This fact has inspired researchers to investigate small non-immunoglobulin based protein frameworks that may offer advantages relative the conventional antibody. In fact, scaffold proteins have been suggested to be able to substitute
Affinity proteins for molecular recognition

for most of the whole antibody-associated properties [106], and have been described as the missing link between small molecule drugs and antibodies since they bridge several of their respective strengths and limitations [167].

Properties of scaffold proteins

Most of the alternative binding-proteins that are used for engineering are naturally involved in protein-protein binding or catalysis, and show a large diversity in structure and function. Ideally, alternative scaffolds should be relatively small and composed of a rigid and stable framework in one single polypeptide chain. The scaffold should tolerate diversification of loops or surface exposed residues for generation of new target-binding variants and without loss of protein structure. Moreover, the scaffold should enable flexible engineering for generation of multivalent and multispecific constructs [168]. Additional desired characteristics include high chemical, proteolytic and thermal stability. The scaffold should also have a low degree of post-translational modifications, and facilitate high yield as well as cost-effective production by chemical synthesis or bacterial production [169]. A fold that is independent of disulfides is also advantageous, as the absence of intramolecular cysteines permits introduction of a unique cysteine for site-specific thiol-based modifications that can be of use in e.g. diagnostic applications [170,171].

In recent years, several scaffold proteins have been investigated for translation into biologicals with drug-like properties. Many candidate-drugs have entered clinical trials, with great expectations to make it into clinics [172]. The majority of these novel scaffolds have been developed against validated disease-targets that have proven to be effective in marketed targeted therapies. The rationale for such development is to increase the likelihood of achieving proof-of-concept as therapeutics in humans, and to decrease possible safety risks in the clinic [172].

The generally small size of alternative scaffold proteins makes them attractive agents for diagnostic imaging. The fast biodistribution enables efficient target-binding and tumor penetration as well as a rapid blood clearance, which allow for generation of high-contrast images [173]. Importantly, properties such as enhanced half-life and effector mechanisms that can be desired in therapeutic applications can be facilitated by engineering principles, e.g. by fusion or conjugation to albumin-binding domains (ABD) [170], Fc-domains [157], FcRn-binding domains [174], polyethylene glycol (PEG) molecules [175], albumin [176, 177], or through glycosylations [178]. It has been suggested that the robustness of several scaffolds may make them amendable to various routes of administration, which is also an important aspect to consider in therapeutic applications [139,172]. In this context, the low molecular mass of the scaffold protein is also an advantage since lower doses are
Affinity proteins for molecular recognition

generally required compared to administration of a larger antibody (for administration of equal molar amounts) [170]. Furthermore, for in vivo applications, smaller proteins and peptides generally tend to be less immunogenic than larger proteins [179]. However, efforts to decrease immunogenicity have been made by predicting and removing T cell epitopes, or fusing the protein to PEG-molecules [31,158,180].

Scaffolds and their classification

Approximately 50 protein scaffolds have been suggested over the years [181]. Generally, these are classified based on their structure (immunoglobulin-like or surface-randomized) and the strategies for engineering of their binding-sites. Immunoglobulin-like folds are based on a rigid framework and diversified loops for recognition, similar to the architecture of the antibody. Surface-randomized scaffolds instead have a compact structure with flat surface or cavity for diversification directly on the secondary structure elements of the protein [167]. Affibody molecules are one such class of molecules with a surface-randomized scaffold that are central to the work in this thesis. A more extensive description of Affibody molecules is provided in a separate section below.

Adnectins, also referred to as monobodies, are based on a single domain Ig-like scaffold that is derived from the tenth human fibronectin type III domain (10Fn3). Fibronectins are natural mediators of protein-protein interactions in humans [182]. The adnectins scaffold is a 94 residue (10 kDa) cysteine-free domain, that resembles the VH domain of an antibody with three randomizable CDR-like loops connecting seven anti-parallel β-strands [183,184]. Target-binding candidates have been generated with phage display, mRNA display, and several other systems [185]. Adnectins for therapy have entered clinical trials for treatment of e.g. glioblastoma (targeting VEGFR2), and for stimulatory effects for T cell therapy in patients with advanced cancer [186,187].

Anticalins, derived from the natural lipocalin protein family that participates in transport and storage of various compounds, are also based on an Ig-like scaffold. Anticalins are characterized by a rigid β-barrel structure (approximately 20 kDa) of eight antiparallel β-strands connected by four flexible loops. The flexible loops form an entry to a ligand-binding cavity, through randomization of 16-24 residues. The binding site of the anticalin molecule enables structural plasticity, and consequently interactions with both small proteins that can enter the barrel pocket, and large proteins that interact with the anticalin surface [188,189]. Anticalins have been developed for therapeutic purposes [189], and recently entered clinical trials for treatment of anemia (targeting VEGF) [190].

The Kunitz domain is a smaller Ig-like scaffold, with natural serine protease inhibition capability. The protein is composed of ~ 60 amino acids organizing into α-helices and
β-sheets that rely on three disulfides for structure [106]. Diversification is performed of residues on loops in the scaffold. The scaffold has successfully been used for generation of protease inhibitors of therapeutic interest [191]. In 2009, a Kunitz domain was the first engineered scaffold protein to reach clinical approval, for treatment of acute hereditary angioedema [192].

Knottins are an other class of cysteine-carrying molecules that are based on an extraordinarily stable scaffold that exists naturally in several organisms. Knottins of 30-50 amino acids fold into a ‘knot’ of antiparallel β-strands, which are stabilized by three disulfide bridges. The proteins demonstrate a very high thermal, chemical and proteolytic stability, which make them attractive for in vivo applications as well as alternative administration routes. Knottins have been generated for therapeutic purposes, and many have also shown promising results in in vivo imaging studies [193].

Designed ankyrin repeat proteins (DARPins) are based on a surface-randomized scaffold with origin in a human ankyrin repeat protein that naturally mediates in a large fraction of protein-protein interactions. The DARPin scaffold is composed of a 33 amino acid domain with a β-turn followed by two antiparallel α-helices and 7 randomized positions. Typically DARPins consist of four to six such connected domains (with an approximate final size of 14-21 kDa) that are flanked by constant C- and N-terminal capping domains, generating an extended concave binding interface that shields the continuous hydrophobic core. However, even larger repeat-proteins, and consequently also larger binding sites, can be generated by connecting a larger number of randomized domains [194-198]. DARPins have been evaluated for different applications, including tumor imaging and therapy [197, 199, 200]. Recently, a therapeutic candidate against VEGF entered initial clinical testing (http://www.molecularpartners.com/).

Affibody molecules

Affibody molecules originate from staphylococcal protein A (SpA), which is displayed on the cell surface of Staphylococcus aureus to bind to Fc and Fab regions of antibodies upon infection in order to escape a potential attack by the immune system (most probably). SpA contains five homologues Ig-binding domains (E, D, A, B, and C), all capable of binding the Fc part of antibodies from different species and subclasses [201]. All five domains have also been demonstrated to interact with the Fab domain of antibodies belonging to the V\textsubscript{H}3 subclass [202]. In 1987, the B-domain was isolated and engineered by two amino acid substitutions in order to increase resistance to hydroxylamine cleavage and to facilitate genetic cloning [203]. The improved protein domain, denoted Z, demonstrated retained Fc-binding, however, a significantly reduced affinity to the V\textsubscript{H}3 Fab region [202]. The Z-
Affinity proteins for molecular recognition

domain consists of a single polypeptide chain of 58 amino acids, which folds into a compact structure of three anti-parallel α-helices. The domain is highly stable, free of cysteines, has the fastest reported folding kinetics for a scaffold protein (0.3 μs) [204], and a high solubility that allows for inexpensive production in a prokaryotic host [92,205]. The small size and rapid folding kinetics also allow for solid-phase peptide synthesis, which results in material of high purity [206].

**Figure 4.2:** Schematic illustration of the Affibody scaffold (Z), derived from the 58 amino acid B-domain of staphylococcal protein A (PDB entry 2B88). Variation of thirteen surface exposed amino acids on helices 1 and 2 of the protein allows for generation of libraries.

The proven capability of molecular recognition of the Z-domain, in combination with several characteristics mentioned above, provide an ideal basis for combinatorial engineering of the binding-surface to generate mutants with binding-capacity for other proteins. In 1995, the
first combinatorial library based on the Z-domain was generated through randomization of 13 surface-exposed positions on helix 1 (seven positions) and 2 (six positions) (Figure 4.2). Nine of these positions are involved in the original interaction to the Fc-domain of an antibody [207]. Helix 3 has been found important for the stability of the molecule [208,209]. Engineering efforts for increased hydrophilicity, thermostability, and amenability to peptide synthesis, has substituted more than 40\% of the original bacterial sequence (including the randomized positions) [210]. Libraries are typically created by use of synthetic oligonucleotides, but other strategies have also been reported including error-prone PCR [211]. Members of the Z library are denoted Affibody molecules. Affibody molecules binding to a range of different targets, such as EGFR [212], HER2 [213], HER3 [214], CD28 [215], TNF-alpha [134], Aβ [216], VEGFR2 [135], HIV gp-120 [217], IL-6 [218], and IGF1R [219] have been isolated from various Affibody libraries using different types of selection platforms such as phage display [207], staphylococcal display [134], ribosome display [220], and protein complementation assay (PCA) [221]. Microbead display [222] and E. coli display [223] are also investigated for selections. The binding-surfaces of generated Affibody molecules comprise a typical protein-protein interaction area of 800-900 Å² [224]. The recognized surface on the target molecule generally tend to be flat and the structures of the Affibody molecules correlate with their library design [225]. However, one interesting exception to this is the Aβ-binding Affibody molecule (denoted Z_{Aβ3}) [226] that undergoes conformational change upon complex formation with the target peptide. Z_{Aβ3} is central to the work of this thesis, and is further described in chapter 6.

Applications for Affibody molecules
The simple and robust structure of Affibody molecules, together with their low molecular weight (~ 6.5 kDa) and lack of cysteines make them suitable for a wide variety of applications, including in bioseparations [203,227–230] and as detection reagents [231]. Affibody molecules are also suitable candidates for in vivo imaging in tumor targeting and have been extensively validated for such purposes [232]. The small size of the Affibody molecule provides good tissue penetration and rapid blood clearance, which in combination with strong and selective interaction enables high-contrast imaging of tumors for cancer diagnostics [170,233]. The fact that the Affibody protein can be efficiently produced by solid-phase peptide synthesis facilitates site-specific incorporation of various functional non-biological groups such as radionuclide chelators [170,206,234]. Promising results have been achieved using HER2 and EGFR specific Affibody molecules labeled with various radionuclides for in vivo tumor imaging [235–238]. A recent tumor-targeting diagnostic investigation of a HER2-binding Affibody molecule has shown promising results in imaging of HER2-expressing breast cancer tumors and metastases in humans [232].

Affibody molecules are also promising agents for therapeutics purposes. Generally, the
short *in vivo* half-life limits the therapeutic effect. However, recent studies have demonstrated that the pharmacokinetics can be modulated by fusion to an albumin-binding domain (ABD) to yield an *in vivo* half-life that is similar to that of albumin itself [170, 173, 239, 240]. In addition, it has been demonstrated that the ABD-fused Affibody molecule reduces kidney-uptake by 25-fold (high kidney uptake is typically associated with smaller proteins) and increases tumor uptake three-fold [173]. Moreover, the ABD reduces the immunogenicity of the fusion partner, and it has been shown that the fusion is well tolerated in repeated administrations [241]. Affibody molecules have been developed for investigations of several potential therapeutic applications, including blocking of protein-protein interactions [120, 218], to mediate cytotoxic effects via fusion to a truncated version of Pseudomonas Endotoxin A [242], and for delivery of payloads such as radionuclides [173, 238]. A $^{177}$Lu-labeled Affibody molecule fused to an albumin-binding domain (ABD) [173], and a $^{186}$RemaSGS-Affibody conjugate has demonstrated the potential for systemic therapy [238]. Recent preclinical studies with ABD-fused Affibody molecules for therapy of both HER3 overexpressing cancer and Alzheimer’s disease have demonstrated promising initial results (the latter is further discussed in chapter 7). Additional studies involving Affibody molecules for therapeutic applications include redirection of adenoviral particles [243] and nanoparticle- or liposome-based drug delivery [244, 245].
Towards treatments for Alzheimer’s disease

Alzheimer’s disease (AD) is the most frequent age-related neurodegenerative disorder, representing one of the largest global health challenges of our time. It is the leading cause of debilitating dementia, with about 35 million people affected worldwide. The disorder is characterized by progressive loss of memory and cognitive functions. A major factor contributing to the skyrocketing prevalence is the unprecedented number of aging persons that is projected to double by 2030 and triple by 2050 [246, 247]. AD has been a field of intense research for several decades. Despite the fact that the pathological features and risk factors of AD have been examined in detail, the underlying disease mechanisms still remain partly unclear. Consequently, methods for correct and early diagnosis, as well as development of curative treatments that stop or reverse the disease progression are still matters of intense research.

This chapter provides an overview of the most established theory for development and progression of the disease, known as the amyloid cascade hypothesis (ACH). Furthermore, an overview of the therapeutic strategies that are presently investigated in clinical trials for treatment of the disease is provided.

Alzheimer’s disease

In 1907, the German psychiatrist Alois Alzheimer was the first to describe the disorder. He presented a case study of a 51-year-old woman with paranoid delusions. At their first meeting, he portrayed her with memory impairment, and reading as well as writing difficulties. As the illness progressed, she developed hallucinations, lost the ability to communicate, suffered serious memory loss, lost motor coordination and spatial disorientation. Postmortem, Dr. Alzheimer described two principal types of brain pathologies: cerebral senile plaques (SP’s) and neurofibrillary tangles (NFT) [248], which still are used as neuropathological signatures for the disorder. SP’s are formed by progressive self-aggregation of amyloid beta peptides (Aβ), which accumulate around neurons. NFTs are formed by hyperphosphorylation of the microtubule-associated Tau protein in cell bodies and axons of neuronal cells.
Towards treatments for Alzheimer’s disease

(Figure 5.1a). These events are initiated years before onset of clinical symptoms [249], and provoke complement activation as well as production of inflammatory mediators [250] to actively eliminate accumulations. As the pathology progresses, the cognitive impairment becomes more severe and leave patients with full-blown dementia. End-stage AD patients are often bedridden and depend on custodial care, with death occurring on average 9 years after diagnosis [251].

Even though substantial evidence indicates that Aβ aggregates and Tau filaments are fundamental to the disease process [252,253], it is still not known whether they are causative or merely disease markers [254]. Based on these pathological hallmarks, several theories on the molecular causes have been suggested, which underpin the development of new potential treatments and identification of diagnostic biomarkers. For 20 years, the amyloid cascade hypothesis (ACH) has dominated the field of AD research [255]. This theory is based on several lines of research that indicate that the primary event in the pathogenic process of AD is the self-aggregation of Aβ peptides, and that this initiates a downstream complex pathological cascade of events, including a rise in NTFs inside neurons and neuronal projections that result in symptoms of the disease [256]. The work in this thesis is based upon the ACH.

The amyloid cascade hypothesis

Aβ production is a natural process in all individuals. Aβ peptides (∼ 4 kDa) are derived from the large transmembrane amyloid precursor protein (APP), which is expressed by a variety of cells throughout the body, with neurons in the brain being the main site for production. The specific biological roles of APP and Aβ peptides still remain elusive, however it has been suggested that APP is involved in neuronal development and survival [257–260] and Aβ monomers are involved in synaptic physiology by regulating synaptic scaling and synaptic vesicle release [260,261]. The metabolism of APP has alternative pathways, resulting in either amyloidogenic- or non-amyloidogenic processing. Processing in an amyloidogenic pathway involves sequential cleavage of APP by two membrane-anchored proteases. Initially, β-secretase (β-site APP-cleaving enzyme, BACE) cleaves the ectodomain of APP. The remaining membrane-associated stub subsequently undergoes an intramembrane cleavage that is mediated by γ-secretase. The γ-secretase site is composed of an ensemble of proteins, including prenecillin-1 (PS1) and prenecillin-2 (PS2), which are responsible for the catalytic activity [260]. The γ-secretase can cleave at different sites (referred to as γ, ζ, and ε) in the transmembrane region, whereby Aβ peptides of varying lengths (38, 40 or 42 amino acids) are liberated (Figure 5.1b). The precise site of this processing event has
Towards treatments for Alzheimer’s disease

an important influence on the self-aggregation properties and the final pathogenicity of Aβ. The most abundantly produced form is Aβ1−40. The longer, 42-amino-acid species is related to toxicity due to its higher propensity to aggregate [252,255,261,262]. Processing of APP in a non-amyloidogenic pathway is catalyzed by α-secretase. The enzyme cleaves APP in the midst of the Aβ domain, whereby release of the 38-42 amino acid family of Aβ peptides is precluded [252].

The exact molecular mechanisms underlying aggregation and its relationship to toxicity remain enigmatic [252,261,263]. In vitro studies suggest that unstructured monomeric Aβ adopts a transiently and partially structured β-hairpin structure, due to the very hydrophobic central (LVFFA, Aβ17−21) and C-terminal regions of the peptide [264]. Next to self-association of the peptides, elongation rapidly proceeds into extended and soluble oligomeric β-strand aggregates that vary in size and shape from dimers up to large spherical or fibril-like transient aggregates (protofibrils) [265,266], that eventually mature to insoluble fibrils. Fibrils of Aβ finally deposit as senile plaques outside neurons in the brains of patients [248,267]. Interestingly, extensive research demonstrates that soluble oligomeric species of Aβ1−42 better correlate with cognitive dysfunction and progressive synapse loss than insoluble fibrils [255,261,268]. Growing evidence also show that early intraneuronal accumulation of Aβ peptides is one of the key events leading to synaptic and neuronal dysfunction [262].

The majority of all AD cases are sporadic with late onset. It has been proposed that the increased accumulation of Aβ species in the brains of patients is due to errors in maintaining Aβ metabolism. Such dysfunction includes overproduction of Aβ peptides due to APP processing errors, increased aggregation, faulty Aβ catabolism, or clearance from the brain. In the context of clearance from the brain, several lines of evidence indicate that receptors that are involved in the transport system of Aβ over the blood-brain barrier (BBB) are affected. Patients with AD generally exhibit an increased influx of circulating Aβ from the periphery into the CNS, due to unregulated expression of RAGE transport receptors (Figure 5.1a) [258]. Furthermore, some patients also have a down-regulated LPR1 receptor and consequently experience a deficient efflux from the brain.

A small fraction of all AD cases (<1%) is linked to early-onset and inherited familial forms of AD (FAD), which is associated with increased Aβ production [255]. One family is related to missense mutations in and around the Aβ region of APP, altering the production and properties of Aβ [262]. In this context, mutations near the β-secretase site may elevate Aβ production [269], mutations near the γ-secretase site may increase the amount of Aβ1−42, and mutations within the sequence may increase the aggregation propensity [252]. Another family of early onset AD is linked to mutations in the genes for PS1 and PS2 (constitute the catalytic site of the γ-secretase complex). These mutations increase the Aβ1−42/Aβ1−40
Figure 5.1: Schematic illustration of pathological cascades in Alzheimer’s disease. **a)** Neuronal cells in the brain are the main producers of amyloid precursor protein (APP). Amyloid plaque build-up interfere with neuronal cell homeostasis. Aβ accumulation may enhance Tau phosphorylation, leading to the formation of neurofibrillary tangles. These pathologies eventually lead to dementia. **b)** Aβ peptides are naturally generated in the metabolism of APP. They are partially embedded in the plasma membrane (or in subcellular organelles), and are produced through consecutive cleavage by β-secretase and γ-secretase at the N-terminal and C-terminal, respectively. Aβ peptides of diverse lengths, typically 40 and 42 amino acids, are generated as a consequence of the multiple catalytic sites of γ-secretase.
ratio by strongly increasing $A\beta_{1-42}$ production with concomitant suppression of $A\beta_{1-40}$ [261]. Familial AD has also been linked to carriers of the $\epsilon 4$ allele of apolipoprotein E (ApoE), which blocks LRP1-mediated clearance of $A\beta$ from the brain [270,271]. The pathological mechanisms for early onset familial AD comprise compelling evidence for the amyloid cascade hypothesis and the development of sporadic AD [262].

**Treatment of AD**

To date, there are no available treatments that prevent, arrest, or reverse the devastating neuropathology and the clinical consequences of the disorder [272,273]. During the last decades, tremendous efforts to understand the molecular aspects have opened up a number of avenues to explore for therapeutic treatments [251,272]. Discoveries have also provided valuable information for development of tools to facilitate early and correct diagnosis, as well as to monitor progression of the disease [274,275]. The importance of early diagnosis is today well recognized, as it allows implementation of treatment before clinical symptoms appear. Traditionally, AD has been diagnosed based on clinical examinations of the patient (according to widely used criteria that include neurophysiological tests to evaluate mental and cognitive functions), and post-mortem brain biopsies [275,276]. Recently, these guidelines for diagnosing AD were revised. Present methods include neuroimaging and measurement of levels of a combination of CSF $A\beta_{1-42}$ and Tau (phosphorylated/non-phosphorylated) since they are early and region-specific proteins that appear years before clinical symptoms [276–281]. Other attractive approaches under investigation are based on biomarker-based tests using blood [282,283]. Such biomarker-based tests provide a direct and convenient mean of studying biochemical changes without the need to perform brain biopsy [279,284]. In addition, considerable effort is devoted to develop protein-based imaging tracer molecules that are capable of crossing the blood-brain barrier to access brain $A\beta$ amyloid loads.

**Current treatments**

Present standard of care for mild to moderate AD and fall into the category of symptomatic relief therapies, as they do not address the causative disease aspects or modify the course of the disease. These approaches are directed at correcting neurotransmitter-specific deficiencies, using chemical drugs, primarily cholinesterase inhibitors. The rationale for this approach is that AD causes a dramatic loss in cholinergic neurons. Hence, by increasing levels of acetylcholine, the remaining cholinergic neurons are kept active. Another type of drug for AD (NMDA receptor antagonists) dampens the action of glutamate in NMDA
receptors, which is believed to reduce toxicity. Even though neither type of drug stops the progressive loss of neurons, depending on the individual and the stage of intervention, these therapies provide short-term symptom improvements [163].

**Future therapeutic Aβ-targeting strategies**

The majority of current research efforts are directed towards developing disease-modifying therapies. Even though several different therapeutic approaches are presently investigated (including targeting of Tau or increasing levels of acetylcholine), the most attractive approach is by targeting Aβ-amyloidosis and its downstream consequences including inhibition or modulation of Aβ-generating proteases, prevention of Aβ aggregation, and promotion of Aβ clearance [251,273,285]. Aβ-immunotherapy has attracted considerable attention during the last years, as it offers the possibility to generate binding-molecules with high selectivity and affinity against specific parts of the Aβ peptide, in order to prevent its aggregation and preserve its solubility [163,251,253,272].

**Immunotherapy**

Active and passive immunization have been the most widely studied approaches to immunotherapy over the past decade [286]. *Active immunotherapy* jump-started the field of AD immunotherapy, and involves a vaccine to stimulate an immune response that generates specific antibodies in the recipient. Such vaccines can be either full-length Aβ peptides or fragments thereof for activation of the immune system to generate α-Aβ antibodies. The Aβ peptides or peptide-fragments are typically conjugated to a carrier protein and may be administered with an adjuvant in order to help stimulate an immune response. In *passive immunotherapy* α-Aβ monoclonal antibodies are generated *ex vivo*, and administered to the patient without requiring the immune system to generate an antibody response [251,280,287]. Both active and passive strategies to immunotherapy have advantages and disadvantages. Passive immunotherapy has been suggested advantageous, as it offers the possibility to deliver known quantities of well-characterized antibodies against very specific epitopes. It is also suggested to provide safer treatment, due to lower risks of irreversible autoimmune complications. Nevertheless, antibodies are associated with high costs and practical limitations, involving infusing or injections at regular intervals for what may need to be lifelong treatment [286].
Mechanisms of action

Antibodies to Aβ are suggested to reduce brain levels of Aβ by four different mechanisms, which are most likely active simultaneously (Figure 5.2): i) α-Aβ antibodies pass the BBB to reach amyloid deposits in the brain and dissolve them on-site [288,289], ii) α-amyloid antibodies pass BBB to reach amyloid deposits in the brain and trigger a phagocytic response by FcR-mediated activation of microglial cells [290–292] iii) α-amyloid specific antibodies reach the brain and bind to neurotoxic oligomeric Aβ species and block their neurotoxic effects without immediate impact on amyloid load [39,293], and iv) α-amyloid antibodies bind free Aβ in the peripheral compartment and act as a sink for soluble Aβ species by shifting the dynamic equilibrium of free Aβ from the brain to the blood [294]. The binding epitope of the antibody is likely to influence the mechanism of action. For example, antibodies that bind soluble forms of the peptide may increase clearance from the brain and hence shift the equilibrium, whereas antibodies binding to deposited amyloids may require microglial activation to achieve plaque reduction [286]. There is no known active transport mechanism for antibodies to cross the BBB into and out of the CNS. However, it has been demonstrated that the neonatal Fc receptor (FcRn) allows trancytosis of a small fraction (∼ 0.1%) of antibodies in the circulation into the CNS [39,286]. Consequently, the mechanisms of how Aβ levels are reduced in the brains of patients in several interventions are not fully understood, however could possibly include the lymphatic system, perivascular spaces and areas within the CNS in which the BBB is leaky [285,286,293]. Clearance of Aβ-antibody complexes from the brain to the blood is suggested to be mediated by reverse transcytosis across the BBB via FcRn and LRP-1 [293].

Present clinical investigations

A number of studies have previously demonstrated the efficacy of both active and passive immunization to reduce brain Aβ burden in proof-of-concept models [295] in mice [291,296–299], monkeys [300–302], and dogs [303]. These preclinical studies have led to the development of several potential treatments that are presently assessed at various stages in human clinical trials (Table I). Although, results from animal studies have proven successful and provided encouraging results, current clinical investigations from both active and passive immunotherapy have identified a number of challenges involving immune-related complications [304–306].

The first study with active immunization was performed using synthetic Aβ1–42 and the saponin-based adjuvant QS-21. The trial was halted early, as 6% of the patients developed adverse immunological responses that lead to meningoencephalitis [304]. It was hypothesized that the vaccine promoted production of Aβ-reactive autoimmune T cells, caused
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Figure 5.2: Suggested mechanisms of amyloid clearance by Aβ-immunotherapy. a) Aβ-specific antibodies reach amyloid deposits in the brain and dissolve them directly through interaction of the antibody with the amyloid deposit, b) Small amounts of Aβ-specific antibodies reach amyloid deposits in the brain and trigger a phagocytic response by microglia, c) Aβ-specific antibodies rapidly bind to oligomeric Aβ species, and block their toxic effects without immediate impact on amyloid load, and d) Amyloid-specific antibodies act as a peripheral sink for soluble Aβ species, which ultimately leads to the resolution of brain deposits by pulling soluble Aβ into the periphery, where it is rapidly cleared.

most likely by the activation of TH1 lymphocytes by the QS-21 adjuvant [273,307]. Even though the study was halted prior to completion, patients that were immunized exhibited a significant reduction of cognitive decline [308]. Several second-generation and new vaccines have been/are currently being tested in clinical trials to prevent plaque deposition and/or enhance Aβ clearance (Table I) [308–310].

Based on the lack of ability to regulate response levels and duration of the treatment with active immunization, much effort has been undertaken to test and develop passive
immunization strategies with humanized α-Aβ monoclonal antibodies (mAbs). Several antibodies that are instigated for passive immunization are originally naturally generated towards different conformations of Aβ upon active vaccination. Many mAbs are presently investigated for passive immunotherapy at various Phases of development and clinical trials (Table I).

**Targeting fibrillar forms of Aβ**

Bapineuzumab is a humanized IgG1 mAb that targets the N-terminal region of Aβ. This region typically remains exposed when fibrils are formed, and can therefore be used in the targeting of fibrillar species. In phase II trials of mild-to-moderate AD, bapineuzumab treatment was associated with a significant number of cases of proinflammatory events, leading to BBB disruption (observed as vasogenic edema) and cerebral microhemorrhage [305,306,311]. Even so, a Phase III program was initiated. As the antibody failed to meet its primary endpoints of improving or stabilizing cognition and functional performance in two 18-month trials [312], all subsequent trials were discontinued (http://clinicaltrials.gov). It has been speculated that the lack of clinical efficiency of bapineuzumab was due to too low amounts of antibody reaching the brain, and/or that the treatment was initiated at a too late stage of the disease process to be able to reverse the neurodegenerative changes that underlie memory loss [273].

**Targeting soluble monomeric forms of Aβ**

Solanezumab is a humanized IgG1 mAb that recognizes the central region of Aβ, and consequently binds soluble monomeric forms of the peptide. In two large Phase III trials, solanezumab was investigated in mild-to-moderate AD patients. Neither study met the pre-specified primary outcomes of improving or stabilizing cognition and functional performance in moderate diseased patients. However, secondary analysis of pooled data from the trials for patients with mild AD, demonstrated a 34% reduction in cognitive decline, indicating that earlier treatment could be beneficial. Solanezumab trials were not associated with the adverse proinflammatory reactions that were seen in trials with bapineuzumab [313–315]. In 2013 a new Phase III trial in patients with mild AD was started, and is expected to read out in December 2016 (http://clinicaltrials.gov).

**Targeting soluble aggregates of Aβ**

Intravenous immunoglobulin (IVIg) is a polyclonal antibody preparation derived from the blood plasma of thousands of healthy donors. IVIg has an established safety-record from human use in immunodeficiency and certain autoimmune conditions. It is thought to promote amyloid clearance and mediate anti-inflammatory effects. Several Phase-trials are on-going (http://clinicaltrials.gov).

**Targeting Aβ with a conformational antibody**

Gantenerumab is the first fully human IgG1 α-Aβ antibody, with a high capacity to specifi-
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<th>Trial Phase</th>
<th>Status</th>
<th>Note</th>
<th>Company</th>
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<tbody>
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<td>AN-1792</td>
<td>Phase IIa</td>
<td>Discontinued, no improvement</td>
<td>Aβ(1-42) peptide with saponin-based QS-21</td>
<td>Janssen, Pfizer</td>
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<tr>
<td>ACI-24</td>
<td>Phase I/II</td>
<td>Completed, not reported</td>
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<tr>
<td>CAD106</td>
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<td>Aβ(1-6) with bacteriophage Qβ virus-like particle</td>
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<tr>
<td>Affitope AD02</td>
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<td>Six synthetic amino acids that mimics the N-terminus of Aβ</td>
<td>AFFiRiS AG</td>
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<th>Trial</th>
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<th>AD population targeted</th>
<th>mAb/Target</th>
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<td>Bapineuzumab (AAB-001)</td>
<td>Two Phase III</td>
<td>Discontinued, no improvement</td>
<td>Mild-to-moderate</td>
<td>Humanized m3D6, binds N-terminal Aβ(1-5), mimics natural antibody triggered by AN-1792</td>
<td>Janssen, Pfizer</td>
</tr>
<tr>
<td>Bapineuzumab (AAB-003)</td>
<td>Phase I</td>
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<td>Mild-to-moderate</td>
<td>Humanized m3D6 with IgG4 backbone</td>
<td>Janssen, Pfizer</td>
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<td>Solanezumab (LY2062430)</td>
<td>Two Phase III</td>
<td>Completed, no improvement in first analysis. Secondary analysis showed improvements in patients with mild AD</td>
<td>Mild-to-moderate</td>
<td>Humanized m266, binds central Aβ(16-24)</td>
<td>Eli Lilly &amp; Co.</td>
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<tr>
<td>IVIg naturally occurring</td>
<td>- Gamunex</td>
<td>Phase II/III</td>
<td>On-going</td>
<td>Mild-to-moderate</td>
<td>Grifols Biologicals Inc.</td>
</tr>
<tr>
<td></td>
<td>- Octagam</td>
<td>Phase II</td>
<td>On-going</td>
<td>MCI and early AD</td>
<td>Octapharma</td>
</tr>
<tr>
<td>Gantenerumab (RO4909832, RG1450)</td>
<td>Phase III</td>
<td>On-going</td>
<td>Mild</td>
<td>Fully human IgG4 backbone, binds fibrils with conformational epitope: N-terminal Aβ(3-12) and central Aβ(18-27)</td>
<td>Chugai Pharmaceutical Co., Hoffmann-La Roche</td>
</tr>
<tr>
<td>Gantenerumab &amp; Solanezumab (DIAN)</td>
<td>Phase II/III</td>
<td>On-going</td>
<td>Preventative/early treatment</td>
<td></td>
<td>Eli Lilly &amp; Co., Hoffmann-La Roche</td>
</tr>
<tr>
<td>Aducanumab (BIB037)</td>
<td>Phase III</td>
<td>On-going</td>
<td>Mild</td>
<td>Naturally occurring in healthy donors</td>
<td>Biogen</td>
</tr>
<tr>
<td>Crenezumab (MABT5102A, RG7412)</td>
<td>Phase II</td>
<td>On-going</td>
<td>Mild-to-moderate</td>
<td>Humanized with IgG4 backbone, binds soluble Aβ and plaques</td>
<td>Genentech</td>
</tr>
<tr>
<td>Crenezumab (MABT5102A, RG7412)</td>
<td>Phase II</td>
<td>On-going</td>
<td>Preventative/early treatment</td>
<td></td>
<td>Genentech</td>
</tr>
<tr>
<td>BAN2401</td>
<td>Phase II</td>
<td>On-going</td>
<td>MCI and early AD</td>
<td>Humanized mAb158, recognizes protofibrils</td>
<td>Biogen, Eisai Co., Ltd</td>
</tr>
</tbody>
</table>
Towards treatments for Alzheimer’s disease

ically bind to cerebral amyloid plaques. It encompasses both N-terminal and central amino acids of Aβ. Gantenerumab acts to reduce amyloid burden through phagocytosis via brain microglial cells and prevent senile plaque formation [316]. In a current Phase III study, monthly subcutaneous administrations are assessed for safety and efficacy (cognition and function), in patients with mild AD (http://clinicaltrials.gov). In addition to the ongoing studies, gantenerumab in combination with solanezumab is investigated in a Phase II/III trial aimed at preventing dementia in people who are on the path to AD due to the inherited autosomal-dominant mutation in APP, PS1, or PS2 (http://clinicaltrials.gov).

Aducanumab is a fully human IgG1 mAb that recognizes a conformational epitope found on aggregated forms of Aβ. The antibody is derived from healthy, aged donors that are cognitively normal. It is hypothesized that the immune systems in these individuals have successfully prevented Alzheimer’s disease due to different operative antibodies (of which Aducanumab is one) (http://www.biospace.com). Based on preclinical work and Phase I data, a Phase III study was enrolled in 2014 with final read out in December 2016. In March this year, first data from the study reported that aducanumab reduced amyloid deposition in the brain in a dose-dependent fashion (http://clinicaltrials.gov).

**Targeting Aβ with reduced effector functions**

Crenezumab is a fully humanized IgG4 mAb that recognizes both monomeric and multiple forms of aggregated Aβ. The IgG4 subclass leads to reduced effector functions, including reduced release of inflammatory cytokines which potentially avoids development of vaso- genic edema. Crenezumab is currently being investigated in Phase II studies in patients with mild-to-moderate AD. In addition to the ongoing trials, crenezumab is also being tested in a Phase II trial aimed at preventing dementia in presymptomatic carriers of autosomal-dominant PS-mutations (http://clinicaltrials.gov).

**Targeting Aβ protofibrils**

BAN2401 is a humanized IgG1 mAb that selectively binds to a unique structure in large and soluble Aβ protofibrils. Binding is suggested to lead to clearance or neutralization of toxicity [317, 318]. Phase IIb is presently ongoing in patients with mild cognitive impairment (MCI) and early AD (http://clinicaltrials.gov).

**Challenges of immunotherapeutic strategies**

The immunotherapy field has grown tremendously in the past decade, and has pointed towards some issues that need to be overcome in order to see long-term clinical safety and efficacy of treatments. The encountered clinical failure is suggested to be caused by several combined factors, including i) too low penetrance of antibodies over the BBB [294], ii) delivery routes [163], iii) Fc-mediated pro-inflammatory adverse events, iv) start-point of
the treatment, and 5) length of the treatment.

Related to the issue of insufficient transfer of the therapeutic protein into the CNS, several innovative strategies have been suggested to boost brain-uptake. Such strategies include the use of chaperone proteins or bi-specific binding-proteins that can piggy-back the therapeutic protein into brain [163]. Bi-specific binding-proteins have been engineered to e.g. the insulin receptor (IR) and the transferrin receptor (TfR), which are both highly expressed on the BBB [163,302,319]. Recent preclinical studies performed by Genentech, using a human TfR knock-in mouse model, demonstrated that antibodies that exhibit specificity for both the TfR (brain uptake) and BACE1 (prevent processing of Aβ) can cross the BBB and reduce brain Aβ levels in a TfR affinity-dependent fashion. In addition, studies demonstrate that the therapeutic antibody robustly and safely can be delivered across the BBB in primates, and reduce Aβ both in cerebral spinal fluid and in brain tissue [302]. Other suggested strategies to increase the brain uptake are by transient opening of the BBB by chemical or radiological means.

Regarding observed Fc-mediated pro-inflammatory adverse events that are caused by conventional therapeutic IgGs, it has been suggested that exchange of the IgG1 framework to IgG4 can reduce the activation of inflammatory events. Smaller binding-proteins that lack the Fc-domain have also been suggested to be attractive alternatives.
The Aβ-binding Affibody molecule Z\textsubscript{Aβ3}

In order to conquer the challenges that have been identified in several of the advanced human trials for treatment of Alzheimer’s disease, the pharmaceutical market is focusing a lot of effort to developing new agents that can potentially overcome the antibody-associated issues.

An Affibody molecule denoted (Z\textsubscript{Aβ3}) was previously generated in-house, to be used for potential depletion of non-aggregated Aβ peptides from the circulation. Z\textsubscript{Aβ3} was also generated for potential use as an agent in future in vivo diagnostic settings, and to be utilized as a reagent in research applications. The first three studies of this thesis are based on the Z\textsubscript{Aβ3} Affibody molecule. Therefore, this chapter provides an overview of the pioneering work of the this Affibody molecule, including how it was generated, its rare structure in complex with the Aβ peptide, and results from the first in vivo animal study.

Selection and characterization

Several Affibody molecules binding to soluble, non-aggregated Aβ were selected by phage display from a large combinatorial Affibody library (3 x 10⁹ members). The selection was performed using a biopanning strategy with biotinylated Aβ\textsubscript{1-40} as target (as it is known to aggregate much slower than Aβ\textsubscript{1-42} [320]). Notably, all selected sequences contained a conserved cysteine in amino acid position 28. In characterization and ranking experiments of the variants, the top-candidate, Z\textsubscript{Aβ3}, demonstrated efficient capture Aβ peptides spiked in plasma and serum samples [216].

The structure of Aβ\textsubscript{1-40} in complex with Z\textsubscript{Aβ3} was studied with NMR. Interestingly, Z\textsubscript{Aβ3} was shown to bind the Aβ peptide as a disulfide-linked homodimer, involving the internal cysteine in the second helix of each of the two subunits. Helix 1 was destabilized in both monomers of the Affibody, which resulted in exposure of the cores of the two Z-domain scaffolds and formation of a large hydrophobic, tunnel-like cavity that served as the binding-
The Aβ-binding Affibody molecule $Z_{\mathrm{A}\beta}$

site for the Aβ peptide. Binding of dimeric $Z_{\mathrm{A}\beta}$ to Aβ was coupled to conformational reorganization and stabilization of both the ligand and the Aβ peptide. The dimeric Affibody molecule underwent conformational transition into two $\beta$-strands and four $\alpha$-helices. The Aβ peptide adopted a $\beta$-hairpin structure that was characterized by two antiparallel $\beta$-strands, formed by residues 17-23 and 30-36. These strands were connected by intramolecular backbone hydrogen bonds. The $\beta$-hairpin of the Aβ peptide within the hydrophobic core was stabilized on each side by short solvent-exposed $\beta$-strands formed by residues 15-18 of each $Z_{\mathrm{A}\beta}$ molecule (Figure 6.1). Importantly, the cavity of $Z_{\mathrm{A}\beta}$ dimer was inaccessible to water, and the nonpolar faces of the Aβ$_{1-40}$ $\beta$-hairpin inside the cavity were thus mostly shielded from the solvent. The N-terminal region of Aβ$_{1-40}$ (positions 1-15), and the terminal regions of both $Z_{\mathrm{A}\beta}$ subunits (positions 1-13 and 57-58) were not well defined by the NMR constraints, implying a lack of ordered structure in those regions [226]. The hairpin-fold of Aβ in complex with $Z_{\mathrm{A}\beta}$ resembled the structure of Aβ within the amyloid fibril [321], which is also characterized by a $\beta$-strand-turn-$\beta$-strand arrangement [267,322].

The equilibrium dissociation constant ($K_d$) of $Z_{\mathrm{A}\beta}$ for Aβ$_{1-40}$ and Aβ$_{1-42}$ was determined to 17 nM [226,323]. Removal of the disordered N-terminus of $Z_{\mathrm{A}\beta}$ increased the affinity approximately 10-fold [324]. Cysteine-to-serine mutants were generated to circumvent multimerization problems, however, the substitution drastically reduced the affinity to Aβ [216].

Inhibition of Aβ aggregation in vitro

Studies have demonstrated that the $Z_{\mathrm{A}\beta}$ Affibody can inhibit amyloid fibril formation by sequestering monomeric Aβ [226,325]. Thioflavin T (ThT) assays have showed that addition of $Z_{\mathrm{A}\beta}$ to Aβ aggregation solutions (in which amyloids are already being formed) stops further aggregation. Inhibition was achieved on a stoichiometric 1:1 basis of Aβ and dimeric $Z_{\mathrm{A}\beta}$, and correlated with the sequestration mechanism that the Affibody exhibits on the aggregation-prone central and C-terminal regions of the Aβ peptide (Figure 6.1) [325].

Furthermore, it has been demonstrated that $Z_{\mathrm{A}\beta}$ can act to dissolve pre-formed aggregates of Aβ by shifting the dynamic monomer-oligomer equilibrium. Large oligomers (protofibrils) of Aβ$_{1-42}$ that travel in the void volume in size-exclusion chromatography columns form fibrils within 10 hours, with monomeric Aβ present transiently in the process. By addition of stoichiometric amounts of dimeric Affibody, the oligomer population was reduced. However, protofibril dissolution was very slow and occurred on the order of several hours.
The Aβ-binding Affibody molecule Z\textsubscript{Aβ3}

\begin{align*}
\text{(Aβ\textsubscript{1-40})} & \quad \text{DAEFRHDSGYEVHHQKLVFFAEDVGSNKGA IIGLMVGGVV} \\
\text{(Z\textsubscript{Aβ3})} & \quad \text{VDNKFNKEMASAGGEIVYLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK}
\end{align*}

\textbf{Figure 6.1:} Amino acid sequences and structure of the complex between the Aβ\textsubscript{1-40} peptide and the Z\textsubscript{Aβ3} Affibody molecule. (a) Aβ\textsubscript{1-40} peptide, with hydrophobic residues in yellow and the Z\textsubscript{Aβ3} Affibody molecule. Residues that adopt β-strand conformation in the β-hairpin upon binding to Z\textsubscript{Aβ3} are indicated by arrows underneath the sequence. The sequence for the Z\textsubscript{Aβ3} Affibody molecule is outlined below the sequence for Aβ. Structural rearrangement of the Affibody molecule upon binding to the peptide is indicated by arrows and cylinders below the sequence. (b) The three-dimensional structure of the Aβ:Z\textsubscript{Aβ3} complex (modified from [226]).

\section*{Inhibition of Aβ aggregation in an \textit{in vivo} fruit fly model of AD}

The potency of the Z\textsubscript{Aβ3} Affibody molecule to inhibit Aβ aggregation and neurotoxicity \textit{in vivo} was evaluated in a Drosophila fruit fly model of AD. Expression of Aβ peptides in the brains of transgenic \textit{Drosophila melanogaster} is toxic to the flies and generates phenotypes with similarities to AD (Aβ aggregation and similar histopathology). Flies transgenic for Aβ\textsubscript{1-40}, Aβ\textsubscript{1-42}, or the very aggregation-prone arctic mutant Aβ\textsubscript{1-42}E22G, were crossed with flies transgenic for Z\textsubscript{Aβ3} monomer, Z\textsubscript{Aβ3} dimer or Z-domain control. The Affibody
The Aβ-binding Affibody molecule ZA33
dimer was genetically generated as a head-to-tail construct, to enhance the rate of dimer formation after translation in vivo.

Expression of Aβ1−42E22G in the brain of flies caused rapid neurodegeneration and resulted in a drastic reduction in the lifespan from 38 to 9 days. However, co-expression of the Affibody dimer prolonged the median life span of the flies to 31 days, indicating that the neurotoxicity of Aβ had been almost entirely abolished since the flies lived almost as long as wild-type controls. Furthermore, the life length of flies expressing Aβ1−42 was completely restored from 28 days to wild-type levels. Immunohistochemistry showed that Aβ aggregates were present in the brains of flies that expressed Aβ but absent in the brains of those that also expressed the ZA33 dimer. Aβ levels in fly brains were also assessed by biochemical means, and showed a large reduction (97%) of Aβ1−42E22G levels in brains co-expressed with the ZA33 dimer. These results correlated well with corresponding reduction in neurotoxicity, as measured by the survival assay [325].

ZA33 as a research tool

The interesting aggregation-inhibitory abilities of ZA33 has facilitated it to be implemented in different research applications. For example, it has been utilized to facilitate recombinant production of Aβ peptides. The majority of all conducted research employs synthetic Aβ peptides. However, recombinant expression in E. coli is generally considered advantageous due to lower costs, fast growth rate, and high expression levels. Various attempts to use recombinant expression and purification of highly amyloidogenic peptides, such as Aβ, has previously proved difficult due to the small size, tendency to aggregate and the toxicity of the formed aggregates. Therefore, strategies based on different fusions to the peptide have been explored to protect it from proteolysis and enhance solubility. However, these approaches have provided an Aβ peptide that is affected by a tag. To circumvent such problems, the ZA33 molecule have been used for recombinant co-expression with the Aβ peptide, to stabilize the target and prevent its aggregation [326].

Furthermore, the discovery of the β-hairpin structure of Aβ in complex with ZA33, which resembles the conformation of the Aβ peptide in amyloid fibrils, has enabled modeling of possible mechanisms for oligomer and fibril formation [322]. The β-strands of the Aβ hairpin within the Affibody dimer form hydrogen bonds and bind each other within the Aβ peptide. In amyloid fibrils, the β-hairpin strands are stabilized by backbone hydrogen bonding to other Aβ molecules. Soluble oligomers are hypothesized to form by hydrophobic stacking of such β-hairpins. A concerted 90° rotation around the strand axes establishes a fibril seed with in-register parallel β-sheets [226,323]. By exploiting the ZA33:Aβ complex, a stable variant of Aβ (denoted Aβcc) has been engineered by alanine to cysteine sub-
The Aβ-binding Affibody molecule ZAβ3

Substitutions at positions 21 and 30, leading to a disulfide-locked hairpin conformation that inhibits the 90° conformational transition and hence is incompatible with fibril formation. As a result, Aβcc arrests aggregation at the protofibril state [321, 322, 327]. While natural protofibrils of Aβ are unstable, heterogeneous and ultimately form amyloid fibrils (a property that impede detailed structural studies) [322], stable Aβcc protofibrils can instead be used to model and investigate oligomer and fibril formation. In study IV of this work, Aβcc was exploited as a tool for development of Affibody molecules recognizing aggregated forms of Aβ.

ZAβ3 as scaffold for generation of new binding-molecules

The advantageous target-sequestering property of ZAβ3 could also be efficient for interactions with other aggregation-prone target-peptides. Therefore ZAβ3 has been explored as a template for engineering of new agents with aggregation-inhibitory capacity. Based on this scaffold molecules binding the aggregation-prone tau protein that is involved in AD pathogenesis [328], and α-synuclein that is involved in pathogenesis of Parkinson’s disease [329] have been generated for potential future therapeutic applications.
Present investigation

In this chapter of the thesis, the work in the five appended publications and manuscripts is reviewed. The objectives of each study are first presented, followed by an overview of the results and a discussion of the studies.

Objectives of the studies

Overall, the studies have aimed to generate and engineer Affibody molecules that target various conformations of the Alzheimer’s-related A\(\beta\) peptide for future use in both research as well as in diagnostic and therapeutic applications for treatment of Alzheimer’s disease.

**Study I**: To investigate combinatorial and rational methods for future engineering of the pre-existing Z\(_{A\beta 3}\) Affibody molecule, using the staphylococcal cell surface display platform.

**Study II**: To improve the affinity of the Z\(_{A\beta 3}\) Affibody molecule to non-aggregated forms of A\(\beta\), using a semi-rational approach and staphylococcal surface display for engineering. Future prospects include animal studies to assess the potential of new Affibody molecules to prevent development of A\(\beta\) pathology.

**Study III**: To assess the capacity of an affinity-matured A\(\beta\)-binding Affibody molecule (denoted Z\(_{SY M73}\)) to prevent development of A\(\beta\) pathology in an APP/PS1 2x transgenic mouse model of Alzheimer’s disease, when treated at an early stage of disease development. Future prospects include expanded animal studies.

**Study IV**: To generate new Affibody molecules that bind aggregated forms of A\(\beta\) (instead of monomeric forms), using combinatorial protein engineering and phage display technology. Future prospects include further characterization of the generated binders and animal studies to assess the potential of the Affibody molecules to prevent development or possibly to reverse A\(\beta\) pathology.
Present investigation

Study V: To develop a fluorescence-based system for high-throughput FACS-screening of protein-based agents with aggregation-inhibition capacity. Future prospects include protein library applications for generation of novel binders to aggregation-prone peptides such as Aβ (Alzheimer’s disease) and α-synuclein (Parkinson’s disease).

Contributions to the different studies

Study I: Designed the study together with coauthors and performed the majority of the experiments. Wrote the manuscript.

Study II: Designed the study together with coauthors and performed the experiments. Wrote the manuscript.

Study III: Participated in design, production and purification of the Affibody proteins. Wrote the manuscript.

Study IV: Designed and performed cloning, protein production and purification of the dimeric Affibody molecules, as well as some SPR experiments. Wrote the corresponding parts of the manuscript.

Study V: Designed the study together with coauthors and performed the experiments. Wrote the manuscript.
Present investigation

Study I

Staphylococcal display for combinatorial protein engineering of a head-to-tail affibody dimer binding the Alzheimer’s amyloid β peptide

As described in the previous chapter, an Aβ-binding Affibody molecule, denoted Z_{Aβ33}, was previously generated [216]. In this study, we investigated different engineering strategies for the Affibody molecule, in order to improve its potential for preclinical investigations for treatment of AD. We assessed rational and combinatorial engineering strategies to improve characteristics such as scaffold properties and binding affinity, while retaining the functional capacity. Staphylococcal cell surface display was investigated as a platform for the engineering efforts, as it has previously been successfully employed for combinatorial approaches of other Affibody molecules.

Characterization of the system

The Z_{Aβ33} Affibody molecule and a cysteine-to-serine mutant (denoted Z_{Aβ33C28S}) were separately generated as head-to-tail dimers, and displayed in fusion to an albumin-binding protein (ABP) on the surface of staphylococcal cells. Incubation with fluorescently labeled Aβ, and HSA for normalization of expression levels, demonstrated that the Affibody molecules were functionally displayed and that target-binding for the first-generation binder was higher than for the mutant, as expected (Figure 7.1a-e). The apparent dissociation constants were determined by incubating Affibody-displaying cells with different concentrations of labeled Aβ_{1-40}, in a range surrounding the K_D value. The original binder demonstrated an apparent K_D value of approximately 100 nM, and the cysteine-to-serine mutant an apparent K_D of 320 nM on the bacterial surface. These on-cell affinities were somewhat lower than reported affinities for the proteins in soluble form. However, in correlation with previous reports, the cysteine-to-serine substitutions had a negative effect on the affinity. The platform was further assessed for engineering purposes by determining the apparent rate of dissociation (k_{off,app}) at pH 7.4 and 5.5, respectively. The rationale for investigating dissociation at the lower pH was to examine the stability of the complex for a potential future therapeutic strategy, in which circulating Affibody:Aβ complexes are hypothesized to be pinocytosed and transported to endosomes in endothelial and antigen-presenting cells. Ideally, the complex should dissociate at the lower pH in the endosomes, whereafter the Aβ peptide should be directed to the lysosome for degradation whereas the
Present investigation

Figure 7.1: a,b) Structure of the dimeric Z\(\text{A}\beta_3\) in complex with A\(\beta_1-40\) (PDB entry 2OTK). A\(\beta_1-40\) - deep purple, the two subunits of the Z\(\text{A}\beta_3\) dimer - orange and green, respectively, and the disulfide bridge - yellow. c) Illustration of the dimeric affibody (Z1 and Z2) fused to an ABP on staphylococci. d,e) Flow-cytometric analysis of staphylococci displaying the two dimeric Affibody molecules (Z\(\text{A}\beta_3\))\(_2\) and (Z\(\text{A}\beta_3\text{C}28\text{S}\))\(_2\). f,g) On-cell flow-cytometric determination of the equilibrium dissociation constants (K\(\text{D}\)) of (Z\(\text{A}\beta_3\))\(_2\) and (Z\(\text{A}\beta_3\text{C}28\text{S}\))\(_2\) for A\(\beta_1-40\). Analysis was performed in triplicate on different days. h-k) On-cell flow-cytometric determination of the dissociation rate constants (k\(\text{off}\)) of (Z\(\text{A}\beta_3\))\(_2\) and (Z\(\text{A}\beta_3\text{C}28\text{S}\))\(_2\) for A\(\beta_1-40\) at pH 5.5 and 7.4. Analysis was performed in triplicate on different days.

Affibody should be directed back to circulation via mechanisms involving interactions with the FcRn (Z-ABD:HSA:FcRn). To investigate potential dissociation at the lower pH, cells were incubated in a concentration of labeled A\(\beta_1-40\) peptide that corresponded to at least threefold the K\(\text{D}\). After washing, a high concentration of non-labeled A\(\beta_1-40\) was added to the samples to minimize rebinding of dissociated labeled A\(\beta\) and cells were sampled at various time points and analyzed using flow-cytometry. Results indicated that neither of
the interactions were pH sensitive, as the $k_{off}$ for both the Affibody constructs at both pH was approximately $10^{-4}\text{s}^{-1}$ (Figure 7.1h-k).

**Characterization of the system for protein library efforts**

The display platform was also successfully assessed for library engineering approaches of the $\text{A}β$-binding Affibody molecule. A randomized library was designed based on the head-to-tail chained dimeric $(Z_{\text{Aβ3}})^2$ Affibody molecule. In order to investigate the potential of substituting the covalent disulfide bond in the interface of the second helices of the two Affibody molecules for a non-covalent interaction to stabilize the dimer, the cysteines were substituted for a diversified interface (Figure 7.2a). The library genes were generated using trinucleotide synthesis, with Slonomics\textsuperscript{R} technology. A protein library of $10^7$ individual clones on staphylococci was produced, and sequence analysis of the unsorted library demonstrated that the displayed library was in accordance with the theoretical design. To verify that the protein library was displayed on the cell surface, cells were labeled with fluorescent HSA and subsequently analyzed by flow-cytometry. The result showed that approximately 80% of the cells in the library expressed recombinant proteins with functional ABP molecules.

In order to isolate new $\text{A}β$-binding molecules, staphylococci corresponding to 100x the library size were incubated with fluorescently labeled $\text{A}β_{1-40}$ and fluorescently labeled HSA, and subsequently subjected to flow-cytometric cell sorting. In five cycles of sorting, clones with the highest target-binding to surface expression ratio were gated and sorted. Stringency was increased in terms of sorting parameters and sorting gates in each cycle (Figure 7.2b). From the last cycle of sorting, two candidates dominated the output. Interestingly, in spite of a library design devoid of cysteines, both clones contained one cysteine each at positions that corresponded to the wild-type positions (Figure 7.2c). Most likely, these residues had been mis-incorporated into the library and enriched for in the selection procedure.

To analyze binding to $\text{A}β$ of the isolated Affibody molecules, the clones were individually subjected to flow-cytometric analysis. The two clones demonstrated target-binding, although weaker compared to the head-to-tail dimeric $(Z_{\text{Aβ3}})^2$.

**Trans-dimerization of Affibody proteins on the cell surface**

We hypothesized that the low target-binding signals for the two new cysteine-carrying clones were not due to the Affibody construct binding of $\text{A}β$ as a head-to-tail dimer but instead caused by trans-dimerization of distinct displayed recombinant proteins on the cell
Present investigation

Surface expression level (FL-4)

Amyloid β binding (FL-1)

Amino acid sequences of the head-to-tail dimeric \((Z_{A\beta 3})_2\) Affibody molecule and the \((Z_{A\beta \text{lib}})_2\) combinatorial library with randomized positions indicated with a dot.

Density plots from the flow-cytometric sorting of the library. Plots show the staphylococcal library before flow-cytometric sorting cycle 1, 2, 3, 4, and 5, respectively, with outlined regions used for gating.

Amino acid sequences of dimeric \((Z_{A\beta 3})_2\) and isolated Aβ-binding Affibody molecules.

Figure 7.2: a) Amino acid sequences of the head-to-tail dimeric \((Z_{A\beta 3})_2\) Affibody molecule and the \((Z_{A\beta \text{lib}})_2\) combinatorial library with randomized positions indicated with a dot. b) Density plots from the flow-cytometric sorting of the library. Plots show the staphylococcal library before flow-cytometric sorting cycle 1, 2, 3, 4, and 5, respectively, with outlined regions used for gating. c) Amino acid sequences of dimeric \((Z_{A\beta 3})_2\) and isolated Aβ-binding Affibody molecules.

surface (Figure 7.3a). To investigate this hypothesis, \(Z_{A\beta 3}\) was displayed as a monomer on the cell surface and analyzed for Aβ-binding using flow-cytometry. A monomeric construct of the cysteine-to-serine mutant was also included in the analysis as a negative control for comparison. The assay demonstrated that the construct containing a cysteine residue retained a low target-binding capacity, whereas the cysteine-to-serine mutant showed no antigen-binding signal (Figure 7.3b-c). These results suggest that the new head-to-tail Affibody molecules with only one cysteine form trans-dimeric complexes on the cell surface, and emphasize the importance of cysteines in the Affibody dimer for efficient binding to the Aβ peptide.
Present investigation

Figure 7.3: a) Schematic illustration of the selected Aβ-binding Affibody molecules containing one cysteine residue each in fusion to an ABP on the staphylococcal surface. Distinct polypeptides on the cell surface form disulfide-linked dimers. b-f) Flow-cytometric analysis of staphylococci displaying: b) monomeric ZAβ3. c) monomeric ZAβ3C28S, d) truncated ZAβ3(15−58), (e) truncated ZAβ3(12−58), and f) original (ZAβ3)2.

Evaluation of future strategies for engineering efforts

For future engineering and affinity maturation efforts, truncated variants of the Aβ-binding Affibody molecule, which have previously been reported to exhibit an increased affinity for the Aβ peptide [324], were investigated for reproducible effects on the cell surface. Two head-to-tail linked Affibody variants, shortened by 11 or 14 residues at the N-terminus were constructed, denoted ZAβ3(12−58) and ZAβ3(15−58), respectively. Only the N-terminal region of the first Affibody domain was truncated in the new constructs, whereas the unstructured N-terminal part of the second domain was retained to serve as a linker. Cells displaying the two truncated binders were analyzed for target-binding, relative to the full length head-to-tail (ZAβ3)2. Fluorescence intensities corresponding to both the surface expression and the Aβ-binding of truncated (ZAβ3(12−58))2 were higher than signals from the original head-to-tail binder (ZAβ3)2, suggesting that deletion of the unstructured part of helix 1 had a positive effect on both expression level and affinity (Figure 7.3d-f).
Present investigation

Study II

A truncated and dimeric format of an affibody library on bacteria enables FACS-mediated isolation of amyloid-β aggregation inhibitors with subnanomolar affinity

In therapeutic strategies that are based on peripherally-administered Aβ-specific agents, it is critical to use agents with high affinity to be able to shift the dynamic equilibrium of free Aβ peptides over the BBB (from the CNS to the blood) [330,331]. Therefore, to increase the potential of the Aβ-binding Affibody molecule as a future therapeutic candidate, we here aimed to significantly improve its affinity to the Aβ peptide, as well as to improve the scaffold properties and decrease the size of the dimeric Affibody molecule. Based on results from the previous study, engineering efforts were conducted with a combined approach of rational design and library technology using the staphylococcal display platform.

Scaffold design and affinity maturation libraries

A new scaffold was designed with the two Affibody domains formatted as a single chain head-to-tail genetic dimer to i) enhance the rate of dimer formation, ii) allow independent engineering of each Affibody domain, and iii) permit display on the surface of staphylococci. In the new scaffold, the unstructured N-terminal sequence was removed from the first domain, while replaced by a hydrophilic flexible serine-glycine linker ((S$_4$G)$_2$) in the second domain to allow for correct folding of the dimeric construct with minimal steric hindrance.

Two approaches for diversification of the head-to-tail dimeric Affibody molecule was employed. The positions for diversification were based on structural analysis of the Z$_{Aβ3}$:Aβ interaction and the sequence output from the phage display selection of the first-generation Affibody molecules [216,226]. In one library, denoted Z$_{ASlib}$, 15 asymmetrically distributed positions were subjected for partial randomization (Figure 7.4a-b). In the other library, denoted Z$_{SYMlib}$, 16 symmetrically distributed positions (eight in each domain) were subjected for partial randomization (Figure 7.4c). In order to retain the functionality of the library, the diversity was restricted to retain the codon for the original amino acid to 50-75% in combination with a mix of codons for 1-4 other amino acids in each randomized position. The genetic library was constructed using trinucleotide synthesis, with Slonomics® technology as it allows diversification with minimal biases compared to degenerate codons.
and error-prone PCR approaches. The designs resulted in theoretical library complexities of $8.1 \times 10^7$ (ZASlib) and $2.3 \times 10^7$ (ZSYMlib) individual oligonucleotides, respectively.

**Figure 7.4:** Illustration of the residues subjected for randomization in the two truncated head-to-tail linked dimeric libraries in complex with the $\alpha\beta_1-40$ peptide (PDB entry 2OTK). Affibody subunits (E) and (F) - blue and cyan ribbons. $\alpha\beta$ - orange. **a-b)** Two suggested topologies for the asymmetric library in complex with the $\alpha\beta$ peptide, as it is not known which of the N- and C-terminal subunits in the head-to-tail dimer that correspond to the E (blue) and F (cyan) subunits of ZAβ3. **c)** Symmetric library.

**Library construction and FACS**

Genetic libraries were inserted into the staphylococcal display vector in fusion to an ABP, and subsequently displayed on the cell surface. Protein libraries with sizes of $1 \times 10^8$ (ZASlib) and $1.3 \times 10^7$ (ZSYMlib) variants were generated, representing the largest published staphylococcal displayed libraries to this date. Sequence analysis of the unsorted libraries was in principle in accordance with the theoretical design. Flow-cytometric analysis of the libraries demonstrated that 60-67% of the clones in each library had surface-exposed
Figure 7.5: a-b) Density plots from the flow-cytometric sortings of the two affinity maturation libraries. Regions used for gating are outlined in each plot. a) Asymmetric library, and b) symmetric library. c-d) Amino acid sequences of the truncated head-to-tail dimeric Z_{A\beta3} Affibody molecule and the FACS-isolated affinity-matured A\beta-binding candidates from c) asymmetric library, and d) symmetric library. Randomized positions are indicated with a dot. The numbers to the right indicate how many times each clone was isolated.
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recombinant library proteins, which was somewhat lower than expected. However, a large fraction of library clones retained target-binding capacity, as expected due to the low mutation frequency (Figure 7.5a-b). In order to isolate new Aβ-binding molecules, cell numbers corresponding to 10-50x the library sizes were incubated with Aβ peptides and subjected to four cycles of flow-cytometric cell sorting. In each cycle, the selection stringency was increased by changing sorting parameters and gates, as well as by decreasing the target concentration (50 nM, 20 nM, 10 nM), resulting in decreasing average signal intensities in subsequent rounds. In the last cycle, an off-rate selection approach was used to favor binders with the slowest dissociation rates (Figure 7.5a-b). From the last cycles of sortings, 51 unique sequences were identified from the output of the asymmetric library, and 55 unique variants were identified from the symmetrical library (Figure 7.5c-d).

Characterization

Thirty-seven clones occurring multiple times in the sequence identification were individually ranked in an on-cell strategy for apparent dissociation rate measurements. All candidates demonstrated considerably slower dissociation of the Aβ peptide compared to the 11 amino acid truncated parental (ZAβ3A12)2 Affibody (Figure 7.6a).

Eight clones from the on-cell assay were recloned and purified as soluble head-to-tail dimeric proteins for further ranking and determination of affinity and dissociation rate using an SPR-based biosensor assay. Each binder was injected at several concentrations over a neutravidin chip surface with immobilized biotinylated Aβ peptides. The head-to-tail dimeric ZSYM73 Affibody molecule demonstrated the highest affinity to Aβ1-40 (KD 336 pM) representing a 50-fold improvement in affinity relative to the published value for ZAβ3 [226,324]. Dissociation was followed during 2 h, and the off-rate for ZSYM73 was determined to 3.2 x 10^-5 s^-1 (Figure 7.6b), which is approximately 15-fold slower than for the first-generation binder.

Further characterization using CD spectroscopy showed that ZSYM73 demonstrated a retained melting temperature of approximately 43°C, as compared to the first-generation ZAβ3 [226]. Furthermore, the Affibody molecule refolded after 90°C heat-treatment, which is an attractive feature for in vivo imaging applications where radionuclide labeling is often performed at high temperatures.

Affibody-mediated Aβ capture from a complex protein mixture

In a potential therapeutic setting, a highly relevant function of the new Affibody molecule would be to capture Aβ1-42 peptides from the circulation, while simultaneously binding
Present investigation

Figure 7.6: a) Off-rate ranking of the sorted binders on the staphylococcal cell surface, analyzed by flow-cytometry. b) SPR sensorgram showing the interaction of the high-affinity ZSY,M73 to biotinylated Aβ1−40 on a neutravidin sensor chip. The Affibody molecule was injected in four concentrations (50, 25, 12.5 and 6.25 nM). c) SDS-PAGE analysis of protein fractions from ZSY,M73-mediated capture of Aβ1−42 peptides from spiked E. coli lysate. The concentration of Aβ spiked into the lysate was 10 ng/mL. Lane 1, SeaBlue2 marker; lane 2, amyloid beta spiked E. coli lysate diluted 1:10; lane 3, pooled eluted fractions (containing approximately 0.5 μg Aβ1−42 peptide), lane 4, 0.5 μg Aβ1−42 control peptide. The upper band in lanes 3 corresponds to the ABD-fused ZSY,M73 ligand and the lower band corresponds to the Aβ peptide (arrow head).

albumin for improved in vivo half-life. Therefore, such a setup was assessed in a chromatography assay. The Affibody molecule was genetically fused to ABD at the C-terminus, and non-covalently bound to HSA-sepharose for subsequent capture of Aβ1−42 peptides at physiological concentrations from spiked bacterial lysate. Several concentrations ranging from 10 ng/mL to 50 pg/mL were assayed, as levels of Aβ peptides vary a lot between patient samples (concentrations of 20 ng/mL to a few pg/mL have been reported [284,332]). After elution, SDS-PAGE analysis demonstrated that both the Affibody molecule and Aβ peptide were eluted, as expected, from the HSA-resin at acidic conditions (Figure 7.6c-d).
These results furthermore demonstrated that the $Z_{SYM73}$ ligand was capable of efficiently capturing $A\beta_{1-42}$ peptides at all assayed $A\beta$-concentrations.

![Figure 7.7](image-url)  
*Figure 7.7:* Modeling of the mutated residues in the dimeric Affibody molecule in complex with the $A\beta$ peptide. The peptide backbone of the Affibody subunits - blue (E) and cyan (F), the $A\beta$ peptide - orange.  

**a)** Substitution of I16R on the E subunit (blue) stabilize $A\beta$ through possible hydrogen interactions of arginine and tyrosines on both the E and F units.  

**b)** Substitution of I16R on the F subunit (cyan) possibly stabilize $A\beta$ through hydrogen bonding to tyrosine, and a salt bridge to $A\beta$.  

**c)** The H32R and H35E substitutions are expected to form stabilizing salt bridges in the interface of helix 2.

**Modeling of the Affibody: $A\beta$ interaction**

To understand how side-chain replacements in $Z_{SYM73}$ could act to increase binding affinity to $A\beta$, potential conformations were modeled with Swiss PDB viewer using the solution structure of the $Z_{A\beta3}$ Affibody in complex with $A\beta_{1-40}$ (PDB 2OTK) as a template. The Affibody molecule contained three replacements in each of the domains, corresponding to I16R, H32R and H35E in both the E and F subunits of $Z_{A\beta3}$. These substitutions caused the two-fold symmetry of $Z_{A\beta3}$ to break in complex with $A\beta$. The asymmetry primarily affected modeling of the I16R replacement. We hypothesized that the I16R replacement on the E subunit provided the complex with additional stabilizing hydrogen bonds together with the tyrosine residues (Y18) on both the E and F subunits to the $A\beta$ ligand (Figure 7.7a). Similarly, I16R replacement on the F subunit enabled hydrogen bonding with Y18 on the E subunit and, more importantly, a salt bridge bond with a glutamate (E22) on the $A\beta$ ligand (Figure 7.7b). The selection of H32R and H35E in $Z_{SYM73}$ were probably based on R32 and E35 forming salt bridges that presumably act to stabilize helix 2, which would be favorable for complex formation (Figure 7.7c).
Study III

Affibody-mediated reduction of amyloid burden and improvement of cognitive decline in an animal model of Alzheimer’s disease

In this study, the high-affinity $Z_{SYM}$ Affibody molecule was further characterized in an APP/PS1 double transgenic (2xTg) mouse model of AD. The phenotype for this strain was manifested by overexpression of aggregation-prone Aβ peptides, and eventually plaque deposition in the brains of the animals at an early age. The Affibody was assessed for preventative treatment, with injections starting at the onset of pathology development.

Design and production of Affibody molecules for injection

It has previously been demonstrated that fusion of Affibody molecules to an affinity-matured albumin binding domain significantly improved the in vivo half-life, and hence the therapeutic effect through binding to serum albumin [120]. Based on such demonstrations and results from study II, in which it was demonstrated that the $Z_{SYM}$ molecule in fusion to an ABD could interact with Aβ and albumin simultaneously, we here subcloned and expressed the high-affinity $Z_{SYM}$ Affibody molecule and a dimer of the control molecule $Z_{Taq}$ in fusion with a C-terminal deimmunized high-affinity albumin-binding domain (ABD$_{094}$). A flexible 10 amino acid linker was included to separate the Affibody and ABD moieties. The resulting proteins were $[Z_{SYM}]$-GAPG4STS-ABD$_{094}$ and $[Z_{Taq}]_2$-GAPG4STS-ABD$_{094}$ (hereinafter denoted $Z_{SYM}$-ABD and $(Z_{Taq})_2$-ABD, respectively). The proteins were produced in *E. coli*, and cultivation was conducted using a multi-fermentor system (Belach Bioteknik AB). Approximately 300 mg of each protein variant was purified using an affinity chromatography column with an ABD-specific affinity ligand immobilized on Sepharose (Affibody AB) followed by size exclusion chromatography in an ÄKTA system (GE-Healthcare). Highly pure proteins were obtained by applying the sample onto Endo-Trap columns to remove potential endotoxins. The molecular weight and purity of the proteins were verified by LC/MS and SDS-PAGE using both reducing and non-reducing conditions (Figure 7.8) and the protein concentration was determined by absorbance measurement at 280 nm.

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Animal model and treatment

To model amyloid deposition and amyloid related pathology, the well-characterized double-transgenic APP/PS1 mouse model was used (2xTg, APP<sub>K670N/M671L</sub> and PS1<sub>M146V</sub>). The 2xTg mice overexpressed mutations in APP and PS1 at the β and γ-secretase cleavage sites, resulting in elevated levels of Aβ production and pathology development at an early age of 3-4 months. The presence of the transgenes (APP/PS1) in each mouse were first confirmed by PCR. Thereafter, twenty animals were divided into two groups that received three weekly intraperitoneal (i.p.) injections of 100 µg (in a volume of 100 µl) of Z<sub>SYM73-ABD</sub> protein or the irrelevant (Z<sub>Taq</sub>)<sub>2</sub>-ABD protein for 13 weeks, beginning at the onset of pathology development. During the treatment, veterinary staff monitored the animals for signs of toxicity, including changes in body weight, physical appearance, and altered behavior. None of the animals showed any signs of toxicity. At 6-7 months, the mice went through extensive behavioral testing and were subsequently sacrificed for tissue analysis.

Behavioral assessment of the mice

Prior to cognitive testing in a radial maze, motor coordination and balance of the animals were tested by exploratory locomotor activity and accelerating rotarod performance tests.
These tests were performed to exclude that any observed effects in the performance of the cognitive tasks were due to differences in sensorimotor abilities. Before all behavioral testing, the mice were adapted to the room with lights on for 15 min. All tests were performed by an individual blinded to the treatment assignment.

**Sensorimotor tests**

Exploratory locomotor activity was recorded in a circular open field chamber, measuring 70 cm in diameter. Prior to testing, the mice were habituated for 15 min in the field. In the trial, a camera mounted above the chamber recorded horizontal movements in the open field in each dimension. Total distance was measured in centimeters traveled and was defined as the sum of sequential movement between interruptions of the animal. The duration of the behavior was timed over 15 min. Results from the study demonstrated that there were no significant differences between the groups in locomotor activity in terms of distance traveled, maximum speed, mean velocity and rest time for the mice (Figure 7.9a).

The animals were also assessed in a rotarod test, in which each animal was placed on a rod apparatus with a diameter of 3.6 cm to assess differences in motor coordination and balance. The mice were habituated to the apparatus for two training trials in order to reach a baseline level of performance, and subsequently tested with increasing speed in three trials. The total time and latency to fall or invert (by clinging) from the top of the

![Graphs showing sensorimotor data](image)

**Figure 7.9:** Sensorimotor testing shows that there is no performance difference amongst the groups. *a)* Open field locomotor activity shown in max and mean velocity ($V_{\text{max}}$ and $V_{\text{mean}}$), distance travelled, and rest time of the $Z_{\text{SYM73}}$-ABD treated and negative control-treated mice. *b)* Rotarod shown in running speed (rpm).
rotating barrel was recorded. In correlation with locomotor activity assessment, rotarod testing did not reveal any differences between transgenic controls versus treated transgenic mice (Figure 7.9b). The results suggest that the Affibody treatment did not influence the behavior of the mice in any negative way.

**Radial maze**
The working memory of the mice was subsequently assessed in a radial maze with eight 30 cm long arms originating from a central space. In addition to the $Z_{SYM73}$-ABD and $(Z_{Taq})_2$-ABD treated mice, an age-matched control group of 10 wild type mice was included in the experiment. After two days of adaptation, water restricted mice were given one training session per day for ten consecutive days. The animals were given controlled access to the arms from a central area from which the animals entered and exited the apparatus. During each session, all arms were baited with saccharine-flavored water, and the animals were permitted to enter all arms until the eight rewards had been consumed. The number of errors (entries to previously visited arms) and time to complete each session were recorded. Results demonstrated that mice treated with $Z_{SYM73}$-ABD, and wild type mice used as controls, performed significantly better compared to the 2xTg mice injected with control Affibody molecule. In fact, there was no difference in performance between the wild type control mice and the $Z_{SYM73}$-ABD treated animals (Figure 7.10). Hence, treatment with $Z_{SYM73}$-ABD significantly improved the cognitive abilities of the transgenic AD model mice.

![Figure 7.10: Cognitive testing of 2xTg mice using a radial arm maze. The number of errors is plotted versus the day of testing. The $Z_{SYM73}$-ABD treated mice demonstrated a restored memory function, relative to $(Z_{Taq})_2$-ABD treated animals.](image)
Histology

Prior to extraction of the brain for analysis of Aβ levels and inflammatory responses, the mice were anesthetized with sodium pentobarbital, and perfused transaortically with phosphate buffer. The right hemisphere of the brain was immersion-fixed in periodate-lysine-paraformaldehyde, snap-frozen and cut in serial coronal sections of 40 µm (approximately 30 sections/brain in total). Sections of cortex and hippocampus of the treated mice were stained with a mixture of the monoclonal 6E10/4G8 antibodies that recognize both pre-amyloids and plaques of Aβ.

**Figure 7.11:** Quantification of Aβ burden in mouse brains. Representative brain sections, immunostained with α-Aβ antibodies 6E10/4G8. Hippocampus of a) negative control-treated and b) ZSYM73-ABD treated mice. c) Bar graph of Aβ levels in dentate gyrus. Cortex of d) control-treated and e) ZSYM73-treated mice. f) Bar graph of Aβ levels in motor cortex of negative control and ZSYM73-ABD treated mice. On average there was a 50% reduction of levels in dentate gyrus and motor cortex, respectively.

Immunostaining demonstrated a significant reduction of Aβ burden in both hippocampus (Figure 7.11a-c) and cortex (Figure 7.11d-f) of the ZSYM73-ABD treated mice, relative to negative control-treated mice. On average there was a 50% reduction of levels in dentate gyrus and motor cortex, respectively.
Present investigation

Figure 7.12: Representative brain sections from a) control and b) \(Z_{SYM73}\)-ABD Affibody-treated 2xTg mice, immunostained with anti-GFAP for quantification of astrogliosis in the hippocampus. Histological quantitation revealed that there was a reduction of astrogliosis in the \(Z_{SYM73}\)-ABD Affibody-treated mice, however this was not significant. c) Bar graph of astrogliosis levels in hippocampus.

Neuroinflammatory response after treatment

An important concern when administering a treatment that targets A\(_\beta\) deposits is the possibility of increased brain inflammation, ultimately resulting in neuronal dysfunction or death. Neuroinflammation, due to an abnormal activation of microglia and astrocytes, is commonly seen in AD brains. Therefore, the extent of astrogliosis was examined in the brains of the animals, using immunohistochemical staining of sections from the right hemisphere. An anti-GFAP (glial fibrillary acidic protein) antibody was used to stain for GFAP, which is a component of the glial intermediate filaments that forms part of the cytoskeleton in astrocytes. Histological observation revealed that there was a larger amount of astrogliosis in the negative control treated group of animals than the \(Z_{SYM73}\)-ABD Affibody-treated 2xTg mice (Figure 7.12 a-b). However, semiquantitative analysis showed that the observed differences were not significant (Figure 7.12 c).
Present investigation

Conclusions on studies I, II, and III

These three studies describe the engineering and development of the previously in-house generated $Z_{A\beta 3}$ Affibody molecule that targets monomeric, non-aggregated $A\beta$ peptides. In the first two studies, $Z_{A\beta 3}$ was engineered for improved traits, including increased binding to the $A\beta$ peptide. Staphyloccocal cell surface technology was used to display large truncated head-to-tail dimeric affinity maturation libraries for subsequent FACS-screening and isolation of high-affinity binders. An Affibody molecule, denoted $Z_{SYM73}$, demonstrated an affinity of approximately 300 pM, corresponding to a 50-fold improvement, as compared to the first-generation binder. In the third study, the new high-affinity Affibody molecule was investigated in a preclinical study aimed at preventing development of disease-related A$\beta$ pathology in a transgenic mouse model of AD. The Affibody molecule was generated in fusion to an ABD, in order to prolong the in vivo half-life and hence its therapeutic effect. The treatment demonstrated that the Affibody molecule was well tolerated by the animals, as demonstrated by the lack of side-effects after the 13 week treatment. Furthermore, treatment significantly improved the working memory and cognitive functions of the mice. This behavioral rescue was associated with a marked reduction in A$\beta$ pathology, as determined histologically (and biochemically). These results demonstrate that treatment with the newly engineered $Z_{SYM73}$ Affibody molecule was in fact beneficial for the transgenic animals. Moreover, this pre-clinical study comprises the first intervention to demonstrate a therapeutic effect of an Affibody molecule through the mere protein-interaction itself, i.e. sequestering of monomeric A$\beta$ peptides, which is encouraging for development of future Affibody-based therapies.

Future perspectives

The positive results from the studies with the new A$\beta$-binding Affibody molecule have led to continued investigations. In an on-going study, with interesting initial results, the $Z_{SYM73}$ Affibody molecule is used to study the A$\beta$ aggregation process.

In addition, the promising results from the mouse study have led to the planning of new animal studies, which will aim to investigate if $Z_{SYM73}$ can be used to treat mice that have already developed pathology, i.e. to assess if the Affibody molecule can act to reverse pathology.

Interesting future engineering projects, which could further improve the performance and possibly also the potential for in vivo applications of the $Z_{SYM73}$ Affibody molecule include
i) improving the stability for labeling approaches and also potential in vivo administration
that generally require a high stability of the binder, \( ii) \) introducing pH-sensitivity into the \( Z_{SYM73}:A\beta \) interaction, so that the natural endosomal pathway could be exploited for degradation of A\( \beta \) peptides while the Affibody molecule would be sent back to the blood for binding of new peptides (via the Z-ABD:HSA:FcRn interaction), \( iii) \) introducing proteolytic functionality into the binder, which would cleave the A\( \beta \) peptide in two harmless parts, and \( iv) \) engineering bispecificity to boost brain uptake by fusing the \( Z_{SYM73} \) Affibody molecule to a domain that binds for example the transferrin receptor (TfR). Such binding-domain could for example be another Affibody molecule or an Affimab (mAbs with Affibody molecules attached to them) Additional interesting targets for generation of bispecific binders are the Tau protein, ApoE, BACE1, PS1, and PS2.
Study IV

Selection of binding-proteins that specifically recognize protofibrillar aggregates of amyloid-β

The aggregation process of Aβ involves a number of intermediate aggregation states, collectively called soluble oligomers, and evidence suggests that there is a direct causative link between soluble Aβ oligomers and synapse dysfunction [333,334]. In order to detect such aggregates in both research as well as diagnostic and therapeutic applications, we here generate a new set of Affibody molecules that recognize protofibrils of Aβ.

Much of the details surrounding oligomer formation and interconversion both in vivo and in vitro remain elusive. Nevertheless, protofibrils of wild type Aβ are instable, and therefore not optimal as target molecule in a selection. Therefore, a previously engineered Aβ_{1-42} variant (denoted Aβ_{42cc}), which forms protofibrils that do not convert into amyloid fibrils [321,322,327] was utilized in the selection. In Aβ_{42cc}, alanine residues 21 and 30 are replaced with cysteine residues, allowing an intramolecular disulfide bond to form. The disulfide locks the peptide in a hairpin conformation, which is compatible with the conformation of Aβ_{1-42} in protofibrils, but incompatible with the conformation observed in fibrils (previously described in chapter 6). Aggregation of Aβ_{42cc} is therefore halted at the protofibril stage, and therefore, Aβ_{42cc} protofibrils can be a suitable mimic of wild type Aβ_{1-42} protofibrils.

Phage display selection

Affibody molecules were selected using phage display with biotinylated Aβ_{42cc} protofibrils as target and streptavidin-coated magnetic beads for capture of binding-variants. Affibody molecules were selected from a naïve phage library containing $1.4 \times 10^{10}$ variants. The library was designed to exclude cysteines exclude the possibility of generating cysteine-containing Affibody molecules that can form dimers, such as the Aβ-monomer binding Affibody molecule that the previous studies are based upon. The selection was performed in two tracks, of which one involved a pre-selection against fibrils of Aβ_{1-42} to remove fibril-binders. Six cycles of selection were performed with increased stringency in-between each round by decreasing the target concentration from 2 μM (monomer based) in the first round down to 0.2 nM in the final round.

744 randomly chosen Affibody-carrying phage clones from cycles four and six were ex-
pressed directly from the phagemid vector in fusion to an albumin-binding domain (ABD), and screened for Aβ_{42cc} protofibril-binding activity in an ELISA assay. 56 protofibril-binders were identified and ranked based on their apparent equilibrium dissociation constants (EC_{50} values). In principle, all Affibody molecules showed a concentration dependent binding response to Aβ_{42cc} protofibrils. Estimated EC_{50} values were in the range of 15 to 90 nM for the top 25 binders.

Validation of target-binding

Based on ELISA-binding performance and sequence homology, five strong Aβ_{42cc} protofibril binders (denoted Z_{Aβ^{42cc−1}}, Z_{Aβ^{42cc−2}}, Z_{Aβ^{42cc−3}}, Z_{Aβ^{42cc−4}}, and Z_{Aβ^{42cc−5}}) were selected for further characterization (Figure 7.13). The five Affibody molecules and one irrelevant negative control Affibody molecule (Z_{Taq}) were subcloned with and without a terminal ABD-fusion, expressed and purified for more detailed characterization.

As it was essential that the selected binding proteins also recognized wild type Aβ_{1−42} aggregates, the binding selectivity of the binders to different aggregated forms of Aβ_{42cc} and wild type Aβ_{1−42} was profiled using a second type of ELISA assay. Affibody-ABD fusions were immobilized to a plate via an anti-ABD antibody followed by incubation with Aβ_{42cc} protofibrils, Aβ_{42cc} monomer, wild type Aβ_{1−42} protofibrils, wild type Aβ_{1−42} fibrils or wild type Aβ_{1−42} monomer. The wild type Aβ_{1−42} protofibrils were generated by incubating Aβ_{1−42} at 4°C overnight and purifying the protofibrils by size exclusion chromatography. An irrelevant Affibody molecule and PBS buffer were used as controls. The bound Aβ that remained after washing was detected using an HRP-conjugated antibody (6E10) that recognized the N-terminus of Aβ (most likely insensitive to the Aβ aggregation state) or a monoclonal antibody that recognizes protofibril conformations over monomeric and low-molecular weight Aβ aggregates (mAb1C3). The results with conformation independent 6E10 antibody detection indicate that three of the five tested Affibody molecules possibly bind wild type protofibrils with an affinity that, at least in the case of Z_{Aβ^{42cc−1}}, is close to that for binding of Z_{Aβ^{42cc}}. Detection using the weakly protofibril conformation-selective mAb1C3 monoclonal antibody indicates that wild type protofibrils remain bound to Z_{Aβ^{42cc−1}}, Z_{Aβ^{42cc−3}} and possibly Z_{Aβ^{42cc−4}} at the time of detection.

As expected, the five Affibody molecules were all shown to bind to Aβ_{42cc} protofibrils. More importantly, Z_{Aβ^{42cc−1}}, Z_{Aβ^{42cc−3}}, and Z_{Aβ^{42cc−4}} also recognized wild type Aβ_{1−42} protofibrils, which was the intended outcome of the selection (Figure 7.14). None of the five binders recognized monomeric or fibrillar wild type Aβ_{1−42}, but all binders displayed some apparent affinity for the Aβ_{42cc} monomer, which was probably an artefact due to aggregation.
Figure 7.13: Sequences of the top 25 Affibody molecules selected for binding to Aβ_{42}cc protofibrils (randomized positions indicated by dots). Z_{Aβ_{42}cc−1}, Z_{Aβ_{42}cc−2}, Z_{Aβ_{42}cc−3}, Z_{Aβ_{42}cc−4}, and Z_{Aβ_{42}cc−5} (arrow heads) were chosen for further characterization.

Affinity determination

The Affibody molecules were characterized by determining the kinetics for association and dissociation using an SPR-based biosensor assay. Each binder was injected in several concentrations over a CM5 sensor chip with immobilized Aβ_{42}cc protofibrils. The Affibody molecules, with and without ABD fusions, all demonstrated similar affinity and kinetics with average dissociation constants for the high-affinity sites of k_d = 5.0 (±2) \times 10^{-4} \, \text{s}^{-1} and K_D = 1.7 (±0.6) \, \text{nM}.

The concentrations of Aβ_{1−42} protofibrils in the brains of patients has not yet been determined, however are speculated to be very low, relative to the total soluble Aβ_{1−42} concentration. Therefore, it would be desirable to generate even stronger binding Affi-
body molecules to ensure the detection of protofibrillar species. It was recently shown that by creating Affibody dimers to repetitively arranged targets, the affinity can be improved 1000-fold as compared to monomers [335]. Hence, the proteins were produced as head-to-tail dimers with flexible (GGGS)$_n$ or (GGGGS)$_n$ linkers of variable lengths. In total, 14 dimeric constructs, with or without ABD-fusions were generated. As expected, all dimers demonstrated slower off-rates, and in some cases also higher affinities of up to 1 - 2 pM (Table II). However, the affinity gain was not as high as expected, based on previous studies. Furthermore, there were in some cases trends of an apparent effect of the linker length on the binding affinity. For instance, dimeric $Z_{A\beta 42cc-4}$ without linker ($Z_{A\beta 42cc-4}-Z_{A\beta 42cc-4}$) demonstrated a $K_D = 0.26$ nM, intermediate length linkers showed $K_D = 0.6$ to 0.7 nM, and the $Z_{A\beta 42cc-4}$ dimer with the longest linker ($Z_{A\beta 42cc-4}-(GGGS)_4-Z_{A\beta 42cc-4}$)
demonstrated weaker binding ($K_D = 1.1 \text{ nM}$). This trend was however not statistically significant over the whole dimer binding data set.

Table II. Kinetics of Aβ$_{42}$cc-binding Affibody molecules, determined by SPR.

<table>
<thead>
<tr>
<th>Binding topology</th>
<th>$K_D$ (nM, mean ± SD)</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$, mean)</th>
<th>$k_d$ (s$^{-1}$, mean)</th>
<th>$K_D$ (nM, mean ± SD)</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$, mean)</th>
<th>$k_d$ (s$^{-1}$, mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZAβ$_{42}$cc_1</td>
<td>1.6 ± 0.06</td>
<td>2.9 × 10$^5$</td>
<td>4.7 × 10$^4$</td>
<td>7.0 ± 0.49</td>
<td>1.2 × 10$^4$</td>
<td>8.6 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$_{42}$cc_2</td>
<td>1.1 ± 0.02</td>
<td>7.2 × 10$^5$</td>
<td>7.9 × 10$^4$</td>
<td>9.7 ± 0.20</td>
<td>5.8 × 10$^5$</td>
<td>5.6 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$_{42}$cc_5</td>
<td>1.4 ± 0.01</td>
<td>2.5 × 10$^5$</td>
<td>4.8 × 10$^4$</td>
<td>15.6 ± 0.10</td>
<td>1.3 × 10$^5$</td>
<td>2.0 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$_{42}$cc_3</td>
<td>2.5 ± 0.14</td>
<td>2.5 × 10$^5$</td>
<td>6.3 × 10$^4$</td>
<td>8.7 ± 0.54</td>
<td>3.4 × 10$^5$</td>
<td>3.0 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$_{42}$cc_4</td>
<td>0.3 ± 0.00</td>
<td>3.4 × 10$^7$</td>
<td>9.7 × 10$^4$</td>
<td>5.3 ± 0.02</td>
<td>2.4 × 10$^4$</td>
<td>1.3 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_4-(G$</em>{3}$S)-ZAβ$_{42}$cc_4</td>
<td>0.6 ± 0.01</td>
<td>3.4 × 10$^7$</td>
<td>1.9 × 10$^4$</td>
<td>4.4 ± 1.40</td>
<td>1.8 × 10$^5$</td>
<td>8.2 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_5-(G$</em>{4}$S)-ZAβ$_{42}$cc_5-ABD</td>
<td>0.1 ± 0.00</td>
<td>6.2 × 10$^4$</td>
<td>6.2 × 10$^3$</td>
<td>2.6 ± 0.08</td>
<td>1.4 × 10$^3$</td>
<td>3.6 × 10$^2$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_4-(G$</em>{4}$S)</td>
<td>0.7 ± 0.01</td>
<td>3.2 × 10$^7$</td>
<td>2.4 × 10$^4$</td>
<td>2.7 ± 0.01</td>
<td>1.1 × 10$^5$</td>
<td>3.1 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_4-(G$</em>{4}$S)-ZAβ$_{42}$cc_4</td>
<td>0.6 ± 0.01</td>
<td>4.0 × 10$^7$</td>
<td>2.4 × 10$^4$</td>
<td>4.5 ± 0.02</td>
<td>7.3 × 10$^4$</td>
<td>3.4 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_4-(G$</em>{4}$S)-ZAβ$_{42}$cc_4</td>
<td>1.1 ± 0.05</td>
<td>2.2 × 10$^7$</td>
<td>2.5 × 10$^4$</td>
<td>3.8 ± 3.80</td>
<td>1.3 × 10$^5$</td>
<td>4.9</td>
</tr>
<tr>
<td>ZAβ$_{42}$cc_5-ABD</td>
<td>1.9 ± 0.07</td>
<td>3.1 ± 10$^5$</td>
<td>6.2 × 10$^4$</td>
<td>24.6 ± 1.20</td>
<td>3.2 × 10$^4$</td>
<td>8.0 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_5-(G$</em>{4}$S)-ZAβ$_{42}$cc_5-ABD</td>
<td>0.1 ± 0.00</td>
<td>3.1 × 10$^5$</td>
<td>3.7 × 10$^4$</td>
<td>14.8 ± 0.50</td>
<td>6.6 × 10$^5$</td>
<td>9.8 × 10$^2$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_5-(G$</em>{4}$S)-ZAβ$_{42}$cc_5-ABD</td>
<td>2.0 ± 0.03</td>
<td>2.5 × 10$^5$</td>
<td>5.2 × 10$^4$</td>
<td>17.5 ± 0.30</td>
<td>1.9 × 10$^5$</td>
<td>3.3 × 10$^2$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_5-(G$</em>{4}$S)-ZAβ$_{42}$cc_5-ABD</td>
<td>2.8 ± 0.04</td>
<td>1.6 × 10$^5$</td>
<td>4.5 × 10$^4$</td>
<td>17.2 ± 0.26</td>
<td>1.3 × 10$^5$</td>
<td>2.3 × 10$^2$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_4-(G$</em>{4}$S)-ZAβ$_{42}$cc_4</td>
<td>2.3 ± 0.01</td>
<td>2.0 × 10$^5$</td>
<td>4.8 × 10$^4$</td>
<td>19.3 ± 0.19</td>
<td>3.2 × 10$^5$</td>
<td>6.2 × 10$^2$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_1-(G$</em>{4}$S)-ZAβ$_{42}$cc_1-ABD</td>
<td>1.7 ± 0.02</td>
<td>3.1 × 10$^5$</td>
<td>5.2 × 10$^4$</td>
<td>5.2 ± 0.14</td>
<td>2.5 × 10$^5$</td>
<td>1.4 × 10$^4$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_1-(G$</em>{4}$S)-ZAβ$_{42}$cc_1-ABD</td>
<td>1.1 ± 0.03</td>
<td>1.4 × 10$^5$</td>
<td>1.6 × 10$^4$</td>
<td>8.4 ± 0.46</td>
<td>1.5 × 10$^4$</td>
<td>1.2 × 10$^2$</td>
</tr>
<tr>
<td>ZAβ$_{42}$cc_3-ABD</td>
<td>0.9 ± 0.02</td>
<td>1.3 × 10$^5$</td>
<td>1.3 × 10$^4$</td>
<td>4.6 ± 0.13</td>
<td>6.6 × 10$^6$</td>
<td>3.1 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_3-(G$</em>{4}$S)-ZAβ$_{42}$cc_3-ABD</td>
<td>0.2 ± 0.01</td>
<td>1.3 × 10$^5$</td>
<td>2.1 × 10$^4$</td>
<td>56.2 ± 5.50</td>
<td>7.1 × 10$^5$</td>
<td>4.0 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_3-(G$</em>{4}$S)-ZAβ$_{42}$cc_3-ABD</td>
<td>0.9 ± 0.03</td>
<td>3.9 × 10$^5$</td>
<td>3.7 × 10$^4$</td>
<td>7.1 ± 0.17</td>
<td>3.3 × 10$^5$</td>
<td>2.3 × 10$^3$</td>
</tr>
<tr>
<td>ZAβ$<em>{42}$cc_3-(G$</em>{4}$S)-ZAβ$_{42}$cc_3-ABD</td>
<td>0.1 ± 0.01</td>
<td>1.5 × 10$^5$</td>
<td>1.6 × 10$^4$</td>
<td>17.2 ± 2.40</td>
<td>1.4 × 10$^5$</td>
<td>2.5 × 10$^2$</td>
</tr>
</tbody>
</table>

**Binding topology**

The sequences of the phage-selected Affibody molecules were, with few exceptions highly homologous, suggesting that they bind the same surface on the protofibrils (Z$_{Aβ_{42}cc-5}$ appears to reflect a different binding surface). The chemical properties of the side-chains of the Affibody molecules were mainly defined to three chemically distinct regions of the Z-domain surface, demonstrating a 'positive-nonpolar-polar' surface pattern. It has recently been demonstrated that the structural model of a hexameric protofibril building block of Aβ$_{42}$cc contains surfaces that might match the selected surface pattern of the selected Affibody molecules, and hence constitute a binding epitope.
Conclusions on study IV

In this study, we have developed Affibody molecules targeting protofibrils of Aβ. Despite the fact that this project is still in an early phase of development, the binders are envisioned for potential future detection of aggregates both in research as well as diagnostic and/or therapeutic applications. Beyond those aims, this study importantly also illustrates the power of protein engineering in several ways, including the engineered Aβ\textsubscript{12cc} variant that can be employed to form stable protofibrils (which do not form fibrils), and potential to select for binders that can discriminate a certain protein aggregate from other aggregated and monomeric form of the same peptide.

Future perspectives

Interesting future projects include more extensive characterization the generated binders, including structural studies to examine the binding sites on the protofibrils.

Other interesting future engineering projects also include strategies for affinity maturation to improve the binding of the Affibody molecules to the protofibrils. Such strategies could include generating affinity maturation libraries based on monomeric binders, homo-dimers, or even heterodimers of interesting candidates.

Inspired by the animal studies from the first studies in the thesis, it would also be compelling to investigate the potential of the Affibody molecules as candidates in a therapeutic setting for AD.
Study V

Development of a fluorescence-based intracellular screening assay for functional selection of protein-based aggregation inhibitors

Affinity proteins for therapeutic purposes are mainly generated based on their affinity to the target, without taking the final and desired mechanism of action into account. Such final function could for example be the capacity to inhibit aggregation of peptides or proteins that are involved in neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease. In this study, we therefore sought to develop a method that is based on direct functional screening for aggregation-inhibition capacity, with focus on protein-based aggregation inhibitors to the Aβ peptide.

Design of the system

It has previously been demonstrated that N-terminal coupling of aggregation-prone peptides to green fluorescent protein (GFP) allowed for monitoring of the aggregation process. Intracellular bacterial expression of the aggregation-prone Aβ peptide resulted in a collapse of both the peptide and GFP, and thereby yielded a readily measurable decrease in fluorescent signal, as compared to a soluble mutant of the Aβ peptide in fusion to GFP [336].

Based on this concept, we here developed an intracellular system for high-throughput FACS-screening of protein-based libraries for aggregation inhibitors. Principally, expression of the GFP-fused aggregation-prone target peptide would result in a fluorescent collapse, whereas co-expression with a protein-based aggregation inhibitor would prevent the peptide from aggregating and thereby reinstate the fluorescent signal (Figure 7.15a). To avoid biases from cell-to-cell variations in protein expression levels amongst different cells in future libraries, the inhibitor was genetically fused to a separate fluorescent reporter molecule (mCherry) that provided a tool for normalization of expression levels using two-color FACS (Figure 7.15b). The method was modeled using the Alzheimer’s-related Aβ_{1–42} peptide and the dimeric (Z_{Aβ3})_2 Affibody molecule that possesses a known inhibitory capacity [226,325]. In addition, a negative control Affibody molecule (Z_{HER2}), and soluble variant of the Aβ peptide (Aβ_{1–42}F19S/L34P) were included as controls. An enhanced variant of GFP (denoted EGFP) was used in this study, as it has been optimized for increased excitation by 488 nm laser light in the flow-cytometer. A two-vector system was
Present investigation

Figure 7.15: Concept of using the intracellular two-color expression system for identification of aggregation inhibitors by flow-cytometric screening. **a)** Genetic fusions of an aggregation-prone peptide to the N-terminus of GFP causes a reduction in GFP signal upon aggregation, as compared to soluble controls. Rescue of the peptide from aggregation restores fluorescence. **b)** In library applications, a low expressing cell will yield lower signal than a high expressing variant even thought being equally capable of inhibiting aggregation. To avoid biases based due to cell-to-cell variations, expression levels are normalized to target-binding capacity. **c)** Investigated expression cassettes for the aggregation-prone Aβ peptide and the Affibody inhibitor, respectively.
Present investigation

exploited, with the expression cassettes for the target and the library on separate plasmids. The rationale for developing a two-vector platform was that it would provide the system with a high flexibility since it allows the user to design the recombinant expression system by using vectors with different promoters, origins of replication and resistance markers. Here, we investigated pET26b+ (T7 promoter, ColE1 ori, kanamycin resistance) for harboring of the aggregation-prone $\alpha\beta_{1-42}$ target peptide, and the pBAD-vector (L-arabinose-inducible areBAD promoter, p15A ori, chloramphenicol resistance) for harboring of the dimeric $(Z\alpha\beta_3)_2$ Affibody molecule (Figure 7.15c).

In order to promote correct folding of the inhibiting Affibody molecule (which depends on an internal disulfide) an *E. coli* strain (denoted BL21 *Shuffle T7*) with engineered oxidizing cytoplasmic environment was investigated [337].

**Proof-of-concept**

Expression of $\alpha\beta_{1-42}$ in fusion to EGFP resulted in a low fluorescence, relative to expression of the soluble variant $\alpha\beta_{1-42}F19S/L34P$, which strongly indicated that the aggregation properties of the peptide correlated with the intensity of the GFP fluorescence. Cells co-expressing $\alpha\beta_{1-42}$-EGFP and $(Z\alpha\beta_3)_2$-mCherry demonstrated an increased fluorescent signal, relative to cells with $\alpha\beta_{1-42}$-EGFP or co-expressed with the $Z_{HER2}$ negative control, indicating that the Affibody inhibited aggregation and allowed EGFP to fold into its native structure (Figure 7.16).

![Figure 7.16: Flow-cytometric analysis for proof-of-concept. Cells co-expressing Aβ-EGFP and negative control ZHER2 results in a reduced fluorescent signal, as compared to the soluble controls EGFP and Aβ_{1-42}F19S/L34P-EGFP. Upon-co-expression with the aggregation inhibiting (Z\alpha\beta_3)_2, an increase in fluorescence is monitored, indicating an inhibition of the aggregation process.](image)
Optimization of cultivation conditions

In order to provide the system with a larger dynamic range, expression levels were optimized by assessing cultivation conditions including temperature for growth, concentration of inducer and time point of induction. Initially, expression of the aggregation inhibitor and the Aβ peptide were induced simultaneously (0.2% arabinose and 0.5 mM IPTG), with flow-cytometric analysis after 16 h cultivation. As previously illustrated, analysis of

Figure 7.17: Evaluation of cultivation conditions. Representative histograms showing fluorescence intensities for a) EGFP expression of cells co-expressing Aβ₁₋₄₂ and Affibody, b) mCherry expression of cells co-expressing Aβ₁₋₄₂ and Affibody, c) Monitoring of mCherry expression over time. Affibody expression was induced simultaneously, or 1h, 2h, 3h, 4h, and 16h prior to induction of Aβ expression d) arabinose titration, d) IPTG titration, d) inhibition of Aβ₁₋₄₂ aggregation at 25°C, e) inhibition of Aβ₁₋₄₂ aggregation at 37°C.

cells that co-expressed Affibody and Aβ₁₋₄₂ peptide demonstrated that the system was capable of inhibiting aggregation, as assessed by the EGFP signal (Figure 7.17a). How-
ever, analysis of the Affibody expression levels within the same cells (based on mCherry signals) demonstrated that both \((Z_{A\beta 3})_2\)-mCherry and \(Z_{HER2}\)-mCherry expression or folding were affected when co-expressed with the A\(_{\beta 1-42}\) peptide, as demonstrated by the reduced mCherry fluorescence (Figure 7.17b). Titration of the arabinose concentration did not yield higher MFI signals. Therefore, analysis of the Affibody molecule expression was investigated over a period of time. The Affibody expression was induced by 0.2% arabinose simultaneously, or 1 h, 2 h, 3 h, 4 h, or 16 h prior to induction of A\(\beta\) expression by 0.5 mM IPTG. Flow-cytometric analysis indicated that cells that were induced with arabinose prior to IPTG yielded higher expression signals for the inhibitor (Figure 7.17c).

![Figure 7.18: Density plots from the flow-cytometric sortings of the mock library containing a 1:10,000 mix of inhibitor:non inhibitor. Leftmost plot shows the library before sorting, with the sorting gate indicated in the plot. The rightmost plot shows the enriched population after sorting and overnight growth. The percentage (mean) of events in the upper right quadrant is indicated in the dotplots.](image)

The arabinose concentration was subsequently titrated at concentrations varying from 0.2-0.8%. Results demonstrated that at 0.6% arabinose, expression reached saturation (Figure 7.17d). The expression of the A\(\beta\) peptide was induced 4 h after the inhibitor with several concentrations of IPTG, varying from 0.05 - 0.25 mM (lower and higher concentrations were also tested). Flow-cytometric analysis demonstrated that the expression was not titratable, as it reached saturation at the lowest concentration (in correlation with the literature) (Figure 7.17f). In addition, expression was evaluated at different temperatures. At 25\(^\circ\)C, cells that co-expressed the A\(\beta\) peptide and the aggregation-inhibiting Affibody molecule or the negative control Affibody molecule yielded similar MFI signals, indicating that the peptide did not aggregate properly at this temperature (Figure 7.17e). At 37\(^\circ\)C, cells containing inhibitor versus cells containing a non-inhibitor instead demonstrated a greater shift in expression levels (Figure 7.17g).
Present investigation

Enrichment factor and cell viability from FACS sorted mock library

As a proof-of-concept, a mock library was generated and screened using flow-cytometry for subsequent isolation of aggregation inhibitors. A large pool of cells co-expressing Aβ1-42-EGFP and ZHER2-mCherry was spiked with cells co-expressing Aβ1-42-EGFP and (Z_{A33})_2-mCherry at a 1:10,000 ratio and screened for the inhibiting population using flow-cytometry. The top <0.01% fraction of the cell population, exhibiting the highest ratio of EGFP/mCherry signals, were sorted in one single round (Figure 7.18). Viability of the sorted cells was investigated by directly spreading the sorted cells onto agar plates for cultivation. A 3,500-fold enrichment was achieved, with a 64±10% cell viability. This mock selection demonstrated that it was possible to enrich for inhibitors that constituted 0.01% of the initial cell population in one single selection round under stringent sorting conditions.
Conclusions on study V

In this study, we have developed a method that allows for generation of protein-based aggregation inhibitors. The platform was based on intracellular bacterial co-expression of the target and inhibitor, with the expression cassettes for the respective proteins on distinct plasmids. A dual-vector platform provides the system with a high flexibility since different promoters, origin of replications and resistance markers can be explored. In addition, the method is based on a two-color system that allows for flow-cytometric cell sorting of positive clones. The method obviates the need for production and purification of targets (which is typically challenging for aggregation-prone proteins/peptides). It also offers label-free screenings and selections, as it is based on fluorescent reporters that are genetically fused directly to the target and library. This precludes the need for biotinylation or labeling of the target protein or library members during the selection. Moreover, the system does not require re-cloning of interesting candidates for the initial characterization, as they are genetically encoded and expressed within the cells.

Future perspectives

A number of elements are essential in the design of recombinant expression systems, including the genetic elements of the expression plasmid (such as ori, transcriptional promoters, and antibiotic resistance markers). In order to provide the system with a large dynamic range and more control of protein expression levels during selections, additional vectors with tightly regulated and inducible promoters are currently investigated for harboring of the Aβ-EGFP expression cassette. Moreover, screenings and selections from libraries of Aβ-sequestering Affibody molecules (libraries used in in study II) are presently performed for generation of molecules that inhibit aggregation of distinct aggregation-prone peptides (involved in other neurodegenerative disorders).

In the future, it would also be interesting to introduce a purification tag, for example the hexahistidine tag, to allow for simplified purification of generated candidates. In addition, even thought E. coli has several attractive features as host organism, including high transformation frequency, rapid growth rate, and is well studied for production of recombinant proteins, it would be interesting to extend this concept to other host organisms such as yeast, to allow for expression of other types of aggregation inhibiting proteins that are not easily expressed or folded in E. coli. We believe that the system has a strong potential as a future complement to the conventional display-based selection and screening technologies for generation of various protein-based aggregation inhibitors.
Concluding remarks

This thesis offers a perspective on what can be achieved by using protein engineering strategies for development of tailor-made binding-proteins. We have successfully employed both rational and combinatorial principles to develop novel aggregation-inhibiting Affibody molecules that target different conformations of the Alzheimer’s-related Aβ peptide, for use in research and therapeutic strategies of AD.

In the first three studies, we applied combinations of these strategies for engineering of the $Z_{Aβ3}$ Affibody molecule, which binds non-aggregated Aβ peptides. The scaffold of the binder was formatted into a truncated head-to-tail linked dimer, and the affinity to Aβ was subsequently increased to $\sim 300$ pM, corresponding to a 50-fold improvement. The new high-affinity binder, $Z_{SY\text{M73}}$, was investigated in a preclinical study aimed at preventing development of Aβ-related pathology in an APP/PS1 transgenic mouse model of AD. The Affibody molecule was fused to an ABD to prolong the in vivo half-life and hence the therapeutic effect. Behavioral assessment and histological evaluation demonstrated that the treatment significantly improved the working memory of the mice, and that this amelioration was associated with reductions in Aβ brain burden. This study comprised the first in vivo intervention to demonstrate a therapeutic effect of an Affibody molecule merely through the protein-interaction itself, whereas previous investigations have involved radionuclide-based strategies to achieve a therapeutic effect. Moreover, this investigation importantly demonstrated that the Affibody molecule was well tolerated by the animals. These results are encouraging and promising for development of future Affibody-based therapies. In the fourth study, novel Affibody molecules were generated with high affinities from a large combinatorial library towards protofibrils of Aβ, for future use in both research and potentially diagnostics and therapy. In the last study, a method for function-based screening of new protein-based aggregation-inhibitors was developed, with potential to be expanded to different types of protein-scaffolds and target peptides/proteins.

In conclusion, the different studies presented in this thesis illustrate the power of protein engineering for generation of promising binding-proteins, through the development of Aβ-binding Affibody molecules.
Bibliography


BIBLIOGRAPHY


