Affibody molecules targeting the epidermal growth factor receptor for tumor imaging applications

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### Abstract

Tumor targeting and molecular imaging of protein markers specific for or overexpressed in tumors can add useful information in deciding upon treatment and assessing the response to treatment for a cancer patient. The epidermal growth factor receptor (EGFR) is one such tumor-associated receptor, which expression is abnormal or upregulated in various cancers and associated with a poor patient prognosis. It is therefore considered a good target for imaging and therapy. Monoclonal antibodies and recently also antibody fragments have been investigated for *in vivo* medical applications, like therapy and imaging. In molecular imaging a small sized targeting agent is favorable to give high contrast and therefore, antibody fragments and lately also small affinity proteins based on a scaffold structure constitute promising alternatives to monoclonal antibodies. Affibody molecules are such affinity proteins that are developed by combinatorial protein engineering of the 58 amino acid residue Z-domain scaffold, derived from protein A.

In this thesis, novel Affibody molecules specific for the EGFR have been selected from a combinatorial library using phage display technology. Affibody molecules with moderate high affinity demonstrated specific binding to native EGFR on the EGFR-expressing epithelial carcinoma A431 cell line. Further cellular assays showed that the EGFR-binding Affibody molecules could be labeled with radiohalogens or radiometals with preserved specific binding to EGFR-expressing cells. *In vitro*, the Affibody molecule demonstrated a high uptake and good retention to EGFR-expressing cells and was found to internalize. Furthermore, successful imaging of tumors in tumor-bearing mice was demonstrated. Low nanomolar or subnanomolar affinities are considered to be desired for successful molecular imaging and a directed evolution to increase the affinity was thus performed. This resulted in an approximately 30-fold improvement in affinity, yielding EGFR-binding Affibody molecules with *K_D*’s in the 5-10 nM range, and successful targeting of A431 tumors in tumor-bearing mice. To find a suitable format and labeling, monomeric and dimeric forms of one affinity matured binder were labeled with $^{125}$I and $^{111}$In. The radiometal-labeled monomeric construct, $^{111}$In-labeled-Z$_{EGFR,1907}$, was found to provide the best tumor-to-organ ratio due to good tumor localization and tumor retention. The tumor-to-blood ratio, which is often used as a measure of contrast, was $31\pm8$ at 24 h post injection and the tumor was clearly visualized by gamma-camera imaging.

Altogether, the EGFR-binding Affibody molecule is considered a promising candidate for further development of tumor imaging tracers for EGFR-expressing tumors and metastases. This could simplify the stratification of patients for treatment and the assessment of the response of treatment in patients.

**Keywords:** Affibody, affinity maturation, phage display selection, EGFR, molecular imaging, protein engineering, tumor targeting

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List of publications

This thesis is based on the following papers, which will be referred to in the text by their corresponding Roman numerals (I-V). The five papers are found in the appendix.


All papers are reproduced with permission from the copyright holders.
List of publications not included in this thesis


“Education is the path from cocky ignorance to miserable uncertainty.”

Mark Twain
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<tbody>
<tr>
<td>ABD</td>
<td>albumin-binding domain</td>
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<tr>
<td>CDR</td>
<td>complementarity determining region</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>$C_H$</td>
<td>constant domain of the antibody heavy chain</td>
</tr>
<tr>
<td>$C_L$</td>
<td>constant domain of the antibody light chain</td>
</tr>
<tr>
<td>CT</td>
<td>computed x-ray tomography</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor (ErbB-1, HER1)</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
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<tr>
<td>Fab</td>
<td>fragment, antigen binding (antibody)</td>
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<tr>
<td>Fc</td>
<td>fragment, crystallizable (antibody)</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>HER2</td>
<td>epidermal growth factor receptor-2 (HER2/neu, ErbB-2)</td>
</tr>
<tr>
<td>His$_6$</td>
<td>hexahistidyl</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal ion affinity chromatography</td>
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<tr>
<td>$K_D$</td>
<td>equilibrium dissociation constant</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>NSCLC</td>
<td>non-small cell lung cancer</td>
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<tr>
<td>PCA</td>
<td>protein complementation assay</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>p.i.</td>
<td>post injection</td>
</tr>
<tr>
<td>scFv</td>
<td>single chain variable fragment (antibody)</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPECT</td>
<td>single-photon emission computerized tomography</td>
</tr>
<tr>
<td>TKI</td>
<td>tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>$V_H$</td>
<td>variable domain of the antibody heavy chain</td>
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<tr>
<td>$V_L$</td>
<td>variable domain of the antibody light chain</td>
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INTRODUCTION

1. PROTEINS – ENGINEERING TOWARDS MEDICAL APPLICATIONS

Almost everything that occurs in a living cell involves proteins. Proteins are present in an amazing variety and exhibit an enormous diversity in their biological functions. Over the years, many proteins purified from natural sources have been employed for a broad range of applications. Various enzymes have been used in the biotechnology and food industries, e.g. lipidases and proteases in washing powder and calf rennin enzyme for cheese making. Native proteins have also been used medically, such as insulin, purified from bovine and porcine pancreas, and used in the treatment of diabetes or factor VIII, recovered from human plasma, in the treatment of hemophilia patients. During the 20th century the advances in life science has moved from understanding basic molecular mechanisms to taking advantage of this knowledge to develop biotechnological tools and medical drugs. With new technologies, proteins have been engineered for altered, improved or even new functions for biotechnological and medically-related applications. The medical importance of proteins is reflected by the fact that the majority of all drug targets are proteins. At present, more than 130 different peptides and proteins are approved for clinical use as drugs themselves by the US Food and Drug Administration (FDA), and many more are in development. Below, some of the most important advances in protein engineering and its implications for protein therapeutics are described. This thesis focuses on the development of engineered proteins as reagents that could become future diagnostic tools for molecular imaging of cancer and potentially even for therapy of cancer patients. In short, Affibody molecules (described further in Chapter 2) have been selected using phage display technology (Chapter 3) and
labeled with suitable radionuclides for molecular imaging (Chapter 6) of a specific tumor-associated receptor, the epidermal growth factor receptor (Chapter 5), that is abnormal or upregulated in a wide range of cancer forms.

1.1 Proteins

Proteins are essential for all living organisms. They are among the most abundant of the biological macromolecules and extremely versatile in their functions. In the human body proteins are involved in a diverse range of biochemical interactions and functions. For example, proteins with catalytic activity, enzymes, are involved in most biochemical reactions, such as the digestion of food or in bone formation. Proteins are also involved in transporting molecules within a cell or from one organ to another, e.g. hemoglobin transport of oxygen. Many proteins provide structure, like keratin in hair and fingernails. Some proteins help to regulate cellular or physiological activity, such as insulin or growth hormones. Furthermore, the immune system comprises a variety of proteins that are responsible for the recognition and inactivation of foreign substances. All proteins with their very different properties and functions are built from the same set of 20 amino acids. Each protein is encoded by a segment of DNA, a gene. The DNA is transcribed into mRNA and translated into a sequence of encoded amino acids. This synthesized polypeptide sequence constitutes the primary structure of the protein. The linear polypeptide is locally arranged to form the secondary structure elements, e.g. alpha-helices and/or beta-sheets, which is folded into a tertiary structure held together by intramolecular bonds and sometimes disulfide cross-links. Some proteins comprise multiple polypeptide chains that interact to create a quaternary structure.

1.2 Protein engineering

In many applications, including biotechnological and medical ones, it is desirable to modify a protein to improve its performance. This process is termed protein engineering. Such modifications may be introduced for a number of reasons, e.g. to increase the affinity for an interacting molecule, to prolong the in vivo half-life, to increase the stability or solubility, to reduce/increase the size, or to facilitate protein purification upon recombinant production. About three decades ago, several techniques for recombinant DNA technology (or genetic engineering) were introduced, which opened for a rapid development in protein engineering. Methods for precise cutting (restriction enzymes) and rejoining (ligases) of DNA pieces into vectors were used to generate synthetic genes for production of recombinant
proteins. The genetic engineering of DNA was further facilitated by a pioneering technique for amplification of DNA segments in a process called the polymerase chain reaction (PCR) invented by Kary Mullis in 1985 (Saiki et al., 1985). A technique for site-directed mutagenesis, developed by Michael Smith (Hutchison et al., 1978; Winter et al., 1982), gave the possibility to alter the protein-coding DNA sequence in a site-specific manner. These two techniques were awarded the Nobel Prize in 1993. The amplification of DNA segments with PCR also revolutionized the development of methods for sequencing of the DNA. In 1977 two new techniques were described, one by Alan Maxam and Walter Gilbert (Maxam and Gilbert, 1977) and another by Fredrik Sanger (Sanger et al., 1977). Automated Sanger sequencing was instrumental in the first genome sequencing projects, and have today been complemented with alternative powerful techniques, such as the pyrosequencing-based 454-technology (Margulies et al., 2005).

Using recombinant DNA technology, proteins with new desired characteristics can be generated. There are different methodological approaches to achieve these modified proteins. One approach is commonly referred to as rational design, which aims to understand protein structure and function well enough to apply the information in designing new properties. Rational genetic engineering principles include specific and controlled modifications of the protein by for example point mutations, insertions, deletions and fusions to other sequences, adding desired functionality. Although rational engineering approaches have proven successful for modification of protein properties and functions (as reviewed for therapeutic proteins by Marshall et al., 2003), it is generally a labor-intensive procedure if a large set of altered proteins needs to be generated and characterized. In contrast to rational design, combinatorial protein engineering approaches rely on the generation of large libraries of protein variants, created by introduction of randomized changes at several sites simultaneously, from where proteins with desired traits are isolated using carefully designed selection methods. Combinatorial libraries can be used to evolve the function and properties of a protein by the procedure of, (i) diversification, (ii) selection and (iii) amplification, which are typically repeated until the desired properties are obtained (Hoogenboom, 2005; Matsuura and Yomo, 2006). Different selection systems for the isolation of engineered peptides and proteins are further discussed in Chapter 3. Furthermore, the growing number of determined protein structures together with improvements in computational tools for predictions will give the possibility to further guide rational design and rational methods for generation of combinatorial libraries (Patrick and Firth, 2005; Lippow and Tidor, 2007).
The introduction of recombinant DNA technology has enabled new strategies for the generation of engineered proteins with therapeutic potential. A vast majority of proteins of medical relevance are presently produced as recombinant proteins from a range of cells and organisms, including prokaryotic (bacteria) and eukaryotic (e.g. yeast, insect cells and mammalian cells) organisms. The system of choice will depend on the properties and intended use of the protein, cell expression levels, production cost and required modifications. The bacterial host *Escherichia coli* (*E. coli*) and different mammalian cell lines (mainly Chinese hamster ovary, CHO and baby hamster kidney, BHK cells) are still the most commonly used. Between 2003 and 2006, 9 out of 31 approved protein therapeutics were produced in *E. coli* and 17 in mammalian cell lines (Walsh, 2006). *E. coli* is advantageous because of its ability to grow rapidly to high cell densities on inexpensive substrates, its well-characterized genetics and the availability of a large number of cloning vectors and mutant host strains (Schmidt, 2004). Mammalian cell culturing is technically complex, laborious and expensive, but will provide correct glycosylation pattern for the production of human proteins. Protein glycosylation patterns can influence properties, such as protein stability, ligand binding, serum half-life and immunogenicity, and is significant in the context of efficacy and sometimes safety for a wide range of biopharmaceuticals. Many protein therapeutics have been associated with immunogenicity problems in humans. Protein products have, however, been engineered that are less likely to provoke an immune response. Recombinant *E. coli*-produced insulin, approved by the US FDA in 1982, was the first commercially available recombinant protein therapeutic (Swartz, 2001). Since then, there has been a remarkable expansion in the number of therapeutic applications for proteins. More than 130 proteins (over 95 of which are produced recombinantly) are currently approved by FDA for clinical use, and many more are in clinical development. Recombinant production of proteins has several benefits compared with recovery of proteins from its natural source. First, recombinant proteins are often produced more efficiently and inexpensively, in potentially limitless quantity and generally in a better controlled process. Second, there is a reduction of exposure to animal or human material, which could carry disease-causing components. Finally, recombinant technology allows for the generation of proteins that provide a novel function or activity.
1.3 Protein therapeutics

The many protein therapeutics approved for clinical use are different in their mechanisms of action and can be organized by function and therapeutic application into groups (Leader et al., 2008). The first group is protein therapeutics with enzymatic or regulatory activity. This include replacement of a protein that is deficient or abnormal, to influence an existing pathway or to provide a novel function or activity. Protein therapeutics in this group are for example Factor VIII and Factor IX for replacement of vital blood-clotting factors in hemophilia patients. The second group - protein therapeutics with special targeting activity, includes the interference with a molecule or organism or the delivery of other compounds or effector functions (described further in Chapter 2 and 5). Many antibody therapeutics against cancer and immunological diseases are within this group. The third group - protein vaccines, includes protein therapeutics for protection against a deleterious foreign agent, treating an autoimmune disease, or treating cancer. One successful example of a protein vaccine is the hepatitis B vaccine. The fourth group - protein diagnostics, includes both in vitro and in vivo diagnostics that are invaluable in the decision-making process that precedes the treatment and management of many diseases. Several cancer imaging agents are included in this group, like ProstaScint for prostate cancer detection and OctreoScan for detection of neuroendocrine tumor and lymphoma (described further in Chapter 6.2). This thesis focuses on the use of protein engineering for the development of suitable diagnostic reagents for in vivo imaging of different forms of cancer (Chapter 7). In the future, there is also the hope to overcome certain limitations and further develop these targeting agents into cancer therapeutics for delivery of e.g. potent therapeutic radioisotopes (this is further discussed in Chapter 8).
2. ANTIBODIES AND OTHER AFFINITY PROTEINS

Most biological processes depend on molecular recognition mediated by proteins, such as the interaction between a receptor and its ligand or an antibody with its antigen. Specific interaction between a binding protein (targeting agent) and the target molecule and the ability to manipulate such interactions has proven important for various biotechnological and therapeutic applications. The concept of using an in vivo targeting agent specific for its target molecule without affecting surrounding tissues, a ‘magic bullet’, was first described by Paul Ehrlich at the beginning of the 20th century. Since then, a number of targeting agents against a wide variety of disease-related target molecules have been described.

There are several properties to consider for an engineered binding protein, depending on its intended use. In biotechnological applications, like separation and detection, the binding protein must first of all have sufficient affinity and specificity. In affinity chromatography, thermal and chemical stability of the capture ligands are also key issues (Skerra, 2007). In medical therapy and in vivo diagnostics the specificity for the target protein is most essential in order not to bind to and affect normal tissues. Good affinity and long tumor residence time are important parameters for a long-lasting effect in therapy and to get good contrast in in vivo imaging. Furthermore, the size of a targeting agent is an important issue for the pharmacokinetics and biodistribution. Large targeting agents, like antibodies (~150 kDa), have a long circulation time (biological half-life) which is a desirable property for therapy. In molecular imaging (see Chapter 6), however, rapid clearance is important. Smaller molecules, with a size below the threshold for kidney filtration (approximately 60 kDa), like certain antibody fragments, scaffold proteins, peptides and small molecules, will be cleared much quicker from the system through excretion via the kidneys (Behr et al., 1998; Holliger and Hudson, 2005). The size of the targeting agent is also influencing the tissue penetration. Different targeting agents, their strengths and weaknesses and applications are discussed below.
2.1 Antibodies

Antibodies, or immunoglobulins (Ig), are probably nature’s most common and widely used affinity reagents. They were first discovered at the end of the 19th century by the German scientist von Behring, an achievement for which he later received the Nobel Prize in 1901. In higher vertebrates antibodies are produced by cells in the immune system as a defense against all kinds of foreign substances and invading pathogens, like viruses and bacteria. The antibodies have two functions; one is to bind specifically to the foreign substance, referred to as the antigen, and the other is to recruit various cells and molecules to destroy the pathogen once the antibody is bound to it. Upon exposure to the antigen, antibodies with suitable binding capacity are recruited for further maturation by incorporation of somatic mutations in the variable genes and only those B-cells which express antibodies of higher affinity for the antigen are expanded. This process of natural diversity and selection of antibodies in the immune system can be mimicked in the generation of new affinity proteins through combinatorial protein engineering and selection of binders using in vitro selection systems (described in Chapter 3).

Antibody molecules have a Y-shaped structure consisting of four polypeptide chains, with two identical shorter light (L) chains and two identical longer heavy (H) chains coupled together via disulfide bonds (Fig. 1). Both the heavy and the light chain consist of a constant part (C) and a variable part (V). The highly variable regions of the heavy and light chain on the tip of the arms combine to form two identical antigen-binding sites containing six hyper-variable loops, referred to as CDRs (complementarity determining regions). The stem of the Y-shaped antibody, the Fc, has several functions. It is responsible for recruitment of cytotoxic effector functions through complement and interactions with Fc receptors (Fcγ) (Ward and Ghetie, 1995). The Fc domain is also providing the long serum half-lives of antibodies through interaction with the neonatal Fc receptors (FcRn) (Roopenian and Akilesh, 2007). The Y-shaped antibodies are bivalent which greatly increases their functional affinity and confers high retention times (also called avidity) upon binding to many cell-surface receptors and polyvalent antigens. Differences in the heavy chain constant region make up five different classes of antibody molecules, called isotypes. One of them is the 150 kDa IgG molecule, which constitutes about 80% of the total serum immunoglobulin pool. The IgG molecule is the format almost exclusively used in therapeutic antibodies and is the one being discussed in this thesis, hereafter referred to as antibody.
Because of their ability to function as affinity reagents, natural and engineered antibodies are widely used in various applications, like separation and detection in biotechnology and in recent years also in medical applications like imaging and therapy. The antibodies generated in a natural immune response or after immunization are heterogeneous, i.e. polyclonal, in their specificity. With the development of the Nobel Prize-rewarded hybridoma technology by Köhler and Milstein in 1975 (Köhler and Milstein, 1975) it was possible to provide monoclonal antibodies (mAbs) with very specific interaction to their respective target antigen. The hybridoma technology involves the fusion of an antibody-producing B-cell with a myeloma cancer cell to produce a hybrid cell providing an endless source of mAbs. These initial mAbs were, however, of murine origin and despite high specificity and affinity, they had some limitations for clinical use, such as immunogenicity in humans, poor ability to recruit immune effector cells and shorter serum half-lives. With the chimerization (Boulianne et al., 1984) and humanization (Jones et al., 1986) techniques, where the entire murine variable regions or the murine CDRs are grafted into the human IgG framework, a much wider use has been possible. The targeting specificity and affinity is retained and with the incorporation of the human Fc part the antibodies are far less immunogenic when administered to humans, than the mouse mAbs. They also retain the biological effector functions of the human antibody and are more likely to trigger the human complement activation and Fc receptor binding. The hybridoma technology, together with humanization of antibodies of animal origin or by the use of transgenic mice (Fishwild et al., 1996), have been very successful technologies in generating therapeutic mAbs. Still it has inherent limitations. First, it is not applicable to antigens that are toxic to the animals or conserved across species and thus very poorly immunogenic. Second, the isolation of antigen-specific antibodies can be slow because the selection conditions in vivo are difficult to control. Finally, the number of isolated antibodies, their recognized epitopes and their specificity and affinity can be unpredictable. A faster, more flexible and more reliable alternative for the generation of therapeutic mAbs is represented by the creation of large, diverse combinatorial libraries of antibody heavy and light chain variable domains (V_H and V_L), followed by the selection of specific binding molecules using display technologies in vitro (described in Chapter 3).
Fig. 1. Schematic overview of an IgG antibody molecule and different antibody fragments. The Y-shaped IgG molecule consists of two identical light chains (L) and two identical heavy chains (H) held together by disulfide bonds. Both the light and the heavy chain consist of a variable (V) (indicated in light grey) and a constant part (C) (indicated in white). On the tip of the arms, the variable regions of the heavy and light chains combine to form two identical antigen-binding sites containing six hyper-variable loops, referred to as CDRs (complementarity determining regions) (indicated as black fields). The stem of the Y-shaped IgG, the Fc, is responsible for recruiting different effector functions and can provide longer half-lives through interactions with Fc receptors. Also shown are different extensively investigated antigen-binding antibody fragments; Fab\(^\prime\)\(_2\) fragment, Fab fragment, scFv fragment, diabody, minibody and single domain antibody (dAb; of either variable heavy or light chain).

Monoclonal antibodies are widely used in therapy today. More than 150 such drugs are in clinical development and 23 (as of January 2008) have been approved for the market (Leader et al., 2008). A majority of these are for oncology use and today nine mAbs are approved for use in cancer treatment (Adams and Weiner, 2005; Reichert et al., 2005; Carter, 2006; Leader et al., 2008) (Table 1). There is an estimate that mAbs will account for 32% of all revenues in the biotech market in 2008 (Hale, 2006). The mAbs available for the clinic use different mechanisms in directing cytotoxic effect to the cells. Most of the antibodies approved are naked or non-conjugated and interact with components of the immune system through
antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC), and many alter signal transduction within the cell or act to eliminate a critical cell-surface antigen (Adams and Weiner, 2005). Monoclonal antibodies can also deliver therapeutic payloads, such as radioisotopes, toxins, or other drugs to directly kill tumor cells or to activate a prodrug specifically within the tumor cells (Adams and Weiner, 2005).

Although mAbs are widely used in many different applications all properties are not always useful or desired. The large size will lead to long half-lives, which is not desired for an imaging reagent that has to be cleared rapidly from the bloodstream to provide a good contrast (see Chapter 6). The Fc-mediated immunological effector functions are only desired for certain applications and an inappropriate activation of Fc receptor-expressing cells, like neutrophils, natural killer (NK) cells and macrophages can lead to unwanted side-effects. The bulky frame will also limit tissue penetration which might complicate some medical applications (Holliger and Hudson, 2005; Beckman et al., 2007). Moreover, there are high manufacturing costs involved in producing correctly glycosylated antibodies because of requirement of mammalian cell culture. Finally, there is also a complex intellectual property situation for the production and use of antibodies.
<table>
<thead>
<tr>
<th>Product name; generic name</th>
<th>Company</th>
<th>Antibody format</th>
<th>Target</th>
<th>Approved indications</th>
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<tr>
<td><strong>Unconjugated mAbs</strong></td>
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<tr>
<td>Rituxan; rituximab</td>
<td>Genetech/ Biogen Idec</td>
<td>Chimeric IgG1</td>
<td>CD20</td>
<td>Non-Hodgkin’s lymphoma and rheumatoid arthritis</td>
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<tr>
<td>Herceptin; trastuzumab</td>
<td>Genentech/ Roche</td>
<td>Humanized IgG1</td>
<td>HER2</td>
<td>Metastatic breast cancer that overexpresses HER2</td>
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<tr>
<td>Campath; alemtuzumab</td>
<td>Genzyme</td>
<td>Humanized IgG1</td>
<td>CD52</td>
<td>B-cell chronic lymphocytic leukaemia</td>
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<td>Erbitux; cetuximab</td>
<td>ImClone Systems/ Bristol-Myers Squibb/ Merck</td>
<td>Chimeric IgG1</td>
<td>EGFR</td>
<td>Metastatic colorectal cancer and head and neck cancer</td>
</tr>
<tr>
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<td>Genentech/ Roche</td>
<td>Humanized IgG1</td>
<td>VEGF</td>
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</table>

2.2 Antibody fragments

In order to circumvent some of the limitations of antibodies, described above, smaller sized antibody fragments have been investigated (Holliger and Hudson, 2005). Initially the generation of smaller antibody fragments were performed by proteolytic digestion of full-length antibodies, giving rise to Fab (~54 kDa) or Fab’2 (~100 kDa) fragments with retained antigen-binding activity (Fig. 1). Later, monovalent (Fab, scFv, single variable V\textsubscript{H} and V\textsubscript{L} domains) or bivalent fragments (Fab’\textsubscript{2}, diabodies, minibodies) were generated recombinantly (Fig. 1). The variable fragment, Fv, consisting of V\textsubscript{H} and V\textsubscript{L} joined together is the smallest entity with intact antigen-binding capacity that can be derived from conventional mAbs. To increase stability and for convenient production, the V\textsubscript{H} and V\textsubscript{L} segments were genetically connected through a flexible polypeptide linker, yielding a single chain Fv (scFv) (Bird et al., 1988; Huston et al., 1988) and this pioneering work has had great impact on the antibody engineering field. The scFv is a useful format for certain applications because of its small size (~27 kDa) and it also enables straightforward production in \textit{E. coli}. The use of antibodies and antibody fragments in medicine and research has mostly been directed to extracellular target proteins. Antibody fragments, mainly in the scFv format, have however also been applied for intracellular expression as so-called intrabodies. These have been studied for the inhibition of intracellular target proteins and interference with a number of relevant disease targets (Lobato and Rabbitts, 2004; Wolfgang et al., 2005). Several recombinant Fabs and scFvs are currently approved by FDA or are in late stage clinical development (Holliger and Hudson, 2005).

In the late 1980s, a small antigen-binding fragment was isolated by Greg Winters group, when a repertoire of isolated murine V\textsubscript{H} domains was screened for binding to lysozyme (Ward et al., 1989). These fragments, called single domain antibodies (dAbs), comprise either the V\textsubscript{H} or V\textsubscript{L} domain and are of much smaller size (~15 kDa) than both Fab and scFv. These first single variable domains were, however, poorly soluble and often prone to aggregate because of exposure of a large hydrophobic area that is normally buried in the contact surface with the other variable domain (Ewert et al., 2003) and did rarely retain the affinity of the parental antibody. The discovery of naturally occurring single domain antibodies from two distinct organisms, the camelids (camels and llamas) (Hamers-Casterman et al., 1993) and cartilaginous fish (wobbegong and nurse sharks) (Greenberg et al., 1995), that were generally soluble, led to their use in research as biotechnological tools and therapeutic reagents (Nuttall et al., 2004; Harmsen and De Haard, 2007; Liu et al.,
Recently, the selection and biologic function of an antagonistic camelid dAb (nanobody) against EGFR in an *in vivo* murine tumor model has been reported (Roovers et al., 2007). However, for *in vivo* administration, humanization may be crucial to reduce immunogenicity, and human single domains might therefore have a certain advantage. Recently, problems of poor stability and solubility have been solved, or at least greatly reduced, for some human V domains by the identification and design of mutations that minimize the hydrophobic interface (Holliger and Hudson, 2005). Selections from libraries based on these enhanced variants have generated numerous binders (Holt et al., 2003; Colby et al., 2004; De Bernardis et al., 2007).

The monovalent Fab, scFv or dAb can be engineered into multivalent molecules, which have characteristics desirable in many applications. Multivalent molecules might show significant increase in functional affinity (termed avidity) and significantly slower dissociation rates for cell-surface or multimeric antigens (Kubetzko et al., 2006). They may also include different binding specificities (Kipriyanov and Le Gall, 2004; Haas et al., 2005; De Bernardis et al., 2007; Herrmann et al., 2008). One example of a multivalent molecule is the diabody (~55 kDa) (Fig. 1), generated by linking of a V\textsubscript{H} and V\textsubscript{L} with a short scFv linker and self-assembly into bivalent dimers (Holliger et al., 1993). The intermediate size and multivalency are favorable properties for tumor-targeting that will provide rapid tissue penetration, high target retention and relatively rapid blood clearance (Adams et al., 1998b; Holliger and Hudson, 2005). Diabodies have shown to give good contrast in *in vivo* imaging of tumors (Sundaresan et al., 2003; Olafsen et al., 2004; Robinson et al., 2005). Bispecific diabodies with dual targeting possibilities have also been generated. Several of these target a tumor associated antigen and the CD3 receptor to recruit T-cell mediated cytotoxicity to the tumor cells. Examples are the targeting of the CD19 receptor on malignant B cells (Cochlovius et al., 2000) and the epidermal growth factor receptor (Asano et al., 2006) that show antitumor activity in *in vivo* tumor models. Minibodies, where IgG1 CH3 domains are used as dimerization domains to express scFvs, are another bivalent intermediate-sized format (~75 kDa) (Fig. 1) (Hu et al., 1996; Olafsen et al., 1998). Minibodies may be ideal for tumor therapy because they achieve a higher total tumor uptake than other smaller antibody fragments and substantially faster clearance and better tumor-to-blood ratios than intact antibodies. Radiolabeled minibodies have demonstrated very good tumor uptake (Hu et al., 1996; Wu and Yazaki, 2000) and high-resolution tumor imaging (Wu et al., 2000).

Several different antibody fragment formats have showed improved pharmacokinetics for tissue penetration and better contrast in molecular imaging,
because of their smaller size. The small single domains have also the possible advantages of providing binding to new target molecules not accessible for antibodies, such as enzyme active sites and viral surface canyons. The Fc domain function to mediate intrinsic cytotoxicity via recruitment of immune effector mechanism can be replaced by a therapeutic payload, like a radioisotope or a toxin.

2.3 Peptides

Derivatives of natural peptide receptor ligands may demonstrate high affinity for the relevant receptor, but there is a limited range of natural peptides to choose from (Reubi et al., 2005). In addition, peptides are generally susceptible to proteolytic degradation and natural peptide receptor ligands may also trigger undesired signaling events when binding to their target. Peptides, linear and cyclic, have been randomized to obtain peptide libraries for potential selection of variants with desired characteristics (Cwirla et al., 1990; Devlin et al., 1990; Smith and Petrenko, 1997). Peptides have been selected that bind several different cancer targets (Weiner and Thakur, 2002; Aina et al., 2007) and that bind targets in other areas, like cardiology and inflammation and infection (Stefanidakis and Koivunen, 2004). However, it has proven difficult to generate peptides with high affinity for its target, most likely due to their flexible nature (Landon and Deutscher, 2003). Peptides, although interesting targeting agents, will not be further discussed here.

2.4 Non-antibody scaffolds

Certain limitations of antibodies as binding molecules in certain applications have become evident, as described above. These limitations thus inspired for the development of alternative protein frameworks, so called “scaffolds”. Candidates for suitable scaffolds should have a structurally rigid core that could carry changes, such as amino acid changes or inserts in loops or side chain replacements on a contiguous surface. In order to get novel binding molecules with different function, typically for specific target binding, the scaffold has to be suited for diversification and selection. Usually, a combinatorial protein engineering approach is used with random mutagenesis of suitable amino acids to generate a synthetic library. This is followed by selection of variants with desired binding activity using different selection strategies (further described in Chapter 3). For new binding molecules to compete with already established antibodies, they need to possess the same or preferably improved properties. There are several aspects to consider for a scaffold protein as described below and that is also thoroughly reviewed by Nygren and Skerra, 2004.
The scaffold protein should preferably be relatively small and be composed of a single polypeptide chain with intrinsic stability, and the stability should also be kept in spite of the randomization. The scaffold should, if possible, not be dependent on disulfide bridges for its stability, as this could limit the use in intracellular applications and make the production more difficult. Furthermore, if the scaffold has no cysteines, a unique cysteine can be introduced to provide the possibility of specific labeling for example. Most of the scaffold proteins are based on naturally occurring binding proteins, which are frequently engineered to improve properties such as stability. In the creation of a combinatorial library the amino acids naturally involved in ligand interaction are the first choice for randomization. The number of positions for variation should be large enough to provide an interface for interaction with the target molecule, but not too many positions should be varied in order to avoid a decrease of the scaffold stability. Immunogenicity of a new scaffold protein needs to be considered if the binding protein is intended for \textit{in vivo} applications, like therapy or imaging. Both “non-human” and engineered “human scaffolds” could elicit an immune response. There are strategies emerging for rational reduction of protein immunogenicity, including PEGylation (Chapman, 2002) and T-cell epitope engineering (Flower, 2003).

There is a broad variety of scaffold proteins being used. These have different structural frameworks and differ in their way of binding to a target molecule. Some use cavities in binding of low molecular weight compounds, and some use flexible loops to bind to enzyme pockets and yet others use extended binding surfaces for the recognition of larger proteins. Although very different mechanisms for binding (both loops and secondary structure interfaces) are being used, binders with high affinity have been generated from many different frameworks (see below). Here, scaffolds have been divided into four groups depending on their binding properties: (i) single loops on rigid framework, (ii) several loop structures forming a continuous surface, (iii) engineered interfaces resting on a secondary structure and (iv) oligomeric domain structures. Examples of scaffold proteins are briefly presented in this thesis and summarized in Table 2. For a more thorough reading on different scaffold proteins there are several recent publications (Nygren and Skerra, 2004; Binz et al., 2005; Hey et al., 2005; Hosse et al., 2006; Skerra, 2007). The main focus here will be on the Affibody scaffold (presented in Chapter 2.5), which is the affinity protein used in all the studies in this thesis.
2.4.1 Single loops on rigid framework

This is a strategy where one single loop on a conserved protein framework is used for diversification. The loop does either possess a natural binding property or is hypervariable in length and substitution of amino acids. Protease inhibitors are one of these natural binding proteins that are small and stable and expose a single loop. Kunitz domain inhibitors are stable proteins consisting of ~60 amino acids that possess three disulfide bonds, and act as reversible inhibitors of serine proteases. Several different Kunitz domain inhibitor scaffolds have been used to select binders (Dennis and Lazarus, 1994; Williams and Baird, 2003; Nygren and Skerra, 2004) (Table 2). Randomization of the first Kunitz domain of human lipoprotein associated coagulation inhibitor (LACI-D1) and selection using phage display generated a potent inhibitor, DX-88, of human plasma kallikrein (Williams and Baird, 2003), which is now in phase III clinical trials. There are also other scaffolds of the type of single loops on rigid framework, including the so-called ‘knottin’ family (Smith et al., 1998; Lehtiö et al., 2000), aptamers (Borghouts et al., 2008) (Table 2) and human serum transferrin (Nygren and Skerra, 2004).

2.4.2 Several loop structures forming a continuous surface

This approach is the same as the one used in nature by antibodies, where several loops on a rigid framework constitute a surface for binding. There are several natural binding proteins that perform binding in the typical way of antibodies but that can overcome some of the limitations of antibodies, e.g. because of their small size. The fibronectin type III domain constitutes a small (94 amino acid), monomeric natural β-sandwich protein that consists of seven strands with three loops connecting the strands in one end of the β-sheet. Fibronectin type III domain has no disulfide bonds and is a common protein involved in molecular recognition. The 10th domain of 15 repeating units in human fibronectin was chosen as a scaffold (Koide et al., 1998) (Table 2). Using phage display (Koide et al., 1998), yeast two-hybrid (Koide et al., 2002) and mRNA display system (Xu et al., 2002; Getmanova et al., 2006) protein binding variants specific for ubiquitin, human estrogen receptor α, TNF-α and vascular endothelial growth factor receptor 2 (VEGFR2), with an affinity in the micromolar to subnanomolar range and with biological activity, have been reported (Koide et al., 1998; Koide et al., 2002; Xu et al., 2002; Getmanova et al., 2006). Other scaffold proteins mimicking the antibody concept include members of the lipocalin family (Beste et al., 1999; Skerra, 2000) (Table 2).
2.4.3 Engineered interfaces resting on a secondary structure

This class of protein scaffolds have a binding interface that is composed of solvent exposed side-chains, located on the rigid secondary structure of the protein. The side-chains can be randomized to modify a pre-existing binding site or, in some cases, generate a new binding area. Repeat proteins, like ankyrin or leucine-rich repeat polypeptides, contain consecutive copies of small (about 20-40 amino acid) structural units that stack together to form a continuous binding surface. These are naturally abundant proteins involved in protein-protein interaction in many biological processes. Ankyrin repeat proteins are composed of 33 amino acid units, where each unit consists of a β-turn and two-anti-parallel α-helices. Normally an ankyrin repeat domain consists of four to six repeats. Designed ankyrin repeat proteins (DARPins) (Forrer et al., 2003) (Table 2) have been used to generate high-affinity binders using ribosomal display towards several targets including maltose-binding protein (Binz et al., 2004), MAP-kinase (Amstutz et al., 2006), intracellular proteinase (Kawe et al., 2006) and caspase-2 (Schweizer et al., 2007). Recently, a 90 pM HER2-binding DARPin was selected in an affinity maturation procedure using error prone PCR in ribosomal display (Zahnd et al., 2007). Other scaffolds using secondary structure surfaces for interaction include the PDZ-domain, affilins, and the Z-domain based on staphylococcal protein A, which is the scaffold protein used for all the studies in this thesis and is further described in Chapter 2.5 (Table 2).

2.4.4 Oligomeric domain structures

Certain protein scaffold approaches take advantage of an oligomeric structure and multiple interactions to form macromolecular complexes with high avidities. One example is the Avimers, artificial multidomain proteins derived from the human A-domains that are found in the low-density lipoprotein receptor (LDLR). The structural conformation of the 39 amino acid domain is determined by three disulfide bonds. Avimers for therapeutically relevant targets have been generated with phage display (Silverman et al., 2005).
Table 2. Examples of protein scaffolds of non-immunoglobulin origin for the generation of novel affinity proteins

<table>
<thead>
<tr>
<th>Name</th>
<th>Scaffold</th>
<th>Class</th>
<th>Species</th>
<th>Size (aa)</th>
<th>Cross-links</th>
<th>Selected references/Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kunitz domain</td>
<td>BPTI LACI-D1 ITI-D2</td>
<td>Single loop</td>
<td>Human</td>
<td>58</td>
<td>3 SS</td>
<td>Dennis et al., 1994; Williams et al., 2003 Dyax</td>
</tr>
<tr>
<td>Knottin (Microbody)</td>
<td>EETI-II AGRP</td>
<td>Single loop</td>
<td>Plant/human</td>
<td>~30</td>
<td>3-4 SS</td>
<td>Smith et al., 1998; Lehtio et al., 2000; NascaCell</td>
</tr>
<tr>
<td>Aptamer</td>
<td>Thioredoxin</td>
<td>Single loop</td>
<td>Bacterial</td>
<td>108</td>
<td>1 SS</td>
<td>Borghouts et al., 2008 Aptanomics</td>
</tr>
<tr>
<td>AdNectin</td>
<td>10^Fn3 (fibronectin III)</td>
<td>Several loops</td>
<td>Human</td>
<td>94</td>
<td>-</td>
<td>Koide et al., 1998; Xu et al., 2002 Compound Therapeutics</td>
</tr>
<tr>
<td>Anticalin</td>
<td>Lipocalin (BBP)</td>
<td>Several loops</td>
<td>Human/insect</td>
<td>160-180</td>
<td>0-2 SS</td>
<td>Beste et al., 1999; Skerra 2000; Pieris Proteolab</td>
</tr>
<tr>
<td>DARPin</td>
<td>Ankyrin repeat</td>
<td>Secondary structure</td>
<td>Designed</td>
<td>67 + n×33</td>
<td>-</td>
<td>Binz et al., 2004; Zahnd et al., 2007 Molecular Partners</td>
</tr>
<tr>
<td>Affibody</td>
<td>Z-domain of protein A</td>
<td>Secondary structure</td>
<td>Bacterial</td>
<td>58</td>
<td>-</td>
<td>Nord et al., 1997; Orlova et al., 2006; Affibody AB</td>
</tr>
<tr>
<td>Affilin</td>
<td>γB-crystallin/ubiquitin</td>
<td>Secondary structure</td>
<td>Human</td>
<td>198</td>
<td>-</td>
<td>Hey et al., 2005 Scil Proteins</td>
</tr>
<tr>
<td>Avimer</td>
<td>LDLR-A domain</td>
<td>Oligomeric</td>
<td>Human</td>
<td>n×40</td>
<td>3 SS + Ca^{2+}</td>
<td>Silverman et al., 2005 Amgen</td>
</tr>
</tbody>
</table>
2.5 Affibody molecules

The Z-domain, based on staphylococcal protein A, is a protein scaffold used in combinatorial library selections to generate so-called Affibody molecules. The staphylococcal surface protein A binds to the Fc portion of immunoglobulins from most mammalian species, including man (Langone, 1982). The interaction between protein A and immunoglobulins is a well studied protein-protein interaction and protein A has been widely used as an immunological tool and as an affinity handle for purification of recombinant proteins through its binding to immunoglobulins (Uhlén et al., 1983). Protein A consists of five small (approximately 58 amino acids) homologous three-helix bundle domains (E, D, A, B, C) (Fig. 2), each with binding ability to the Fc (and also Fab) portion of IgG (Moks et al., 1986). The region mediating binding to the Fc part of immunoglobulins involves two of the three helices and covers a surface area of 800 Å², similar in size to the surfaces involved in many antigen-antibody interactions (Rees et al., 1994). The B-domain of protein A was engineered to increase the chemical stability and termed Z (Nilsson et al., 1987). This resulted in the loss of the native Fab binding (Jansson et al., 1998), but with retained capability to bind IgG Fc-regions. Protein A is known to be highly soluble, and both proteolytically and thermally stable (Ståhl and Nygren, 1997) and the protein A derived Z-domain has inherited the properties of solubility and stable and fast folding.

The Affibody molecule library was constructed from combinatorial randomization of 13 solvent accessible residues (Fig. 2), including those involved in the Fc-binding of domain Z, thereby destroying the native Fc-interaction (Nord et al., 1995). The Affibody library was subcloned into a phagemid vector allowing for phage selection of binders (Nord et al., 1995). The first isolation of Affibody molecules was performed by Nygren and co-workers in 1997 (Nord et al., 1997). Specific binders to three target proteins (Taq DNA polymerase, human insulin, human apolipoprotein A-1 variant) were selected from an Affibody library (~4x10⁷ variants) presented on phages and the affinities were in the μM range. Since then a larger Affibody library of 3x10⁹ variants (Grönwall et al., 2007a) has been used in panning to select binding molecules, mostly yielding affinities in the mid to low nanomolar range, against many different target proteins, including HER2 (Wikman et al., 2004), transferrin (Grönwall et al., 2007b), amyloid beta peptide (Grönwall et al., 2007a), EGFR (Friedman et al., 2007), HIV gp120 (Wikman et al., 2006), and CD28 (Sandström et al., 2003). The Affibody libraries have typically been displayed on phage, where the phenotype is linked to the genotype via genetic fusion of the
library to a phage surface protein (phage selection is further discussed in Chapter 3.1). Efforts to increase the affinity have been successful in several cases (Gunneriusson et al., 1999; Nord et al., 2001; Orlova et al., 2006; Friedman et al., 2008). The affinity maturations have been achieved either by helix shuffling (Gunneriusson et al., 1999) or sequence alignment and directed combinatorial mutagenesis using a single oligonucleotide covering helix 1 and 2 (Nord et al., 2001; Orlova et al., 2006; Friedman et al., 2008) as will be discussed in Present Investigation (paper IV).

**Fig. 2.** Schematic figure of the five Ig-binding domains (A-E) of staphylococcal protein A and the Z-domain. The Z-domain is a 58 amino acid three-helix bundle derived from the B-domain. Thirteen solvent-exposed residues on helix one and two have been randomized to create a so-called Affibody library.
The Affibody molecules are described to have a number of attractive properties useful in a variety of settings, such as biotechnological applications (Nygren and Skerra, 2004), and potentially for therapy and molecular imaging (Nilsson and Tolmachev, 2007; Orlova et al., 2007a; Tolmachev et al., 2007a). They have a small size (~7 kDa) and most of the selected Affibody molecules contain no cysteines and have proven to be highly soluble and stable. Affibody molecules are small enough for solid-phase peptide synthesis (Nord et al., 2001; Engfeldt et al., 2005; Engfeldt et al., 2007a), hence facilitating the introduction of desired fluorophores and also of chemical groups for direct immobilization or for radiolabeling. The lack of cysteines makes the Affibody molecules suitable also for intracellular applications, as well as providing the opportunity for introduction of a unique cysteine for site-specific labeling, e.g. with a fluorophore, or for immobilization on a solid surface. The solvent-exposed termini of the Z-domain will allow for independent folding of fused proteins, and hence, multimeric constructs can easily be constructed by head-to-tail genetic fusions. This can be used to increase the functional affinity (avidity) as has been seen for several Affibody molecules (Steffen et al., 2005; Friedman et al., 2007). The structure of a complex between the Z-domain and a Z$_{SPA-1}$ Affibody molecule (isolated using its ancestor protein staphylococcal protein A as target in the selection) was determined by x-ray crystallography (Högbom et al., 2003) as well as by NMR (Wahlberg et al., 2003). The binding surface of the Z-domain was found to adopt when binding to Z$_{SPA-1}$ to increase the total interaction surface and ten out of thirteen residues allowed for variation in the combinatorial library were involved in the binding.

In recent years the Affibody molecules have been used for a wide variety of applications (Table 3); like the use as detection reagents (Andersson et al., 2003; Renberg et al., 2005; Renberg et al., 2007), to inhibit receptor interaction (Sandström et al., 2003), in separation (Nord et al., 2000; Andersson et al., 2001; Rönnmark et al., 2002; Grönwall et al., 2007b), as purification tags (Hedhammar and Hober, 2007), for structure determination (Hoyer et al., 2008) and to engineer adenovirus tropism (Magnusson et al., 2007; Belousova et al., 2008). Furthermore, a high-affinity Affibody molecule directed against HER2 (Orlova et al., 2006) has been investigated thoroughly in several preclinical and pilot clinical studies and is an interesting candidate for the development of diagnostic and perhaps even therapeutic agents (Baum et al., 2006; Tolmachev et al., 2006; Engfeldt et al., 2007a; Orlova et al., 2007b). Affibody molecules against another member of the same receptor family, the epidermal growth factor receptor 1 (EGFR), have also been investigated for development of primarily diagnostic agents and this work is presented further in the Present Investigation (Chapter 7) of this thesis.
Table 3. Applications of Affibody molecules

<table>
<thead>
<tr>
<th>Application</th>
<th>Target protein</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>IgA, apolipoprotein A-1</td>
<td>Two-site Affibody/antibody ELISA to avoid false-positive signals</td>
<td>Andersson et al., 2003</td>
</tr>
<tr>
<td>Protein microarray</td>
<td>Taq DNA polymerase, IgA, IgE, IgG, insulin, TNF-α</td>
<td>Capture ligands on protein microarrays</td>
<td>Renberg et al., 2005; Renberg et al., 2007</td>
</tr>
<tr>
<td>Inhibition of receptor interaction</td>
<td>CD28/CD80</td>
<td>Interference of CD28 and CD80 receptor interaction</td>
<td>Sandström et al., 2003</td>
</tr>
<tr>
<td>Affinity purification</td>
<td>Taq DNA polymerase, apolipoprotein A-1, RSV G-protein, factor VIII</td>
<td>Ligands in affinity chromatography for capture of recombinant proteins from cell lysates</td>
<td>Nord et al., 2000; Andersson et al., 2001; Nord et al., 2001</td>
</tr>
<tr>
<td>Depletion</td>
<td>IgA, transferrin, Aβ peptide</td>
<td>Protein recovery by affinity chromatography from human plasma or serum</td>
<td>Rönmark et al., 2002; Grönwall et al., 2007b; Grönwall et al., 2007a</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>Ion exchange chromatography media</td>
<td>Novel purification tag for general use as fusion partner to different target proteins</td>
<td>Hedhammar et al., 2007</td>
</tr>
<tr>
<td>Gene therapy (vector engineering)</td>
<td>HER2</td>
<td>Engineering of adenovirus tropism</td>
<td>Magnusson et al., 2007; Belousova et al., 2008</td>
</tr>
<tr>
<td>Molecular imaging</td>
<td>HER2, EGFR</td>
<td>Radiolabeled targeting agent for cancer diagnosis</td>
<td>Orlova et al., 2007b; Tolmachev et al., 2007a; Baum et al., 2006; Friedman et al., 2008</td>
</tr>
<tr>
<td>Radioimmunotherapy</td>
<td>HER2</td>
<td>Radiolabeled targeting agent for cancer therapy</td>
<td>Tolmachev et al., 2007b</td>
</tr>
<tr>
<td>Structure determination</td>
<td>Alzheimer amyloid beta peptide</td>
<td>Stabilizing complex formation for structure determination</td>
<td>Hoyer et al., 2008</td>
</tr>
</tbody>
</table>
3. SELECTION SYSTEMS

All living cells and organisms are believed to have evolved primarily by consecutive rounds of diversification, selection, and subsequent amplification of variants with competitive advantage. Recent advances in the field of molecular biology have allowed us to mimic this process at the molecular level, and to evolve the functions of proteins. Since proteins are difficult to sequence and cannot be amplified themselves, the selection based on their properties must simultaneously select the genes encoding them. Hence, a key to all in vitro selection systems is the genotype-phenotype linkage. The selection is typically performed from a large library of different protein variants, physically linked to their encoding DNA, and the protein exhibiting the desired properties is selected, its gene amplified and brought to further rounds of selection. Proteins can be selected for desired properties, such as affinity, stability, solubility, intracellular functionality and catalytic activity. Important issues to consider in combinatorial protein engineering are the size and the functional quality of the library. A larger library will cover more of the theoretically possible sequence variants and have a higher probability of containing desired clones (Bradbury and Marks, 2004). Library constructions will not be discussed in detail in this thesis, but is described for the construction of an affinity maturation library in Chapter 7.4.

There are several different selections systems used routinely today. The main groups are cell-based display systems, cell-free display systems and non-display systems. These are described below. The choice of selection system may depend on several factors, including the type of protein scaffold, the library size, the desired properties of the selected proteins and in particular the inherent properties of the target molecule, e.g. if it is possible to express by recombinant means, if it is functional inside cells, if it can be immobilized to solid support or not. Directed evolution has become a very popular strategy for improving or altering the biophysical properties of proteins, and even for generating proteins with novel functions. In the work this thesis is based on, phage display is used to select proteins based on their affinity for the target protein.
3.1 Phage display technology

Phage display is the most commonly used in vitro method to select engineered peptides, proteins and antibodies (Table 4). Phage display has successfully been used in selection of antibodies from naïve, immunized or synthetic antibody libraries (Rader and Barbas, 1997; Bradbury and Marks, 2004; Hoogenboom, 2005). In addition, several non-immunoglobulin scaffolds have been isolated by phage display (Nygren and Skerra, 2004) as described in Chapter 2 and it is also the selection method used for the studies in this thesis.

The concept of molecular display technologies is the ability to physically link the genotype and phenotype to allow simultaneous selection of the gene that encodes a protein of desired function. This was successfully applied for the first time in 1985 when George Smith displayed peptides on the surface of phages (Smith, 1985) and thereafter selections were described for several peptide libraries displayed on phage (Cwirla et al., 1990; Scott and Smith, 1990). With the PCR technology the amplification of antibody variable genes was possible and the display and selection of antibody fragments, like scFv (McCafferty et al., 1990) and Fab (Garrard et al., 1991; Hoogenboom et al., 1991) on phage did soon follow. Phage display was first developed for the M13 filamentous phage and even though several alternative phage systems have been developed, such as bacteriophage T4, T7, and lambda (Mikawa et al., 1996; Houshmand et al., 1999), M13 still remains the most extensively studied and most commonly used phage. Filamentous phages have the shape of flexible rods about 1 μm long and 6 nm in diameter and have a single-stranded viral DNA (Fig. 3A). The most abundant surface protein, of which there are 2,700 copies surrounding the phage rod, is the major coat protein pVIII. Located at one tip of the phage are five copies each of the minor coat proteins pIII and pVI and at the other tip reside the minor coat proteins pVII and pIX. Filamentous phages are viruses that infect strains of E. coli that display a threadlike appendage, the F pilus.

Both phage and phagemid vectors using different surface proteins have been used for library display. Display in fusion to pVIII will give a more multivalent display and generally only short peptides are tolerated by the phage (Kretzschmar and Geiser, 1995). Most of the work in combinatorial protein engineering (e.g. display of antibody libraries) has, hence, been conducted using fusions to pIII (Benhar, 2001; Bradbury and Marks, 2004). The different phage-display systems are illustrated for the coat protein pIII in Figure 3B and are similar for e.g. pVIII. In a phage “type 3” vector (Fig. 3B), there is a single phage chromosome bearing a single gene III which can be genetically fused to a foreign DNA insert and encode a
single type of pIII molecule. The inserted protein is then theoretically displayed on all five pIII molecules on the virion. In a phage “type 33” vector (Fig. 3B), the phage genome carries two gene III encoding two different types of pIII molecules;

![Diagram of M13 phage and different types of phage display systems](image)

**Fig. 3.** Phage and different types of phage display systems. A. The wild-type M13 bacteriophage. The major coat protein pVIII covers most of the virion. Located at one tip of the virion are five copies of the minor coat protein pIII and pVI and at the other end pVII and pIX. B. The foreign protein to be displayed is genetically fused to a phage coat protein. In type 3 systems, the foreign gene is fused to gene III in the phage genome, which theoretically results in the display of the foreign protein on all pIII. In type 33, two copies of the gene encoding pIII are present in the phage genome, one which is fused to the foreign gene, resulting in the expression of a mixture of wild-type coat protein and the coat protein fused to the foreign protein. In type 3+3, the foreign gene is fused to the gene III in a phagemid vector that contains the phage ori of replication and packaging signal but lacks genes encoding other phage proteins. A helper phage provides all phage proteins and thus allows the assembly of complete phage particles that display a mixture of wild-type coat protein and the coat protein fused to a foreign protein.
one carries a foreign DNA insert and the other is wild-type. This results in a mosaic virion with coat proteins comprised of both wild-type and recombinant pIII molecules. In a phagemid “type 3+3” system (Fig. 3B) there are two gene III on separate genomes. The wild-type version is on a phage, called the helper phage, while the recombinant version is on a special kind of plasmid called a phagemid. The phagemid contains the origin of replication for both M13 and *E. coli* and the M13 phage packaging signal. It is, however, unable to generate functional phage particles unless the *E. coli* carrying the phagemid also contains a helper phage, which supplies all necessary proteins for phage replication and assembly including the wild type pIII. The different antibiotic resistance genes carried by the phage and phagemid allow one to select for bacteria that contain both the phagemid and the helper phage. From a practical point of view, it is easier to create large libraries with phagemid systems as the plasmid is smaller (e.g. preparing DNA, transfection efficiency). There is, however, slightly greater difficulty in using phagemid libraries in selection because of the need of helper phages. Only 1-10% of the phagemids will have a copy of the displayed protein compared to three to five copies displayed on each phage (Clackson and Wells, 1994). This means that in the selections using phage instead of phagemid, avidity effects could potentially result in the selection of binders with lower affinity. Additionally, affinity maturations could be difficult because of avidity effects. Phage display vectors are usually introduced into *E. coli* cells by electroporation, which is particularly well-suited for making very large libraries. Phage display libraries with complexities over $10^{10}$ individual variants have been reported (Sblattero and Bradbury, 2000; Hoet et al., 2005).

In general a typical phage display selection procedure, often referred to as panning, is performed as follows (Fig. 4). The phage library is exposed to the target protein to allow phage library proteins to bind their target. This is followed by washing to remove unspecific binders and recovery of target protein-bound phage by elution. Subsequently, the eluted phage is used to infect *E. coli* cells to amplify selected clones and phage particles are rescued by superinfection of helper phage to create a new phage library that can be used in a new round of selection. Ideally, only one round of selection would be needed to get specific binders of high affinity, but because of unspecific binding, usually two to five rounds of selection and amplification are necessary. The capturing step of panning can be performed in many ways. Immobilized target protein on a solid support, such as paramagnetic beads or microtiter plates, is one commonly used strategy. Another frequently used strategy is selection in solution using biotinylated target protein followed by capturing of the phage in complex with the target protein on streptavidin-coated solid support, as has been used in the studies in this thesis (paper I and IV).
are also strategies involving panning on fixed prokaryotic cells (Bradbury et al., 1993) and on mammalian cells (Cai and Garen, 1995), enrichment on tissue sections (Van Ewijk et al., 1997), and selections using live animals and even patients (Pasqualini and Ruoslahti, 1996; Arap et al., 2002; Krag et al., 2006). The phage bound to target protein can be eluted in different ways, including incubation with low pH or alkaline buffers, by proteolytic cleavage or by competitive elution with target protein specific ligands to isolate epitope specific binders.

**Fig. 4.** Schematic overview of the phage display selection procedure. Libraries of proteins are displayed on phage particles as fusions to phage coat proteins. Each phage displays a unique protein and encapsulates the corresponding encoding DNA. Highly diverse libraries can be represented as phage pools and target protein-specific clones can be selected by binding to immobilized antigen followed by washing to remove non-binding phage. Retained phage can be amplified by infection of a bacterial host and phage particles can be rescued by superinfection of helper phage, creating a new phage pool. The amplified pool is typically cycled through 2-5 rounds of selection to further enrich for target-binding clones. Individual binding clones can be subjected to screening, e.g. by ELISA, for binding to the target protein for ranking of binding. The sequences of the displayed proteins are identified by DNA sequencing.
Typically, $10^{13}$ phages are used for the first round of selection if the library size is $10^8-10^{10}$ individual variants. Since the display efficiency is less than 10% there will only be 10-100 copies of each protein displayed on the phage at the start of selection. Therefore, the first round of panning should not be too stringent to ensure that all binding molecules are recovered for subsequent amplification. In later cycles the washing steps should be increased substantially in order to increase the stringency. The enrichment of binders with a higher affinity can also be favored by decreased target protein concentration. The selection can also be performed under conditions that permit selections for function (e.g. stability) as well as binding capability, e.g. by selection under reducing conditions, in the presence of proteases, or at elevated temperature.

After the selection procedure, the isolated variants are often screened for target protein binding, typically in an enzyme-linked immunosorbent assay (ELISA) (Bradbury and Marks, 2004) and then identified by sequencing of their co-selected DNA. Soluble protein is generally preferred in the screening assays and in order to avoid time-consuming subcloning procedures, expression and purification of the enriched library members, direct production of soluble protein from phagemid vectors is generally conducted. This is achieved by the insertion of an amber codon in the vector between the inserted DNA and gene III (Hoogenboom et al., 1991), enabling small-scale production of soluble protein. However, the amount of produced protein might be clone-specific and variation in protein concentration between individual clones is often high, which complicates quantification (Bradbury and Marks, 2004). After verification of target protein binding, the genes are subcloned into expression vectors for production of larger amounts of protein in order to study the binding characteristics and biophysical properties of the variants.

For some applications, like therapy and in vivo diagnosis, antibodies isolated from the first selection do not have sufficient affinity. In these cases selected library members can be subjected to directed evolution strategies in order to reach desired affinities and specificities. Single chain Fv fragments and other antibody derivatives are often affinity-matured by randomizing the complementarity-determining regions (CDRs) separately in different libraries, to thereafter combine the obtained mutations identified in improved variants (Schier et al., 1996; Rader and Barbas, 1997). Diversity may be introduced using a variety of methods, such as error-prone PCR, DNA shuffling, or oligonucleotide-directed mutagenesis (Bradbury and Marks, 2004). Directed evolution can be performed in a similar way for scaffold proteins and affinity maturation of an Affibody molecule is described in paper IV.

The main advantages of phage display technology are that it is simple to use, robust and highly versatile, where the selection process can be adopted to many
specific conditions. There are also some limitations with phage display, such as the inability to select under conditions different from the cellular environment and the transformation efficiency that usually limits the library size to $10^{10}$. These limitations may be circumvented by choosing one of the other selection systems described below.

### 3.2 Other selection systems

#### 3.2.1 Cell display systems

Although phage display is currently the most established method for \textit{in vitro} selection of peptides and proteins from combinatorial libraries, there are several alternative cell-based selection systems for bacteria, yeast, and mammalian cells available. The main advantage of cell-based systems compared to phage display is the increased particle size which enables the use of flow cytometry for analysis and sorting. The incubation between a cell-displayed library and the target protein is typically performed in solution with a fluorophore labeled target protein. Due to the multivalent display of library protein on the cell surface together with flow-cytometric sorting, the relative affinity of each library member for the target protein can be quantified during flow-cytometric-based screening. Expression levels typically vary in the expression system but this can be taken into account by using a two-color labeling system, with one fluorophore for expression and another for target protein binding (Boder and Wittrup, 1997). In this way binding data from each library member can be normalized against differences in the surface expression levels of individual cells and the discrimination based on affinity will not be biased.

Bacterial display, using the gram-negative bacterium \textit{E. coli}, was the first cell surface display system (Francisco et al., 1993; Little et al., 1993; Georgiou et al., 1997). The success of \textit{E. coli}-surface display is dependent on the capability of the library protein to be properly transported across the outer membrane to become localized in an accessible form on the cell surface. This could be influenced by the size and biochemical properties of the protein to be displayed, its folding efficiency and the disulfide content. Only a few scaffolds, mostly peptides and a few scFv libraries, have been used for library screening with bacterial display (Daugherty, 2007). To improve the display and screening of scFvs and also of full-length antibodies, a new system has been developed allowing for anchored periplasmic expression (APEx) (Harvey et al., 2004; Harvey et al., 2006; Jeong et al., 2007; Mazor et al., 2007). APEx is based on the anchoring of proteins to the periplasmic side of the inner membrane, followed by disruption of the outer membrane before
incubation with fluorescently labeled antigen and flow-cytometric sorting. One advantage with this system is that the library members only have to traverse one membrane before it is displayed, which can simplify the translocation of certain libraries compared to other cell display systems. A limitation is, however, that after each round of sorting, the selected clones have to be PCR-rescued, recloned and retransformed into cells. Despite the recent advances with scFv and full-length antibody display, *E. coli* display is perhaps better suited for display of peptide libraries (Bessette et al., 2004; Rice et al., 2006) and smaller scaffold proteins (Silverman et al., 2005). The potential utility of the gram-positive bacterium *Staphylococcus carnosus* has been established recently (Wernerus et al., 2003; Löfblom et al., 2005; Löfblom et al., 2007). Flow-cytometric sortings generated high affinity Affibody molecules specific for TNF-α from a large naïve Affibody library previously pre-enriched through one cycle of phage (Kronqvist et al., 2008).

Yeast surface display was introduced 10 years ago (Boder and Wittrup, 1997). In this display system recombinant peptides or proteins are displayed on the surface of the yeast strain *Saccharomyces cerevisiae* as fusions to the α-agglutinin yeast adhesion receptor, which is located on the yeast cell wall. In addition to the advantages of bacterial cell-surface display, yeast is an eukaryotic organism and might therefore be better suited for functional expression of mammalian proteins, such as antibody fragments (Table 4). The lower transformation frequencies of yeast compared to *E. coli* generally generates a smaller functional library size of about 10⁷ members. Yeast surface display has been used for de novo selections of nanomolar affinity antibodies from a large (10⁹ clones) non-immune human scFv library (Feldhaus et al., 2003). The limited library size in combination with the possibility for fine discrimination in affinity between single library members using flow-cytometric sorting has led to the use of yeast display mainly for improving affinity and increasing stability and expression (Gai and Wittrup, 2007). Recently, directed evolution using yeast display has showed a remarkable 200,000-fold (Jin et al., 2006) and 3,000,000-fold (Buonpane et al., 2007) increase in affinity.

Although highly efficient, microbial display technologies are limited by protein folding, postranslational modifications, and different codon usage from those in mammals. Problems can occur when antibodies selected in e.g. phage display are transferred into mammalian expression systems, which are widely used in the pharmaceutical industry to produce active therapeutic antibodies. Therefore, a mammalian expression system, which can be used in antibody selections and affinity maturations, as was recently reported successful for scFvs (Ho et al., 2006), can be useful.
3.2.2 Cell-free display systems

Cell-free display systems offer two main advantages over phage and cell display. First, large libraries (typically $10^{12}$-$10^{13}$ different sequences (Hoogenboom, 2005)) can be made rapidly because there is no need for transformation of a library into a host. Second, additional mutations can be introduced at every round because a PCR step is included in each selection cycle rather than an in vivo amplification step. Both ribosome and mRNA display apply an in vitro transcription/translation system that circumvents growth in and transformation of cells. Libraries are selected using methods similar to phage display, because nucleic acid-protein complexes are too small for detection by flow-cytometric sorting. Ribosomal display is the most successfully and widely used of the cell-free display methods (Table 4) and was first described in 1994 in a paper by Mattheakis and coworkers (Mattheakis et al., 1994). In ribosome display, the selection is based on the mRNA-ribosome-protein complexes formed as a result of in vitro translation of library members and their attachment to the ribosome and to the encoding mRNA. After incubation with target protein and washing steps, the library enriched for target-binding is recovered by destabilizing the ribosomal complexes and isolating the mRNA. A reverse transcription reaction followed by PCR provides the DNA template for the next round. mRNA display is similar to ribosome display but relies on a covalent linkage between the mRNA and the library member in the mRNA-protein complex. Ribosomal and mRNA display have been proven valuable methods for selecting high affinity reagents from diverse libraries (Lipovsek and Plückthun, 2004; Yan and Xu, 2006) In particular ribosome display has been used for affinity maturation of binders down to low pM affinities (Zahnd et al., 2004; Zahnd et al., 2007). There are also several other methods using in vitro translation for direct linking of protein and DNA, including CIS display (Odegrrip et al., 2004), DNA display (Tabuchi et al., 2001), covalent DNA display (Bertschinger et al., 2007) and microbead display by in vitro compartmentalization (Sepp et al., 2002).

3.2.3 Non-display systems

There are selections systems not depending on display of libraries. These in vivo selection systems usually rely on the reconstitution of a protein activity upon binder-target interaction (e.g. enzyme activity, fluorescence or transcriptional activity) and can be selected e.g. on fluorescence intensity in flow-cytometric sorting or on growth survival. The main advantage of these systems is the high-throughput in screening, that could be achieved by direct screening on e.g. growth survival, and
that the work of purifying the target protein is not required. However, there are challenges, like non-specific intracellular interactions and difficulties in discrimination on affinity, that has to be overcome (Koch et al., 2006). The yeast two-hybrid system (Pörtner-Taliana et al., 2000) and protein fragment complementation assay (PCA) (Koch et al., 2006) have been used for selections of novel binding proteins, but are yet not systems widely used for selections.

**Table 4.** The three most established selection platforms; phage display, yeast display and ribosome display are presented and compared.

<table>
<thead>
<tr>
<th>Selection system</th>
<th>Valency of display/ Typical max. library size</th>
<th>Selection scope/ Main application</th>
<th>Main strength</th>
<th>Main weakness</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell display</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Phage</td>
<td>Monovalent or multivalent 10^10 to 10^11</td>
<td>Versatile/ mAbs from naïve, immune or synthetic libraries Affinity maturation Stability improvement</td>
<td>Technically robust Easy to use Versatile</td>
<td>Large libraries difficult to make</td>
</tr>
<tr>
<td>Yeast</td>
<td>Multivalent 10^7</td>
<td>Flow sorting/ Affinity maturation Expression increase Stability increase</td>
<td>On-cell kinetic screening More functional expression of human proteins due to folding advantages</td>
<td>Flow sorting expertise and equipment needed Transformation efficiency</td>
</tr>
<tr>
<td><strong>Cell-free display</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosome</td>
<td>Monovalent 10^12 to 10^13</td>
<td>Limited/ Affinity maturation Stability increase</td>
<td>Intrinsic mutagenesis Fastest of all systems</td>
<td>Functional expression difficult Technically sensitive</td>
</tr>
</tbody>
</table>

Modified from Hoogenboom 2005.
4. CANCER

Cancer is one of the most common diseases and a major public health problem in the western world today. There are over 10 million new cases of cancer reported every year (Parkin et al., 2005) and currently one in four death in the United States is due to cancer (Jemal et al., 2007). Lung, colorectal, breast and prostate cancer are some of the most common cancers in the western world (Parkin et al., 2005). Whereas lung cancer is also the most common cause of death, the prognosis for colorectal, breast and prostate cancer is generally rather good (Parkin et al., 2005). The number of diagnosed cases of cancer has increased over the years (Parkin et al., 2005) and the aging population is believed to be responsible for a part of the increase (Jemal et al., 2007). The mortality has during the same time decreased somewhat (Jemal et al., 2007) and this indicates improvements in the diagnosis and treatment of cancer.

Cancer is a multistep process often starting from a local benign tumor and in unfortunate cases ending with a malignant invasive tumor that metastasize to various organs. During this process cancer cells acquire new properties, which are necessary for the full malignant phenotype. In order to proliferate and divide continuously, cancer cells must circumvent the normal process of undergoing apoptosis. Moreover, to grow beyond a certain size the tumor needs nutrients and oxygen and promotes the formation of new blood vessels. The final step involves the ability to migrate and invade distant tissues, i.e. forming metastases. These hallmarks of cancer are thoroughly reviewed by Hanahan and Weinberg (Hanahan and Weinberg, 2000).

4.1 Cancer treatments

4.1.1 Cancer treatments today

Cancer treatments today mainly depend on surgery, external radiation therapy and chemotherapy. Surgery and external radiation therapy are the major treatment modalities for primary tumors and large metastases. Chemotherapy is used for
treatment of disseminated* tumor disease (e.g. breast, prostate and colorectal cancer). Some of these cancer treatments can also be complemented with hormone treatment, e.g. in breast cancer treatment with the antiestrogenic agent Tamoxifen (Cheung, 2007). There are also an increasing number of mAbs (Leader et al., 2008) and small molecule tyrosine kinase inhibitors (Arora and Scholar, 2005; Rocha-Lima et al., 2007) approved for clinical use in cancer therapy.

4.1.2 New approaches for cancer therapy

The classical treatment modalities; surgery, external radiation therapy, chemotherapy and hormone treatment are probably here to stay. They are constantly being improved but they also have certain inherent shortcomings. Many times there are cancer cells remaining after surgery and some tumors and metastases can be difficult to reach with that method. External radiation therapy and chemotherapy are associated with side effects because of the effect on normal tissues. Much research is now focusing on new approaches for cancer therapy that involve more specific targeting of the cancer disease. Complementary treatment modalities that are being explored include immunotherapy (Blattman and Greenberg, 2004), anti-angiogenesis therapy (Ruegg and Mutter, 2007), apoptosis regulation (Reed, 2004), differentiation therapy (Spira and Carducci, 2003), signal transduction therapy (Klein et al., 2005), nucleic acid-based therapies (Walsh, 2005) and targeted radionuclide therapy (using for example mAbs (Wu and Senter, 2005) or non-immunoglobulin proteins). Since tumors are heterogeneous in their nature and every cancer is a constantly evolving entity, combinations of different treatment modalities are more likely to succeed than single-strategy treatments.

4.2 Cancer detection

An early detection of cancer has normally a large impact on the possibility of curing the disease. The introduction of screening programs for various cancer forms in the population has been debated over the years. Many countries in the western world now offer screening programs for breast cancer (mammography) and cervix cancer. There is also a screening method available for prostate cancer, i.e. the PSA screen. The PSA test has, however, been widely debated due to the number of false positives (and also false negatives).

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* Disseminated means that tumor cells have spread from the original tumor to the bloodstream or lymphatic system and can form metastases at distant sites.
Modern clinical cancer treatments require precise information on the tumor location, the size of the tumor and if the tumor is disseminated. These questions are being answered through the application of traditional anatomical imaging methods, such as computed x-ray tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US). Newer approaches also involve radionuclide imaging with single-photon emission computerized tomography (SPECT), and positron emission tomography (PET), which will be further described in Chapter 6.3.
5. TUMOR TARGETING

In the case of disseminated tumor disease a systemic treatment is required. The efficiency of a systemic treatment using cytotoxic drugs is limited because of the toxicity to healthy tissues. The solution to this problem lies in the potential to increase the specificity of the treatment. This can be achieved by directing the therapy to molecular targets that are more highly expressed or accessible in tumor cells than in normal cells. An increasing number of clinically approved treatments are developed on the basis of knowledge about the molecular targets within tumors, the mechanism of the function of such targets and their abundance in malignant tissues. At present, there are nine monoclonal antibodies approved by FDA for cancer therapy targeting cancer-associated targets (Table 1) and several more have shown promising results in clinical trials (Adams and Weiner, 2005). Other tumor-targeting therapy strategies are also being explored. These include nucleic acid-based therapeutics, such as antisense-based products, small interfering RNA, gene therapy-based products and aptamers (Walsh, 2005). Furthermore, small molecule enzyme inhibitors (Arora and Scholar, 2005), affinity peptides (Aina et al., 2007), cancer vaccines (Curigliano et al., 2006) and non-immunoglobulin scaffolds (Baum et al., 2006; Zahnd et al., 2006; Nilsson and Tolmachev, 2007) are also explored for tumor specific targeting.

5.1 ErbB receptor family

The epidermal growth factor receptor family, ErbB receptor family, has shown to be involved in the development of many human cancers and to play an essential role in most of the hallmarks of cancer development described in Chapter 4 (Holbro et al., 2003). The ErbB receptor family is involved in a complex signaling network resulting in cancer-related cellular responses, such as proliferation, migration, differentiation and apoptosis (Citri and Yarden, 2006). In addition, there are also indications of crosstalk between the ErbB receptor network and other signaling pathways, including the estrogen receptor (ER), the G protein-coupled receptors (GPCRs), and the insulin like growth factor-1 receptor (IGF1R) (Yarden and Sliwkowski, 2001; Hynes and Lane, 2005). The flexibility of the ErbB receptor
signaling network and the crosstalk with other signaling pathways have to be considered in ErbB-receptor targeting and cancer treatment, where the development of biomarker analyses to assess specific molecular targets and tumor response will be important. Furthermore, the use of combination therapies will be important in treating these cancers.

The ErbB family of tyrosine kinase receptors (RTKs) include four members; EGFR (also ErbB1 or HER1), ErbB2 (or HER2), ErbB3 (or HER3), and ErbB4 (or HER4). Henceforth, the ErbB receptor family members will be referred to as: EGFR, HER2, HER3 and HER4. All ErbB receptors have in common an extracellular ligand-binding domain, a single membrane-spanning region and a cytoplasmic protein tyrosine kinase domain (Fig. 5). The ErbB receptors are expressed in various tissues of epithelial, mesenchymal and neuronal origin. Under normal physiological conditions, activation of the ErbB receptors is controlled by the expression of their ligands, which are members of the epidermal growth factor (EGF) family. The ErbB receptors are important during development and in normal adult physiology (Burden and Yarden, 1997). Furthermore, ErbB receptors have been implicated in the development of many human cancers, including breast, prostate, urinary bladder, colon, head and neck, non-small cell lung cancer (NSCLC) and glioma (Yarden and Sliwkowski, 2001; Holbro et al., 2003). Patients that have alterations in ErbB receptors tend to have a more aggressive disease, associated with parameters predicting a poorer clinical outcome, although conflicting results have been reported (see Chapter 7.1) (Nicholson et al., 2001; Arteaga, 2002; Holbro et al., 2003). These receptors have therefore been extensively studied for tumor targeting.

Ligand binding to the ErbB receptors induces the formation of homodimers (between monomers of the same receptor e.g. EGFR/EGFR) or heterodimers (between the bound receptor and another member of the ErbB receptor family e.g. EGFR/HER2). This results in the activation of the intrinsic kinase domain through phosphorylation of specific tyrosine residues within the cytoplasmic tail (Fig. 5). These phosphorylated residues serve as docking sites for a range of proteins, which leads to the activation of intracellular signaling pathways. These include the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 kinase (PI-3K) pathways. The downstream signaling regulates various aspects of cell function, including cell proliferation, programmed cell death (apoptosis), angiogenesis, adhesion and motility (Yarden and Sliwkowski, 2001; Holbro et al., 2003). In abnormal cells, these pathways become dysregulated, leading to cell hyperproliferation, migration and metastases (Fig. 5).
Fig. 5. Overview of the ErbB receptor signaling system. A. Homodimerization of EGFR receptors after ligand (L) activation leads to phosphorylation (P) of the intrinsic kinase domain (K). Activation of the signaling pathway, e.g. through the MAPK and PI-3K pathways, regulates many cell functions, such as growth, adhesion, migration, differentiation, and apoptosis. B. Ligand binding to EGFR facilitates heterodimerization with HER2, the most common co-receptor. C. Although HER3 has no active tyrosine kinase (K), ligand binding initiates signaling via heterodimerization with HER2. D. The mutant EGFRvIII lacks an extracellular ligand-binding domain and does not dimerize, but can still initiate signaling via constitutive activation.

There are more than 11 ErbB receptor-specific ligands, each of which contains an EGF-like domain that confers binding specificity. The ligands, including EGF, transforming growth factor-α (TGF-α), and the neuregulin family, can be divided into three groups depending on their specificity for EGFR, HER3 or HER4. No naturally existing ligands for HER2 has, however, been found (Citri et al., 2003). The HER3 receptor is an impaired kinase due to substitutions in critical residues in its kinase domain (Citri et al., 2003). Hence, HER3 has to form a heterodimer with another receptor to be involved in signaling (Fig. 5). HER2 has a central role in the family since it is the preferred dimerization partner for the other ErbB receptors (Citri et al., 2003; Holbro et al., 2003).
Publications describing the crystal structure of the extracellular domain of the EGFR, HER2 and HER3 (Cho and Leahy, 2002; Burgess et al., 2003; Cho et al., 2003; Ferguson et al., 2003; Garrett et al., 2003) have given new insights into the ligand binding and dimerization of the receptors. The extracellular domain is composed of four subdomains (I-IV), where the domain I and III are known to be involved in ligand binding (Burgess et al., 2003) (Fig. 6). Moreover, studies also reveal that a receptor-receptor interaction is directly promoted by the domain II dimerization arm. In an inactive state the EGFR and HER3 receptors have a “tethered” configuration stabilized by interactions between domain II and IV (Fig. 6). Ligand binding to the receptor will promote an “untethered” extended configuration exposing the dimerization arm for receptor-receptor interaction (Fig. 6). The structure of HER2 is radically different from the others. HER2 has a fixed conformation that resembles the ligand-activated state, where the domain II-IV interaction is absent and the dimerization loop in domain II is exposed (Cho et al., 2003; Garrett et al., 2003) (Fig. 6). The structure of HER2 is consistent with the findings of HER2 being the preferred dimerization partner, since it is constitutently active. This open structure also supports the theory that no natural ligands exist for HER2.

**Fig. 6.** Schematic representation of the ErbB receptor structure and activation. The extracellular domain (subdomain I-IV), the transmembrane region and the intracellular tyrosine kinase domain are depicted. The tethered, inactive EGFR becomes activated upon ligand binding. The receptor is extended and the domain II dimerization arm is exposed. The constitutively active HER2 can then form a heterodimer with EGFR by receptor-receptor interaction via the dimerization arm. The heterodimer now becomes phosphorylated (P) and is active for signaling.
There are two major classes of ErbB receptor-based therapeutics; i.e. extracellular domain-binding antibodies and small-molecule tyrosine kinase inhibitors (TKIs). Three mAbs targeting ErbB receptors are approved for clinical use (Table 1). These bind different epitopes of the receptors and have different modes of action, such as inhibiting dimerization or ligand binding or removal of the receptor from the surface, thereby influencing downstream signaling and engagement of the immune effector system. The TKI agents compete with ATP binding to the tyrosine-kinase domain of the receptor, which inhibit tyrosine kinase activation and subsequently lead to blockade of signaling pathways. Description of the different targeting agents and inhibitors of the ErbB receptor family and their modes of action has been reviewed elsewhere (Baselga and Arteaga, 2005; Hynes and Lane, 2005; Bublil and Yarden, 2007; Jakobovits et al., 2007; Rocha-Lima et al., 2007), and will not be further discussed in this thesis.

5.1.1 EGFR

EGFR was the first cell-surface receptor to be linked directly to human tumors (see review by Jakobovits et al., 2007). Many carcinomas are known to be promoted by EGFR activation, which can result from mutation of the receptor, gene amplification leading to EGFR overexpression, or from EGFR stimulation through autocrine loops (Burgess et al., 2003; Hynes and Lane, 2005). EGFR expression is abnormal or upregulated in many cancers, including non-small-cell lung cancer (NSCLC), head and neck, colon, ovary, breast, pancreatic, urinary bladder cancers and gliomas (Harari, 2004; Perez-Soler, 2004; Rocha-Lima et al., 2007). Targeting of EGFR, both for diagnosis and treatment, is therefore important and the rational behind this thesis (see Present Investigation).

Three mutant EGFR variants have been defined, with EGFRvIII the most commonly detected in human solid tumors (Moscatello et al., 1998). The EGFRvIII variant (also known as de2-7 EGFR) has exons 2-7 missing and, consequently, the domain II dimerization arm (Fig. 5). This mutation yields a constitutively active receptor (Moscatello et al., 1998) that is not found in normal cells, but is present in a wide range of solid tumors. EGFRvIII is detected in 40% of high-grade gliomas, where it frequently exhibits gene amplification, and less frequently in breast, ovarian cancer and non-small-cell lung cancer (Arteaga, 2002; Hynes and Lane, 2005).

Two mAbs targeting the EGFR, cetuximab and panitumumab, are approved for therapeutic use so far and several more are in clinical trials (Adams and Weiner, 2005; Baselga and Arteaga, 2005; Rocha-Lima et al., 2007; Leader et al., 2008). Cetuximab and panitumumab prevent ligand binding and interfere with ligand-
dependent receptor activation. These have shown clinical activity in a variety of solid tumors including colon, head and neck and non-small-cell lung cancer. Cetuximab has been found to show good response in combination with chemotherapy and also to reverse resistance to chemotherapy in several studies on colon cancer patients (Baselga and Arteaga, 2005). Several antibodies have also been developed that specifically target the mutant EGFRvIII (Hills et al., 1995; Wikstrand et al., 1995; Johns et al., 2002). One of these, the mAb 806, binds both the EGFRvIII and a subset (~10%) of wild-type overexpressed EGFR that is in a transition state between tethered and untethered form (Johns et al., 2002; Johns et al., 2004). The mAb 806 has recently entered into phase I clinical trials (Scott et al., 2007). Furthermore, there are two TKIs, gefitinib and erlotinib, approved for NSCLC treatment (Baselga and Arteaga, 2005; Rocha-Lima et al., 2007).
6. MOLECULAR IMAGING IN CANCER

6.1 Molecular imaging

The detection of cancer in an early stage is crucial for patient management and survival. In most cases, an early detection where tumors in general are relatively small and have not yet started to disseminate is associated with a more than 90% 5-year survival rate (Weissleder, 2006). Conventional anatomic imaging techniques (e.g. computed x-ray tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US)) typically detect tumors larger than one centimeter in diameter, at which point they already may consist of more than $10^9$ cells (including circulating and microscopic metastatic deposits) (Weissleder, 2006). Specific targeting of cancer-associated targets with radiolabeled targeting agents (e.g. antibodies, peptides, non-immunoglobulin proteins), and subsequent visualization using gamma- or PET-camera, are examples of molecular imaging. Molecular imaging allows for sensitive and specific monitoring of key cancer-related molecular targets in vivo and is expected to play an important role in future cancer diagnosis because it may allow an earlier and more informative characterization of the patients’ disease. Many new cancer therapeutics, like mAbs (described in Chapter 5), are typically very expensive, and often efficacious only in a subgroup of patients and furthermore sometimes associated with side-effects. Molecular imaging is a non-invasive diagnostic method that could provide important information by demonstrating the presence of appropriate molecular targets and could furthermore also be useful to assess the response after therapy. In this thesis (paper III and V) gamma-camera imaging using radionuclide labeled Affibody molecules have been used.

6.2 In vivo imaging agents

The clinical utility of molecular imaging is determined by its accuracy, which in turn is dependent on a high specificity and sensitivity of the imaging agent. An imaging agent should bind specifically to a tumor-associated molecular target with minimal non-specific binding to non-target molecules. The sensitivity is mainly determined by the ratio of radioactivity concentration in the tumor versus healthy tissues. An
imaging agent should therefore preferably be of small size, in order to increase tissue penetration for accumulation in the tumor. Furthermore, a small targeting agent will be cleared quicker from the blood and other compartments in equilibrium with blood by kidney excretion, which is of importance to provide a good contrast of radioactivity in tumor versus normal tissues and organs (Batra et al., 2002). Targeting agents that provide good contrast within a reasonable short time are desired for the use in the clinic in order to keep the clinical protocols short, ideally providing images within one day. Other factors that can influence the accuracy are the level of antigen expression, the processing and retention of the targeting conjugate by the cancer cell, vascularization of the tumor as well as the resolution and sensitivity of the imaging device.

The most important properties of a targeting agent for imaging may be summarized in the following way:

- High specificity (i.e. absence of cross-reactivity with non-target molecules)
- High target binding affinity (low to subnanomolar binding is typically desired)
- Small size (enabling fast distribution to the tumor and quick clearance from the blood and other compartments)
- High structural stability for radionuclide labeling

There are today a few imaging agents in clinical use for diagnosing cancer and several of these are antibodies (Leader et al., 2008). ProstaScint (anti-PSA mAb) and OncoScint (mAb specific for tumor-associated glycoprotein) are two indium-111-labeled mAbs for detection of prostate cancer and colon/ovarian cancer, respectively (Leader et al., 2008). CEA-scan (anti-CEA Fab) and Verluma are two technetium-labeled Fab fragments, one for detection of colon and breast cancer and the other for small-cell lung cancer detection and staging (Leader et al., 2008). There are also radionuclide labeled peptides for cancer imaging, like OctreoScan, where an indium-111-labeled somatostatin analogue (octreotide) is used for neuroendocrine tumor and lymphoma detection (Leader et al., 2008). Several new targeting agents have the potential for the development of radionuclide imaging agents. Antibody fragments, like the camelid single domain (nanobody) targeting EGFR yielded tumor-to-background ratios of approximately 7 at 3 h post injection in a murine model (Huang et al., 2008). Diabodies targeting e.g. the carcinoembryonic antigen (CEA) have been successfully used for tumor targeting and imaging applications in murine tumor models (Olafsen et al., 2004). Non-immunoglobulin scaffolds could also be used to develop radiolabeled tracers. This has been considered for example for the designed
ankyrin repeat protein binding to HER2 in murine models (Zahnd et al., 2006). Furthermore, Affibody molecules specific for HER2 have shown successful tumor targeting and imaging both in preclinical (Tolmachev et al., 2006; Engfeldt et al., 2007a; Orlova et al., 2007b) and pilot clinical studies (Baum et al., 2006). This thesis will describe the initial efforts towards the generation of EGFR-targeting Affibody molecules suitable for molecular imaging of tumors and potentially also therapy.

6.3 Radionuclide imaging techniques

Radionuclide imaging may be divided into (i) single photon imaging, including planar gamma-camera imaging and single-photon emission computerized tomography (SPECT), and (ii) positron emission tomography (PET). Both planar imaging and SPECT are based on a gamma-camera device, but in SPECT the gamma-camera is rotated around the patient, avoiding the problem with interfering radioactivity from tissues near the area of interest (Tolmachev et al., 2007a). Proteins and peptides in clinical use for tumor imaging are labeled with $^{111}$In and $^{99m}$Tc (Britz-Cunningham and Adelstein, 2003; Leader et al., 2008). PET is a more recent development. The physical features of this method enables a better registration efficiency, better spatial and temporal resolution, and more accurate quantification in comparison with single photon detection. PET utilizes coincident detection of the pair of photons which are formed after annihilation of positrons emitted by nuclides attached to the tracers (Tolmachev et al., 2007a). PET combined with CT will also give detailed anatomical fusion images with much higher degree of accuracy. The most commonly used PET tracer is the $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG), which is used for the assessment of glucose metabolism, e.g. in the heart and the brain. Tumors often have a high metabolic activity and $^{18}$F-FDG can, hence, also be used for imaging of such tumors. It has been demonstrated that $^{18}$F-FDG-PET provides valuable information in diagnosing and staging of, for example, lung cancer (Erasmus and Patz, 1999; Goldsmith and Kostakoglu, 2000). An important limitation of $^{18}$F-FDG imaging is nonspecific uptake of the tracer at inflammatory sites, which thus can give false positive diagnosis. The choice of radionuclide depends on the application and the properties of the targeting agent, as described in Box 6.4.

In conclusion, the PET technique is superior to the available gamma camera techniques when it comes to sensitivity, resolution and quantification. However, SPECT may at present be a preferred technique for molecular imaging, because of
higher availability of diagnostic devices, and higher availability and lower cost of radionuclides (Tolmachev et al., 2007a).

Box 6.4 Radionuclides

The choice of radionuclide depends upon what targeting concept is to be considered. General factors such as decay half-life, type and spectrum of energy of emitted radiation, cost and availability of the nuclide must be considered, and the nuclide must furthermore match the properties of the targeting agent. Different radiation can be used for different applications, like diagnostic imaging of tumors via gamma-camera planar imaging, SPECT or PET, or even therapy of single cells, micrometastases, or solid tumors.

Gamma and positron-emitting radionuclides are used for tumor specific imaging (Table 5). $^{111}$In and $^{99m}$Tc are the most common radionuclides for SPECT and $^{18}$F, $^{11}$C, $^{13}$N and $^{15}$O for PET. The half-lives of $^{11}$C, $^{13}$N and $^{15}$O are, however, generally too short to label peptides and proteins and other radionuclides with longer half-lives, e.g. $^{68}$Ga, are therefore studied for PET imaging of targeting radiopharmaceuticals (Tolmachev et al., 2007a). The cellular degradation and release of proteins labeled with radionuclides will differ between radiohalogens (e.g. $^{125}$I) and radiometals (e.g. $^{111}$In). Radiohalogenated catabolites are, after lysosomal degradation, often uncharged lipophilic molecules that quickly release from cells. Catabolites with radiometals, on the other hand, are positively charged and thus hydrophilic and usually trapped in a bulky chelate and will remain within the cytoplasm for a long time. This provides good tumor retention of the radioactivity, but it also causes a higher accumulation of radioactivity in the kidneys for radiometal-labeled peptides and proteins with a size below the ~60 kDa, the cut-off for glomerular filtration (Behr et al., 1998).

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Physical half-life</th>
<th>Emission</th>
<th>Main application</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{11}$C</td>
<td>20 m</td>
<td>Positron</td>
<td>PET</td>
</tr>
<tr>
<td>$^{99}$Br</td>
<td>16 h</td>
<td>Positron, gamma</td>
<td>PET</td>
</tr>
<tr>
<td>$^{18}$F</td>
<td>110 m</td>
<td>Positron</td>
<td>PET</td>
</tr>
<tr>
<td>$^{68}$Ga</td>
<td>68 m</td>
<td>Positron, gamma</td>
<td>PET</td>
</tr>
<tr>
<td>$^{123}$I</td>
<td>13 h</td>
<td>Gamma</td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{124}$I</td>
<td>4.2 d</td>
<td>Positron, gamma</td>
<td>PET</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>2.8 d</td>
<td>Gamma</td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>6 h</td>
<td>Gamma</td>
<td>SPECT</td>
</tr>
</tbody>
</table>

m – minutes, h – hours, d – days

Three types of radionuclides are suitable for therapy, alpha-emitters, beta-emitters and Auger electron-emitters. Beta particles might be optimal for treatment of larger tumor cell clusters, but not single cells and smaller cell clusters, because of its longer radiation range (Carlsson et al., 2003; Tolmachev et al., 2004a). The longer range of beta particles will lead to cross-fire irradiation, where radiation from one targeted cell will also irradiate surrounding cells. The benefit with cross-fire irradiation is that it is not necessary to target each and every cancer cell, but this also means that surrounding normal tissue will be damaged. Radionuclides emitting alpha particles are options for treatment of small cell clusters or single disseminated tumor cells (Carlsson et al., 2003; Tolmachev et al., 2004a). These have a short range and higher energy deposition is localized to a much smaller volume, which can cause severe multiple DNA damages and DNA fragmentation and lead to cell death after only a few decays (Tolmachev et al., 2004a). It should be noted that Auger electron emitting radionuclides, due to their very short radiation range, must be internalized and in close proximity to the cell DNA to be efficacious.
PRESENT INVESTIGATION

7. GENERATION OF EGFR-BINDING AFFIBODY MOLECULES

In this section I will summarize the results that this thesis and the five papers included are based on. The objective has been to isolate novel Affibody molecules with binding capacity to the epidermal growth factor receptor (EGFR), which is abnormal or upregulated in many cancer forms, and to further develop these into candidate tracers for in vivo visualization of EGFR expression in tumors and metastases.

In paper I, the first Affibody molecules targeting EGFR were isolated and characterized. These were found to bind specifically with moderate high affinity to soluble EGFR and also to native EGFR on cells. Since the proposed main application of these Affibody molecules was targeting of cancer cells in vivo, the cellular processing, biodistribution and tumor targeting ability of the EGFR-binding Affibody molecules were of high interest. In paper II, the cellular uptake, internalization and retention were therefore studied. This was followed by radiolabeling of the best Affibody molecule and a biodistribution study in tumor bearing mice in paper III. Since a high affinity is considered to be desired for successful molecular imaging, a directed-evolution effort to yield high affinity EGFR-binding Affibody molecules was conducted in paper IV. Finally, in paper V, the effect of using a dimeric format and different radionuclide labeling of EGFR-binding Affibody molecules was studied in biodistribution and gamma-camera imaging in tumor bearing mice.
7.1 Phage display selection of Affibody molecules binding to the EGFR (I)

The epidermal growth factor receptor 1, EGFR, is abnormal or upregulated in many human cancers, including breast, prostate, urinary bladder, colorectal, ovary, NSCLC, pancreatic, head and neck cancer and glioma (Harari, 2004; Perez-Soler, 2004; Rocha-Lima et al., 2007). Activation of EGFR initiates signaling resulting in cellular responses, such as proliferation, differentiation, migration and inhibition of apoptosis. Targeted therapies, like monoclonal antibodies (cetuximab and panitumumab) and tyrosine kinase inhibitors (gefitinib and erlotinib), are used to prevent EGFR signaling in combination with other treatments, such as chemotherapy. These treatments have shown encouraging results in several clinical trials (Rocha-Lima et al., 2007). At the same time, the need for better stratification of the patients that would benefit from EGFR targeted therapy is emphasized. Although EGFR is generally considered to be predictive of poor patient prognosis in cancers, conflicting results have been reported (Nicholson et al., 2001). It has been described that EGFR expression levels are highly valuable prognostic indicators of the clinical outcome for patients with head and neck, ovarian, cervical, bladder and esophageal cancer. It is considered to have moderate prognostic value for gastric, breast, endometrial, and colorectal cancer and relatively low prognostic value for non-small cell lung cancer (Nicholson et al., 2001). Molecular imaging of specific cancer-associated molecular targets should add important information in the choice of treatment for a patient and would furthermore allow for the assessment of response to treatment.

Promising tumor imaging results have previously been reported for a HER2-binding Affibody molecule in a xenografted mouse model (Orlova et al., 2006), followed by thorough investigation in preclinical and pilot clinical studies (Baum et al., 2006; Tolmachev et al., 2006; Orlova et al., 2007b). These studies suggested that the main advantages associated with Affibody molecules as tumor targeting agents are their (i) robustness, (ii) low-unspecific binding giving high tumor-to-blood and tumor-to-organ ratios, (iii) suitable biological half-life to allow high-contrast imaging in reasonable time and (iv) ability to be labeled in a site-specific manner using straightforward, well established methods, since Affibody molecules can be conveniently produced either by bacterial production or by chemical synthesis (Engfeldt et al., 2005).

In paper I, phage display in vitro selection technology was used to isolate novel Affibody molecules with specific binding to the extracellular domain (ECD) of the
epidermal growth factor receptor (EGFR). An Affibody phagemid library (described in Grönwall et al., 2007a) was used in the biopanning procedure. The selection was performed in solution where phage that bound to biotinylated EGFR-ECD was captured on streptavidin-coated paramagnetic beads. After four rounds of biopanning randomly picked clones were collected and subjected to an ELISA to exclude Affibody clones that bound the streptavidin-solid phase matrix. DNA sequencing of 192 clones, followed by sequence cluster analysis and exclusion of clones containing amber stop codons and cysteine residues led to the identification of nine sequences of Affibody molecules that were of interest for further characterization. For subsequent studies, the nine Affibody variants were produced and purified as His$_6$-tagged fusion proteins.

![Fig. 7. Biosensor binding studies. A. Sensorgram obtained after injection of the purified Z$_{\text{EGFR:942}}$ (circles), Z$_{\text{EGFR:948}}$ (squares) and Z$_{\text{EGFR:955}}$ (triangles) Affibody variants over sensor chip flow-cell surfaces containing amine-coupled EGFR-ECD (filled circles/squares/triangles) or HER2-ECD (open circles/squares/triangles). B. Sensorgram showing the dissociation phase of the dimeric Affibody molecules. (Z$_{\text{EGFR:942}}$)$_2$ (filled circles), (Z$_{\text{EGFR:948}}$)$_2$ (filled squares) and (Z$_{\text{EGFR:955}}$)$_2$ (filled triangles) obtained after the injection over an EGFR-ECD flow-cell surface. Monomeric Affibody molecules, Z$_{\text{EGFR:942}}$ (open circles), Z$_{\text{EGFR:948}}$ (open squares) and Z$_{\text{EGFR:955}}$ (open triangles) are shown as a reference. For clarity, the response of the dissociation is normalized to 1 in the starting point of the dissociation phase.](image-url)

Binding to EGFR-ECD was analyzed with real-time biospecific interaction analysis (BIA) using a Biacore biosensor instrument. First, the Affibody molecules were injected over separate flow-cell surfaces containing the amine-coupled EGFR-ECD and control proteins HER2 and IgG. Out of the nine candidates, three Affibody molecules, Z$_{\text{EGFR:942}}$, Z$_{\text{EGFR:948}}$, and Z$_{\text{EGFR:955}}$, demonstrated significant binding to EGFR-ECD (Fig. 7A). No significant cross-reactivity to HER2 (Fig. 7A) or IgG
could be seen, despite a 44% sequence homology between EGFR-ECD and HER2-ECD (Earp et al., 1995). Since high affinity and good tumor retention are important parameters in generating an imaging agent with good contrast, an effort to improve the affinity for subsequent studies was made. Dimeric Affibody constructs were generated to increase the functional affinity, as has been demonstrated before (Steffen et al., 2005) and monomeric and dimeric proteins were analyzed by real-time BIA analysis and subjected to kinetic analysis in order to determine the kinetic binding constants. All three monomeric Affibody molecules showed affinity in the same order of magnitude (130-185 nM). Dimerization resulted in improved apparent affinities mostly from improvements in the dissociation rate (Fig. 7B). Although the affinities were similar, a rather clear difference could be observed in the dissociation rates (Fig. 7B). Since Z\textsubscript{EGFR:955} was found to have the slowest dissociation, it could be expected that this Affibody molecule would perform well in cellular binding assays.

![Graph](image1)

**Fig. 8.** Flow cytometric analysis and confocal microscopy image of the binding of fluorescently labeled EGFR-binding dimeric Affibody molecules to native EGFR on A431 cells. A. (1) Negative control Affibody, (2) (Z\textsubscript{EGFR:942})\textsubscript{2}, (3) (Z\textsubscript{EGFR:948})\textsubscript{2}, and (4) (Z\textsubscript{EGFR:955})\textsubscript{2}. B. Confocal microscopy image of A431 cells exposed to the (Z\textsubscript{EGFR:955})\textsubscript{2} Affibody molecule. The fluorescently labeled (Z\textsubscript{EGFR:955})\textsubscript{2} Affibody molecule was incubated with A431 cells for 1 h on ice (left) and 2 h at 37°C (right).

One important step in verifying the utility of the EGFR-binding Affibody molecules as tumor targeting agents was to confirm their binding to native EGFR present on EGFR-expressing cells. All cell assays were performed with dimeric Affibody constructs, to allow for more sensitive cell binding. Prior to flow-
cytometric analysis, the Affibody molecules ((Z_{EGFR:942})_2, (Z_{EGFR:948})_2, and (Z_{EGFR:955})_2) were site-specifically fluorophore labeled using a unique C-terminal cysteine residue, followed by incubation with human epithelial cancer cell line A431 cells. All three Affibody molecules demonstrated significant binding to the A431 cells. No non-specific binding was observed, neither to a control neuroblastoma cell-line SH-SY5Y (data not shown), nor for an unrelated control Affibody to A431 cells (Fig. 8A). This indicated that the anti-EGFR Affibody molecules bound specifically to EGFR. Since a significant effort was made to use equimolar amounts in the analysis, the flow-cytometric analysis could be used to estimate how the different Affibody molecules were found to rank in their binding performance to native EGFR. With this experimental set-up, the Affibody molecules were found to rank accordingly (Z_{EGFR:955})_2 > (Z_{EGFR:948})_2 > (Z_{EGFR:942})_2 (Fig. 8A). The binding of the Affibody molecules to native EGFR on cells was supported by results from immunofluorescence microscopy studies. These studies were performed using (Z_{EGFR:955})_2, since this was the binder performing best in previous cell studies and that had the best off-rate. Incubation of fixed A431 cells with a fluorescently labeled Affibody molecule gave specific staining of A431 cells in confocal microscopy study (Fig. 8B, left figure). Intracellular staining was observed after incubation at physiological conditions (37 °C) for 2 h (Fig. 8B, right figure), supporting the specific binding of the EGFR-binding Affibody molecules for native EGFR and suggesting internalization of the Affibody molecules.

In order for the EGFR-binding Affibody molecules to be used in molecular imaging they should ideally be radiolabeled without compromising their binding capacity. To study this, the three Affibody molecules were indirectly radiolabeled with $^{125}$I to primary amine groups and they all showed retained binding to native EGFR on A431 cells. Furthermore, the three Affibody molecules were found to bind EGFR in a competitive manner, since they inhibited the binding of each other in a cell-binding assay, where excess of non-labeled Affibody molecule could block the binding of another radiolabeled Affibody molecule to A431 cells. This cellular assay suggests that (Z_{EGFR:955})_2, (Z_{EGFR:948})_2 and (Z_{EGFR:942})_2 have the same, or at least overlapping binding sites. The randomized amino acid residues, however, show very low degree of similarity between the three binders and it is therefore difficult to conclude whether they truly bind the same epitope.

In conclusion, the novel dimeric EGFR-binding Affibody molecule $Z_{EGFR:955}$ demonstrated significant binding to the EGFR-ECD. In cellular assays, fluorescently labeled Affibody molecules were bound and internalized into EGFR-expressing human epithelial cancer cell line A431 cells. Radiolabeling of Affibody molecules did not impair their binding to native EGFR on cells. Further studies of the cellular
processing, biodistribution and tumor targeting in tumor-bearing mice would add useful information on the properties and capacity of the EGFR-binding Affibody molecules.
7.2 Cellular studies of binding, internalization and retention of radiolabeled EGFR-binding Affibody molecule (II)

Receptor targeting agents may affect the receptor on an extracellular and intracellular level upon binding. This may in turn change the cellular processing of the radiolabeled agent, among many other things. Both natural ligands and monoclonal antibodies are known to alter the cellular processing (Wiley, 2003; Citri and Yarden, 2006; Jaramillo et al., 2006). For example, in the ErbB family, ligand activation of EGFR will lead to accelerated internalization and enhanced degradation of the receptor and also induce a substantial degradation of ErbB2 if the levels of EGFR and ErbB2 are similar (Wiley, 2003). The cellular processing of the natural ligand EGF and the mAb 225 (the mouse mAb corresponding to cetuximab) have been compared and it was reported that internalization, degradation and recycling processes were very different for the two EGFR-targeting molecules (Jaramillo et al., 2006). Thus, understanding the fate of the radiotracer is important as different radionuclides may give very different results depending on the cellular processing.

In paper II, the cellular binding, internalization and retention of the EGFR-targeting Affibody molecule \((Z_{\text{EGFR:955}})_2\) was investigated using the human epithelial carcinoma cell line A431. For comparison, the monoclonal antibody cetuximab and the natural ligand EGF were included. Cetuximab (~150 kDa) and the studied Affibody molecule (~15 kDa) are both dimers, but differ significantly in size. There is no large size difference between the EGF and \((Z_{\text{EGFR:955}})_2\) (~6 kDa vs ~15 kDa), however, EGF is a monomer and a natural ligand for EGFR (Harari, 2004). These three EGFR-binding substances have different molecular structures and differences in cellular uptake and processing patterns would therefore not be surprising.

The Affibody molecule \((Z_{\text{EGFR:955}})_2\), cetuximab and EGF were labeled with \(^{125}\text{I}\) to study their binding, cellular uptake and retention to EGFR-expressing A431 cells. Direct labeling with \(^{125}\text{I}\) on tyrosines was used for EGF and cetuximab and indirect labeling with \(^{125}\text{I}\) on primary amine groups for the Affibody molecule, since it had been reported that Affibody molecules better preserve their target binding capacity when an indirect labeling method is used (Steffen et al., 2005). In order to confirm specific binding after radiolabeling, A431 cells were incubated with an excess of nonradioactive substance followed by incubation of the corresponding radioactive
substance. The remaining radioactivity after blocking was very low, suggesting preserved binding specificity to A431 cells after radiolabeling.

The cellular uptake of $^{125}$I-labeled-($Z_{\text{EGFR:955}}$)$_2$ (Fig. 9A) and $^{125}$I-labeled-cetuximab (Fig. 9C) reached its maximum after 4–8 h of incubation. After 24 h of incubation the cell-associated $^{125}$I that remained was approximately 80% for $^{125}$I-labeled-($Z_{\text{EGFR:955}}$)$_2$ and approximately 50% for $^{125}$I-labeled-cetuximab. For $^{125}$I-labeled-EGF the $^{125}$I uptake peaked already after 2 h and the cell-associated $^{125}$I activity was very low after 24 h (Fig. 9B). These differences could be due to differences in the degradation of the substances and thereby differences in the release of $^{125}$I. The similar uptake patterns for ($Z_{\text{EGFR:955}}$)$_2$ and cetuximab could possibly also be associated with the fact that both are bivalent substances whereas EGF is monovalent.

**Fig. 9.** Time-dependent uptake of $^{125}$I when delivered with A. $^{125}$I-labeled-($Z_{\text{EGFR:955}}$)$_2$, B. $^{125}$I-labeled-EGF and C. $^{125}$I-labeled-cetuximab. The maximum $^{125}$I uptake for the three agents was obtained after 4–8 h for $^{125}$I-labeled-($Z_{\text{EGFR:955}}$)$_2$ and cetuximab and after 2 h for $^{125}$I-labeled-EGF. The remaining cell-associated radioactivity for $^{125}$I-labeled-($Z_{\text{EGFR:955}}$)$_2$ and $^{125}$I-labeled-cetuximab was 80% and 50%, respectively, after 24 h of incubation, while for $^{125}$I-labeled-EGF, almost all cell-associated radioactivity was gone after 24 h. Mean values and standard deviations are shown.
The cellular binding and internalization of all three molecules were studied in fluorescence confocal microscopy. All three substances were internalized after 2 h of incubation at physiological conditions (Fig. 10). In the case of EGF, internalization could be seen already after 5 min. This was less obvious for the Affibody molecule and cetuximab. A negative control Affibody molecule did, however, not bind or internalize (data not shown). The internalization of all three substances was further studied using CypHer5E-labeled \((Z_{\text{EGFR:955}})_2\), cetuximab and EGF. CypHer5E emits red fluorescence only at low pH, which is the case when internalized in acidic compartments such as endosomes or lysosomes. After 2 h incubation at physiological conditions, all three substances emitted red fluorescence as expected. The amount or fraction of internalized substances could not be estimated since these methods only give qualitative and not quantitative results. However, the pictures in Fig. 10 taken after 2 h of incubation indicated that EGF has a more granular fluorescence pattern in the cytoplasm than \((Z_{\text{EGFR:955}})_2\), suggesting a possible different cellular distribution. Cetuximab had an intermediate granular fluorescence pattern. It is interesting to note that the EGF:EGFR complex is more pH sensitive compared to the cetuximab:EGFR complex (Li et al., 2005; Jaramillo et al., 2006). The stability of \((Z_{\text{EGFR:955}})_2\):EGFR complex at various pH is not known.
Fig. 10. Cell binding and internalization of fluorescently-labeled \((Z_{\text{EGFR:955}})_2\), EGF and cetuximab in A431 cells using confocal microscopy. Fluorescently-labeled \((Z_{\text{EGFR:955}})_2\), EGF and cetuximab were incubated with A431 cells for 5 min or 2 h at 37°C. After the short incubation time (5 min), the green fluorescence was mainly membrane associated for \((Z_{\text{EGFR:955}})_2\) and cetuximab, while after the longer incubation time (2 h), internalization seemed to occur. In the case of EGF, internalization seemed to be initiated already after 5 min.

The cellular retention of \(^{125}\text{I}\) after delivery as \(^{125}\text{I}\)-labeled-\((Z_{\text{EGFR:955}})_2\), \(^{125}\text{I}\)-labeled-cetuximab and \(^{125}\text{I}\)-labeled-EGF was studied at various time points (Fig. 11). A431 cells were incubated with the radiolabeled compounds at 37°C until maximum uptake was reached. Subsequently, the cells were washed, incubated with medium and the radioactivity was measured at various time points. The remaining cell-associated \(^{125}\text{I}\) activity after 48 h of incubation delivered as \(^{125}\text{I}\)-labeled-\((Z_{\text{EGFR:955}})_2\) or \(^{125}\text{I}\)-labeled-cetuximab was rather good (~20% and ~25%, respectively) (Fig. 11). \(^{125}\text{I}\)-labeled-EGF-mediated delivery gave a much faster \(^{125}\text{I}\) release, and almost all cell-associated radioactivity had disappeared after 24 h of incubation (Fig. 11). The retention pattern of \(^{125}\text{I}\) delivered by the Affibody molecule was interesting. The EGF:EGFR complex is known to be internalized via clathrin-coated vesicles (Wiley, 2003; Le Roy and Wrana, 2005). Internalized receptors can, after internalization, be recycled back to the surface or be ubiquitylated and degraded in the lysosomes (Wiley, 2003; Le Roy and Wrana, 2005). Instead of being excreted from the cell like \(^{125}\text{I}\) delivered by EGF, it appeared that a fraction (20-25%) of the \(^{125}\text{I}\) delivered by the Affibody molecule was still associated to the cells
for at least 48 h. Cetuximab also gave a slow release of $^{125}$I. These differences in retention of the radioactivity cannot be fully explained considering the available results. It is possible that the observed differences in $^{125}$I retention partly can be due to different intracellular compartmentalization of the targeting substances after their internalization. Furthermore, differences in labeling could possibly also influence the retention.

![Graph showing cellular retention of $^{125}$I delivered as $^{125}$I-labeled-($Z_{EGFR:955})_2$, $^{125}$I-labeled-EGF or $^{125}$I-labeled-cetuximab. The remaining cell-associated radioactivity after 48 h of incubation was 20% for $^{125}$I-labeled-($Z_{EGFR:955})_2$ and 25% for $^{125}$I-labeled-cetuximab, while almost all cell-associated radioactivity had disappeared after 24 h of incubation with $^{125}$I-labeled-EGF. Mean values and standard deviations are shown.]

**Fig. 11.** Cellular retention of $^{125}$I delivered as $^{125}$I-labeled-($Z_{EGFR:955})_2$, $^{125}$I-labeled-EGF or $^{125}$I-labeled-cetuximab. The remaining cell-associated radioactivity after 48 h of incubation was 20% for $^{125}$I-labeled-($Z_{EGFR:955})_2$ and 25% for $^{125}$I-labeled-cetuximab, while almost all cell-associated radioactivity had disappeared after 24 h of incubation with $^{125}$I-labeled-EGF. Mean values and standard deviations are shown.

The targeting of different epitopes on ErbB could potentially influence receptor processing and downstream signaling of the cell (as discussed in Chapter 5.1). It was therefore of interest to study the site of binding for $(Z_{EGFR:955})_2$ on EGFR. An attempt to determine the position of binding for $^{125}$I-labeled-$(Z_{EGFR:955})_2$ on EGFR was made by studying whether an excess of nonradioactive EGF or cetuximab could disturb the binding of the Affibody molecule. Both agents could block binding of $^{125}$I-labeled-$(Z_{EGFR:955})_2$, which indicated that all three substances have the same or compete for the same binding site. However, the nonradioactive Affibody molecule $(Z_{EGFR:955})_2$ could not completely block the binding of $^{125}$I-labeled-EGF (~10% binding remained) or cetuximab (~40% binding remained). There are several hypotheses that could explain this that are not further discussed here, see Nordberg, et al. 2007. The EGF ligand is known to bind to both domain I and III (Burgess et al., 2003; Ferguson et al., 2003) and cetuximab to domain III (Li et al., 2005). The
results of the blocking experiment could therefore give an indication about where on EGFR the Affibody molecule binds and it seems reasonable to speculate that the binding site is on domain III. In order to more definitely determine the binding position of (Z_{EGFR:955})_2 to EGFR, other methods, such as x-ray crystallography, could be used.

To conclude, the dimeric EGFR-binding Affibody molecule has a cellular uptake pattern similar to cetuximab. The cellular retention is rather good, similar to cetuximab and with a much slower release than for EGF. The Affibody molecule binds specifically to the cells and is internalized by A431 cells. Further cellular studies looking at the intracellular signaling and cell proliferation would also be of interest.
7.3 In vivo and in vitro uptake of $^{111}$In, delivered with the Affibody molecule $(Z_{\text{EGFR:955}})_2$, in EGFR expressing tumour cells (III)

Gamma-camera imaging is frequently used in nuclear medicine applications. There are several imaging agents, radiolabeled with $^{111}$In or $^{99m}$Tc, approved for clinical use (Britz-Cunningham and Adelstein, 2003; Leader et al., 2008). $^{111}$In was chosen as a candidate radionuclide for the development of a radiolabeled EGFR-binding Affibody molecule for gamma-camera imaging. In paper III, the in vitro and in vivo properties of an $^{111}$In-labeled-$(Z_{\text{EGFR:955}})_2$ Affibody molecule were studied with the intention to evaluate the capacity of visualizing EGFR expression in tumors and metastases using a gamma-camera.

Prior to investigating the binding of $^{111}$In-labeled-$(Z_{\text{EGFR:955}})_2$ in vivo, the uptake, specificity and retention was studied in vitro. The Affibody molecules were conjugated to CHX-A"-DTPA on primary amine groups and labeled with $^{111}$In. The $^{111}$In-labeled-$(Z_{\text{EGFR:955}})_2$ showed a rapid cellular uptake of $^{111}$In and ~90% of the cell-associated $^{111}$In remained after 24 h. The uptake pattern of $^{111}$In was similar to $^{125}$I uptake when delivered as $^{125}$I-labeled-$(Z_{\text{EGFR:955}})_2$, as described previously in paper II. The cellular retention analysis showed an initial fast dissociation of $^{111}$In during the first 4 h, which was probably due to $^{111}$In-labeled-$(Z_{\text{EGFR:955}})_2$ molecules detaching from the receptors at the cell surface without being internalized. After 4 h, approximately 38% of the $^{111}$In was associated to the cells for at least 72 h and this fraction was probably internalized. A similar retention pattern was also observed for $^{125}$I-labeled-$(Z_{\text{EGFR:955}})_2$ (paper II). However, the retention after several hours of incubation was lower in the $^{125}$I case. Radiometals are known to be trapped in lysosomes, resulting in good cellular retention (Shih et al., 1994; Orlova et al., 2000), and this is a reasonable explanation for the higher level of retention of $^{111}$In in comparison to $^{125}$I.

To analyze the uptake in vivo, a biodistribution study of $^{111}$In delivered as $^{111}$In-labeled-$(Z_{\text{EGFR:955}})_2$ was performed in Balb/c nu/nu mice carrying xenografted A431 tumors. Four hours post injection (p.i.) the tumor uptake was 3.8±1.4% injected dose per gram (ID/g) (Fig. 12). The tumor uptake of a control Affibody, $^{111}$In-labeled-$(Z_{\text{abeta3-C28S}})_2$, was low (Fig. 12), indicating EGFR-specific uptake of $^{111}$In-labeled-$(Z_{\text{EGFR:955}})_2$. The radiotracer had a rapid blood clearance, with a concentration less than 0.4±0.1% ID/g in the circulation after 4 h, at which time the tumor-to-blood ratio was 9.1 (Fig. 12). This tumor-to-blood ratio was found to compare well with...
literature data where radiometal-labeled anti-EGFR antibodies or the EGF-ligand were used. This is further discussed in paper V.

![Biodistribution graph](image)

**Fig. 12.** Biodistribution of $^{111}$In, delivered as $^{111}$In-labeled-(Z$_{EGFR:955}$)$_2$, expressed as percent injected dose per gram tissue in tumour (A431 cells) bearing nude mice, 4 and 8 h post injection. The mice injected with the control Affibody molecule, $^{111}$In-labeled-(Z$_{abeta3-C28S}$)$_2$, were analyzed 4 h post injection. Mean values and standard deviations are shown.

The uptake of $^{111}$In in the kidneys and liver was high when delivered as $^{111}$In-labeled-(Z$_{EGFR:955}$)$_2$ (Fig. 12). The high kidney uptake was probably a consequence of kidney clearance and tubular reabsorption, which is typical for proteins and peptides smaller than approximately 60 kDa (Behr et al., 1998). Since radioactive metals are residualizing, the $^{111}$In will be trapped in the kidneys (Behr et al., 1998). The high liver uptake is most likely due to the normal expression of EGFR in liver tissue (Gusterson et al., 1984; Damjanov et al., 1986) and an obvious cross-reactivity of $^{111}$In-labeled-(Z$_{EGFR:955}$)$_2$ with murine EGFR. This is supported by the fact that liver uptake could be blocked by administration of a 10-fold excess of unlabeled (Z$_{EGFR:955}$)$_2$, and that the control Affibody molecule was not accumulating in the liver (Table 6). The uptake in the kidneys could not be inhibited this way. On the contrary the uptake of $^{111}$In in kidneys increased when the uptake in liver was blocked (Table 6). The kidney uptake of $^{111}$In was high also for the control Affibody molecule (Table 6).
Table 6. Uptake in liver and kidneys, 4 h post injection, of $^{111}$In delivered as $^{111}$In-labeled-(Z$_{\text{EGFR:955}}$)$_2$ without or with blocking of non-labeled (Z$_{\text{EGFR:955}}$)$_2$.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Liver</th>
<th>Kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{111}$In-(Z$_{\text{EGFR:955}}$)$_2$</td>
<td>16.5±2.2</td>
<td>86.4±14.4</td>
</tr>
<tr>
<td>$^{111}$In-(Z$_{\text{EGFR:955}}$)$<em>2$ + excess (Z$</em>{\text{EGFR:955}}$)$_2$</td>
<td>1.8±0.1</td>
<td>165.2±28.3</td>
</tr>
<tr>
<td>$^{111}$In-(Z$_{\text{abeta3-C28S}}$)$_2$</td>
<td>0.5±0.0</td>
<td>153.9±15.2</td>
</tr>
</tbody>
</table>

Numbers are percentage injected dose per gram tissue (% ID/g). Mean values from four mice ± standard deviations are given.

The tumors in the right fore leg of the mice were clearly visualized in gamma-camera 4 h p.i., even though they were quite close to the kidneys and liver, that also gave a strong signal in the gamma camera (Fig. 13). An excess of unlabeled (Z$_{\text{EGFR:955}}$)$_2$ could block the $^{111}$In-labeled-(Z$_{\text{EGFR:955}}$)$_2$ mediated gamma-camera signal of the tumor, which supported the biodistribution results regarding receptor specificity (Fig. 13).

The results presented here show that an EGFR-targeting Affibody molecule could be successfully radiometal-labeled with retained binding capacity and that it could specifically bind to EGFR-expressing A431 cells in tumor-bearing mice. This suggests that $^{111}$In-labeled-(Z$_{\text{EGFR:955}}$)$_2$ could be a good starting molecule for lead optimization by affinity maturation.

Fig. 13. Gamma-camera images after injection of $^{111}$In-labeled-(Z$_{\text{EGFR:955}}$)$_2$ in tumour-bearing mice. The images were taken 4 h post injection. The tumour areas are marked T. The receptors in the mouse to the right were blocked with an excess of non-labeled (Z$_{\text{EGFR:955}}$)$_2$. 
7.4 Directed evolution to low nanomolar affinity of a tumor-targeting EGFR-binding Affibody molecule (IV)

Low nanomolar or even subnanomolar affinities are considered to be desired for successful molecular imaging (Adams et al., 1998a; Behr et al., 2001). As described before, the accuracy in molecular imaging is determined by high sensitivity and specificity. The sensitivity is depending on the contrast, which is the ratio of radioactivity concentration in the tumor and in normal organs and tissues at the time of imaging. Smaller targeting agents are cleared more quickly from normal organs and tissues than bulky immunoglobulins, resulting in lower background signal and thus better contrast. High radioactivity concentration in tumors depends on the affinity of the tracer to its molecular target. For this reason, relatively high affinity is normally important for obtaining high contrast images. In paper IV, the aim was to improve the affinity of the EGFR-targeting Affibody molecules studied in paper I-III.

Efforts to increase the affinity for Affibody molecules have been successful in several cases (Gunneriusson et al., 1999; Nord et al., 2001; Orlova et al., 2006). The affinity maturations have been achieved either by helix shuffling (Gunneriusson et al., 1999) or sequence alignment and directed combinatorial mutagenesis using a single oligonucleotide (Nord et al., 2001; Orlova et al., 2006). The small size of the Affibody molecule offers a convenient affinity maturation principle. Unlike complex multistep affinity maturations of antibodies (Luginbühl et al., 2006; Pavoni et al., 2006), a single oligonucleotide could here be used to generate the affinity maturation library in one step. This was performed in a similar fashion as a previously described affinity maturation of a HER2-binding Affibody molecule (Orlova et al., 2006).

The affinity maturation library was based on a primary set of EGFR-binding molecules. The sequences of three EGFR-binding Affibody molecules previously studied (Friedman et al., 2007) and a fourth sequence from additional screening, sequencing and characterization were aligned, and it was found reasonable to keep five positions fixed, three positions partially randomized with bias for certain amino acids, and five positions targeted for randomization using NNG/T degenerated codons (Fig. 14A). A single 129-nucleotide oligonucleotide with degenerated codons, encoding helices 1 and 2 of the Z-domain, could be used to generate the new affinity maturation library in a single step. The library consisted of approximately 1 x 10⁹ members, which theoretically covered all the possible variants. Phage
selections were performed with decreasing concentrations of target protein. To further increase the probability to select the strongest EGFR-binding Affibody variants from the library, we added an excess of nonbiotinylated target protein to prevent rebinding of binders with fast off-rate. In addition, an excess of first-generation EGFR-binders (Friedman et al., 2007) was added in one incubation step to compete with second-generation binders, in order to favor the strongest binders.

Based on results from several assays; (i) ELISA for EGFR-binding performance, (ii) DNA sequencing and sequence clustering, (iii) real-time biospecific interaction analysis screening for EGFR-binding using Biacore, and (iv) solubility and stability studies, 14 Affibody variants (Fig. 14B) were selected for further characterization.

An initial ranking of binding affinities for EGFR was performed on the 14 selected Affibody variants with real-time biospecific interaction analysis using Biacore. The different Affibody variants were separately injected over sensor chip flow-cell surfaces containing the immobilized target protein EGFR-ECD and control proteins HER2, and HER3, respectively. Five binders (Z_{EGFR:1853}, Z_{EGFR:1868}, Z_{EGFR:1877}, Z_{EGFR:1907}, and Z_{EGFR:1908}) that demonstrated the best binding affinity, slow dissociation rate and no unspecific binding to HER2 or HER3 (Fig. 15), and which furthermore stained membranes of A431 cells in immunofluorescence assay, were selected for further characterization. In a kinetic analysis, using Biacore, the on- and off-rates and the equilibrium dissociation constant, K_D, were determined for the five candidate second-generation Affibody molecules. The affinity maturation process yielded many improved but similar binders. For the best binders, the affinity was found to be increased approximately 30-fold in the affinity maturation, mostly due to improvements in the off-rate, yielding Affibody molecules with K_D’s in the 5-10 nM range.
Fig. 14. Sequence alignment of first and second generation binders. A. The strategy for affinity maturation. Amino acid sequence of the wild-type Z-domain aligned to amino acid sequences of four first-generation EGFR binders (Friedman et al., 2007). The 13 randomized amino acid residues (Q9, Q10, N11, F13, Y14, L17, H18, E24, E25, R27, N28, Q32, and K35) are indicated by dots. Horizontal bars indicate amino acid identities. The design of the constructed library aimed for affinity maturation, $Z_{\text{libEGFR:mat}}$, is presented in a single letter code, with positions selected for variation in boldface (X = any amino acid). Note that a bias for asparagine and arginine residues is introduced at positions 17 and 18 and that a bias for serine or valine residues is introduced at position 35. The three $\alpha$-helices in the wild-type Z-domain are boxed.

B. Sequences of selected second generation Affibody variants. Amino acid sequence of the wild-type Z-domain aligned to amino acid sequences of 14 different second-generation Affibody variants selected in the affinity maturation against EGFR-ECD to demonstrate amino acid sequence differences. The Affibody variants in boldface are the ones subjected to a more thorough characterization.
Fig. 15. Biosensor binding study using surface plasmon resonance. Sensorgrams obtained after injection of the affinity-matured Affibody variants: $Z_{\text{EGFR}:1853}$ (filled triangles), $Z_{\text{EGFR}:1868}$ (open diamonds), $Z_{\text{EGFR}:1877}$ (open triangles), $Z_{\text{EGFR}:1907}$ (open squares), and $Z_{\text{EGFR}:1908}$ (open circles), as well as the first-generation binder $Z_{\text{EGFR}:955}$ (filled squares) over a sensor chip flow-cell surface containing amine-coupled EGFR-ECD. The Affibody molecules were also injected over a HER2 and HER3 immobilized flow-cell surface. None of the binders showed any cross-reactivity to HER2 or HER3, exemplified by the sensorgram of $Z_{\text{EGFR}:1907}$ (filled diamonds) over a sensor chip flow-cell surface containing amine-coupled HER2-ECD.

Conserved specificity during the affinity maturation was confirmed in a dot blot assay, where $Z_{\text{EGFR}:1907}$ demonstrated highly specific binding to human and mouse EGFR, among many serum proteins and other receptors of the same family. Immunofluorescence microscopy study of fluorophore-labeled $Z_{\text{EGFR}:1907}$ further confirmed that the Affibody molecule was binding the cell surface receptor in its natural context.

The increased affinity was confirmed using flow cytometry, where two of the second-generation binders ($Z_{\text{EGFR}:1853}$ and $Z_{\text{EGFR}:1907}$) were compared with a first-generation binder, $Z_{\text{EGFR}:955}$. The Affibody molecules were site-specifically labeled with a fluorophore via a unique C-terminal cysteine. Upon flow-cytometric analysis, after incubation of the fluorophore-labeled Affibody molecules with EGFR-expressing A431 cells, the second generation Affibody molecules showed an approximately 20-fold increase in fluorescence intensity compared to the first.
generation binder (Fig. 16A). Furthermore, different concentrations of fluorophore-labeled $Z_{\text{EGFR:1907}}$ were incubated with A431 cells and the mean fluorescence intensity from flow-cytometric analysis was analyzed, calculating the $K_D$ from an equilibrium binding curve (Fig. 16B). The $K_D$ of 2.8 nM supported the affinity estimated by the Biacore analysis.

As even minute differences in protein sequence can alter the biodistribution and excretion pattern of radiolabeled proteins for molecular imaging (Engfeldt et al., 2007b; Tran et al., 2007), a biodistribution investigation of all five Affibody molecules was conducted in the evaluation of the new EGFR-binding Affibody molecules. The Affibody molecules were labeled with the radiometal $^{111}$In via
benzyl-DTPA chelator to primary amine groups and studied for biodistribution and tumor targeting in A431 xenografted mice (Fig. 17). All five binders successfully targeted the A431 tumors, with an accumulated 4-6% injected radioactivity per gram (IA/g) of tumor tissue 4 h post injection (p.i.), which was significantly higher than the uptake of a non-EGFR-specific $^{111}$In-labeled-$Z_{taq}$ Affibody molecule. At this time point, the radioactivity concentration in the tumors exceeded the concentration in all other organs and tissues except for liver and kidneys. The radioactivity uptake in the liver, as well as in the spleen and colon, is most likely receptor mediated. Earlier studies (Tolmachev et al., 2004b) demonstrated that binding of a radiolabeled natural ligand, EGF, to these tissues is saturable. The accumulation of radioactivity in the kidneys is associated with the renal excretion and reabsorption of Affibody molecules, as discussed in Box 6.4 and paper III. Though renal excretion may complicate the imaging of targets in the vicinity of the kidneys, it is generally a positive factor, as it enables a rapid clearance of radioactivity from nonspecific compartments. This problem is also shared with many diagnostic radiometal-labeled peptides.

![Fig. 17. Biodistribution of $^{111}$In-labeled Affibody molecules 4 h p.i. in mice bearing A431 xenografts. Each data point represents an average from four animals±standard deviation and is expressed as the percentage of injected radioactivity per gram of organ or tissue.](image-url)
As has been discussed before, EGFR is distributed on various normal tissues and expressed to rather high levels especially in liver hepatocytes and skin epithelium (Gusterson et al., 1984; Damjanov et al., 1986). This can be a problem in directed cancer therapy with cytotoxic agents. For medical imaging, the imaging agent is normally applied in non-toxic quantities, but there can be a problem with poor contrast if the tumors or metastases are in the vicinity of e.g. the liver. EGFR targeting is, however, well studied and some solutions to this challenge have been found (Divgi et al., 1991; Cuartero-Plaza et al., 1996). It seems possible that at high protein doses (more than 40 mg of antibody or 1 mg of a natural ligand EGF), it is possible to obtain high contrast of EGFR-expressing tumors, possibly due to saturation of EGFR in normal tissues. Hence, a careful dose-assessment study would be required in the future for EGFR-targeting Affibody molecules to obtain an optimal imaging contrast. Another strategy to avoid normal tissue targeting is the targeting of the mutant EGFR, EGFRvIII, which has successfully been achieved with the mAb 806 (Scott et al., 2007) (see also Chapter 5.1.1).

In conclusion, a directed-evolution effort resulted in Affibody molecules with a 30-fold improvement in affinity, generating low nanomolar binders that target the human EGFR. The radiolabeled Affibody molecules had preserved binding specificity and showed successful accumulation of radioactivity in xenografted A431 tumors. These binders were considered promising candidates for future development of \textit{in vivo} imaging tracers.
7.5 Affibody molecules for EGFR targeting *in vivo*: aspects of dimerization and labeling chemistry (V)

In previous work (paper IV) a low nanomolar EGFR-targeting Affibody molecule, Z\textsubscript{EGFR:1907}, was generated (Friedman et al., 2008). This Affibody molecule showed specific targeting of EGFR *in vitro* and *in vivo* with an accumulation of approximately 6% of the injected radioactivity per gram of tumor tissue 4 h post injection (p.i.). This was considered a promising candidate for further development of an EGFR-targeting Affibody molecule for molecular imaging. However, further optimization was desired to primarily improve the imaging contrast. This contrast depends on many characteristics of the targeting agent, such as binding strength, properties of the radiolabel during cellular processing, tumor localization rate, clearance from normal tissues, and biological properties of the target. One important parameter is the level of retention of radioactivity at the malignant cells, when non-bound targeting agent is cleared from blood and healthy tissues. The cell retention depends on the dissociation rate of the tracer from the target and on cellular processing of tracer-target complex. Di- or multimerization of a targeting protein usually improves the dissociation rate (slower off-rate) and in a number of examples, an enhanced tumor localization of tracers outweighed the negative effect of an increased size (Wu et al., 1996; Adams et al., 1998b; Batra et al., 2002; Adams et al., 2006). The selection of a labeling nuclide is also important, since it influences the retention of radioactivity after internalization of conjugates in malignant cells (Orlova et al., 2000) and in normal tissues, e.g. excretory organs (Kenanova et al., 2007). In paper V, the aim was to find a suitable format and labeling of the anti-EGFR Affibody molecule Z\textsubscript{EGFR:1907} for radionuclide imaging of EGFR expression in malignant tumors. Thus, a monomeric and a dimeric form of the Affibody molecule, labeled with a residualizing radionuclide, \textsuperscript{111}In, and with a non-residualizing radionuclide, \textsuperscript{125}I, were compared *in vitro* and *in vivo*.

The monomer Z\textsubscript{EGFR:1907} (8.1 kDa) and the dimer (Z\textsubscript{EGFR:1907})\textsubscript{2} (14.6 kDa) were analyzed with real-time biospecific interaction analysis using a Biacore instrument to study the kinetics and determine the affinity constants. The K\textsubscript{D} for the monomer was approximately 5 nM. Dimerization into (Z\textsubscript{EGFR:1907})\textsubscript{2} resulted, as expected, in a substantial improvement of the dissociation rate (Fig. 18A). The equilibrium dissociation constant, K\textsubscript{D}, could, however, not be determined due to poor complete curve-fitting. Instead, different concentrations of fluorescently labeled monomer and dimer were incubated with A431 cells and the K\textsubscript{D}’s were estimated from equilibrium-binding curves of mean fluorescence intensities, 2.8±0.1 nM for
$Z_{\text{EGFR:1907}}$ (Fig. 18B) and $1.6 \pm 0.1$ nM for $(Z_{\text{EGFR:1907}})_2$ (Fig. 18C). The $K_D$ values for the monomer were thus concordant using the two methods for affinity determination. The $K_D$ for the dimer was, however, only two-fold better than the monomer. Thus, it seemed that we had two molecules with similar $K_D$ but different molecular weight and off-rate characteristics, factors that could have an impact on tumor localization and cell retention of a tracer and therefore influence the total tumor uptake.

**Fig. 18.** Binding and affinity analysis of $Z_{\text{EGFR:1907}}$ monomer and dimer using surface plasmon resonance in Biacore and flow cytometry. A. Biacore sensorgram obtained after injection of the $Z_{\text{EGFR:1907}}$ monomer (open triangles), and $Z_{\text{EGFR:1907}}$ dimer (open squares) over a sensor chip flow-cell surface containing amine-coupled EGFR-ECD. B. and C. Flow-cytometric analysis and affinity measurements of EGFR-binding Affibody molecules to EGFR on A431 cells. The Affibody molecules were labeled site-specifically using a C-terminal cysteine with Alexa Fluor 488. Equilibrium-binding curve for $Z_{\text{EGFR:1907}}$ monomer B and dimer C. Different concentrations, ranging from 0.1 to 100 nM, of fluorescently labeled $Z_{\text{EGFR:1907}}$ monomer and 0.05 to 50 nM of dimer were incubated with A431 cells in room temperature for 1 h and kept on ice until analysis. The experiment was performed in triplicate showing the mean values and with standard deviations indicated. The data was analyzed with GraphPad Prism 5, calculating the $K_D$ from an equilibrium binding curve using a non-linear regression, one-site specific model.
The increased retention of the dimer was also seen on living cells during comparative evaluation of $^{125}$I- and $^{111}$In-labeled monomer and dimer in vitro (Fig. 19). There were only relatively small differences between the radioiodinated and radiometal-labeled conjugates, indicating that the internalization rate of the Affibody molecule-EGFR complex is relatively slow both for monomer and dimer. The increased cellular retention in the case of the dimer showed that a slow off-rate was important to keep a high level of cell-associated radioactivity. Thus, the results of the in vitro studies were in favor of the use of the dimeric form. However, it was unclear whether the $^{111}$In or $^{125}$I labeling would provide the best contrast in vivo, since this is dependent on processing by healthy tissues. Hence, an in vivo study was performed.

**Fig. 19.** Cellular retention of radioactivity after interrupted incubation of EGFR-expressing A431 cells with EGFR-targeting Affibody molecules. Left panel: tracers labeled with $^{125}$I; Right panel: tracers labeled with $^{111}$In. Data are presented as average value from three Petri dishes ± standard deviation. Error bars might be not seen because they are smaller than point symbols.

The monomeric and dimeric form of $Z_{\text{EGFR:1907}}$ were labeled with radiometal $^{111}$In via benzyl-DTPA chelator and with indirect radioiodination of $^{125}$I, both labelings directed to primary amine groups on the Affibody molecule. The $^{125}$I- and $^{111}$In-labeled monomer and dimer were studied for their biodistribution in A431 tumor bearing BALB/c nu/nu mice. All conjugates, both EGFR-specific and the negative control Affibody molecules showed a rapid blood clearance (< 2% ID/g in blood 4 h p.i.), and predominantly renal excretion of the radioactivity with subsequent reabsorption, which was manifested in a high radioactivity concentration
in the kidneys. There were, however, significant differences in the biodistribution of the conjugates. The tumor uptake (4 h p.i.) of all EGFR-specific conjugates was significantly higher than the control non-EGFR specific Affibody molecules that had been labeled the same way. This was an evidence of in vivo specificity of tumor accumulation. The uptake of EGFR-specific conjugates was also significantly higher in organs and tissues with normal physiological expression of EGFR, such as liver, spleen and gastrointestinal tract, which demonstrated reactivity of the Affibody molecules with murine EGFR. There was significantly higher tumor accumulation of monomeric Z_{EGFR:1907} in comparison with dimeric (Z_{EGFR:1907})_{2} at 4 h p.i., for both labels. For this reason, tumor-to-organ ratios were generally higher for Z_{EGFR:1907} (Fig. 20). Still, a slow blood clearance of radioactivity resulted in moderate tumor-to-organ ratios even for monomeric Z_{EGFR:1907}. At 24 h p.i. the contrast was appreciably improved due to clearance from the blood and non-tumor compartments (Fig. 20). The effect of radiometal was more pronounced in vivo than in vitro, where the tumor-associated radioactivity decreased less for $^{111}$In-labeled monomer and dimer than for $^{125}$I-labeled monomer and dimer over time. In conclusion, the comparison of monomer and dimer labeled with $^{111}$In and $^{125}$I showed that in spite of a better tumor retention of radioactivity for the dimer, the better tumor localization of the smaller monomer together with good cellular retention of the radiometal label enabled $^{111}$In-labeled monomeric Z_{EGFR:1907} to provide the best tumor-to-organ ratios (Fig. 20). The tumor-to-blood ratio for $^{111}$In-Z_{EGFR:1907} was 31±8 at 24 h p.i. Better visualization of EGFR-expression in A431 by monomer than by dimer was also confirmed in a gamma-camera study (Fig. 21).

![Fig. 20. Tumor-to-organ ratios at 4 h (left panel) and 24 h (right panel) post injection of radiolabeled anti-EGFR Affibody molecules in Balb/c nu/nu mice bearing EGFR-expressing A431 xenografts. Data presented as an average for four animals and standard deviation.](image-url)
Fig. 21. Imaging of EGFR expression in A431 xenografts in Balb/c nude mice using $^{111}$In-labeled-$Z_{1907}$ and $^{111}$In-labeled-($Z_{1907})_2$. Planar gamma-camera images were collected 24 h after administration of tracers. Arrows point to tumors (T) and kidneys (K).

Earlier, both anti-EGFR monoclonal antibodies (Senekowitsch-Schmidtke et al., 1996; Morales-Morales et al., 1999; Reilly et al., 2000; Perk et al., 2005; Cai et al., 2007) and the natural ligand EGF (Senekowitsch-Schmidtke et al., 1996; Hnatowich et al., 1998; Orlova et al., 2000; Reilly et al., 2000; Wang et al., 2002; Babaei et al., 2005; Velikyan et al., 2005), and recently an anti-EGFR nanobody (Huang et al., 2008), have been proposed and evaluated as targeting agents for radionuclide imaging of EGFR expression. A general concern has been the normal expression of EGFR in healthy organs and tissues, such as liver, spleen and gastrointestinal tract, but EGFR expressing tumors were imaged successfully. Generally, the small (6 kDa) radiolabeled EGF provided better tumor-to-organ ratios (imaging contrast) in comparison with bulky monoclonal antibodies and enabled imaging after shorter period of time after injection. These data are consistent with other observations that smaller targeting agents, e.g. antibody fragments, provide better contrast due to more rapid extravasation and tumor penetration on one hand and more rapid blood clearance on the other. However, an agonistic action of EGF may be of concern. It would be interesting to compare imaging properties of the $^{111}$In-labeled-$Z_{EGFR:1907}$ Affibody molecule with other anti-EGFR tracers. For a true comparison, tracers should be studied simultaneously in gamma-camera. However, looking at the literature will at least give an indication on how the EGFR-targeting
Affibody molecule is performing compared to other anti-EGFR tracers. It is apparent from literature that $^{111}$In-labeled-Z$_{\text{EGFR:1907}}$ provide better contrast than any full-size antibody-based tracers. Tumor-to-blood ratio, which is often used as a measure of contrast, was below 3 at 24 h p.i. for any of the anti-EGFR antibodies described in papers that the author is aware of (Senekowitsch-Schmidtke et al., 1996; Reilly et al., 2000; Perk et al., 2005; Cai et al., 2007). Tumor-to-blood ratio was also higher for $^{111}$In-labeled-Z$_{\text{EGFR:1907}}$ (T:B = 31) than for EGF labeled with $^{111}$In (T:B = 2.6) (Reilly et al., 2000) or $^{123}$I (T:B = 23) (Wang et al., 2002). For comparison, an EGFR-specific nanobody labeled with $^{99m}$Tc showed a tumor-to-background ratio of 7.4 at 3 h p.i. (Huang et al., 2008).

As has been discussed earlier (paper III and IV), a possible limitation of $^{111}$In-labeled-Z$_{\text{EGFR:1907}}$ is the high accumulation of diagnostic radioactivity in the kidneys and in tissues with a normal high EGFR expression, e.g. the liver. This problem is, however, shared with other anti-EGFR tracers. The EGFR-targeting Affibody molecule could, nevertheless, be considered as a potential tracer for visualizing, for example, primary tumors and metastases of the lungs, since that is quite a large area not close to the liver or kidneys.

In conclusion, the $^{111}$In-labeled monomeric form of Z$_{\text{EGFR:1907}}$ appears to be the most promising EGFR-binding Affibody molecule variant for imaging. It shows promises for clinical applications, mainly in visualizing tumor and metastases that are not in close vicinity to the kidneys or the liver.
8. CONCLUSIONS AND FUTURE PERSPECTIVES

The studies presented in this thesis have been focused on the development of an EGFR-binding Affibody molecule with specific in vivo tumor targeting properties and with the potential development as an imaging tracer of EGFR-expressing tumors. In paper I, moderate high affinity EGFR-binding Affibody molecules were isolated with phage display technology. They were found to bind specifically to EGFR, both immobilized in biosensor studies and on EGFR-expressing cells. Further cellular assays, in paper II and III, showed that the EGFR-binding Affibody molecules could be labeled with a radiohalogen or a radiometal with preserved specific binding to EGFR-expressing cells. In vitro, the Affibody molecule demonstrated a high uptake and good retention to EGFR-expressing cells and was found to be internalized (paper II). Furthermore, successful targeting of EGFR-expressing tumors in tumor-bearing mice was seen with gamma-camera imaging and biodistribution studies gave a tumor-to-blood ratio of 9.1 at 4 h post injection (p.i.), which was considered promising (paper III). In order to further improve the tumor imaging contrast, a directed evolution effort to increase the affinity was performed in paper IV. The affinity was improved approximately 30-fold and the Affibody molecules showed successful targeting of the A431 tumors with 4-6% of the injected radioactivity per gram accumulated in tumor tissue at 4 h p.i. Finally, in paper V, tumor targeting and biodistribution in tumor-bearing mice were studied for monomeric and dimeric constructs that were radiolabeled either with the radiohalogen $^{125}$I or with the radiometal $^{111}$In. The radiometal-labeled monomeric Affibody construct, $^{111}$In-labeled-$Z_{\text{EGFR}:1907}$, was found to provide the best tumor-to-organ ratio, due to high tumor localization and good tumor retention. The tumor-to-blood ratio was 31±8 at 24 h p.i. and the tumor was clearly visualized by gamma-camera imaging. Altogether, based on the presented results the $Z_{\text{EGFR}:1907}$ Affibody molecule should be considered a promising candidate for further development of a tumor imaging tracer for EGFR-expressing tumors.

Inherent challenges exist in the development of EGFR-based targeting agents due to EGFR expression in normal tissues, such as liver, skin and the
gastrointestinal tract. Strategies to overcome this include the targeting of mutant forms of EGFR, common in certain cancers (discussed in Chapter 5.1.1) (Johns et al., 2002; Jungbluth et al., 2003; Scott et al., 2007).

A possible way to improve specificity and thereby possibly enhance the imaging contrast and therapeutic efficacy is by simultaneous targeting of two different receptors. By using a bispecific targeting agent, the targeting of normal tissues that only express one receptor, or low levels of both receptors, would be much smaller than the targeting of tumor cells. Other groups have developed antibody-based molecules for simultaneous targeting of two cancer-associated targets, such as EGFR and insulin growth factor receptor (IGFR) (Lu et al., 2005) or carcinoembryonic antigen (CEA) and HER2 (Dorvillius et al., 2002). There has been little evidence so far that bispecific antibody-based targeting agents can be employed to enhance the tumor targeting specificity. However, recently a bispecific scFv against HER2 and HER3 has showed to enhance target selectivity and mediate tumor cell growth inhibition (personal communication with Gregory Adams, Fox Chase Cancer Center, USA). Several studies of cancer patients suggest that simultaneous targeting of the EGFR and HER2 could be valuable. Variable expression levels but rather frequent expression of both HER2 and EGFR has been reported in a number of studies in disseminated bladder (Wester et al., 2002; Memon et al., 2006), colorectal (Kluftinger et al., 1992; Porebska et al., 2000) and prostate cancer (Hernes et al., 2004; Bartlett et al., 2005). The prognostic value of the co-expression of EGFR and HER2 in breast cancer patients has also been studied and demonstrated a worse prognosis for the co-expression than for expression of either EGFR or HER2 (Osaki et al., 1992; Tsutsui et al., 2003). These data suggest that simultaneous targeting of EGFR and HER2 could improve the therapeutic efficiency. Recently, a bispecific-Affibody molecule targeting HER2 and EGFR has been constructed by head-to-tail fusion on gene level of an HER2- and an EGFR-binding Affibody molecule. A flexible 20 amino acid linker was introduced between the Affibody molecules. The bispecific Affibody molecule has shown successful simultaneous binding to HER2 and EGFR in a sandwich-type assay format using real time biospecific interaction analysis in Biacore. Furthermore, fluorophore labeled bispecific-Affibody molecule showed efficient binding to HER2-expressing BT474 cells and EGFR-expressing A431 cells, respectively, in flow-cytometric analysis. When blocking SKOV3 cells (expressing both HER2 and EGFR) with non-labeled HER2- or EGFR-specific Affibody molecules, specific binding of both moieties of the bispecific Affibody was demonstrated. It will be interesting to further study this bispecific Affibody molecule and see whether simultaneous targeting of
HER2 and EGFR can enhance the selective targeting and possibly decrease the cell growth of HER2- and EGFR-expressing cells.

In the work presented in this thesis, the targeting of EGFR has been directed to the extracellular domain on the outside of the cell. The targeting of EGFR-ECD with antibodies, such as cetuximab and panitumumab, prevent ligand-binding and will subsequently affect the downstream signaling. An alternative strategy to the interference with ligand-dependent receptor activation on the outside of the cell is by targeting and retaining the receptor inside the cell, e.g. in the endoplasmatic reticulum (ER). By doing this, the receptor expression on the cell surface can be reduced and the intracellular signaling affected. In a study by the group of Nancy Hynes, HER2-ECD-binding scFvs including an ER tag have shown the retention of HER2 in the ER and inactivation of cell growth (Beerli et al., 1994). The Affibody molecule would be a suitable protein scaffold for this kind of application, because of its small size, stable structure and lack of cysteines in the scaffold, which would allow functional binding intracellularly in mammalian cell expression (Magnusson et al., 2007). Since signaling can be mediated via different homo- and heterodimers (Hynes and Lane, 2005; Citri and Yarden, 2006) and coss-talk with other receptors is also known to take place (Yarden and Sliwkowski, 2001; Hynes and Lane, 2005), dual or multiple targeting and ER retention would also be interesting to investigate.

Tumor targeting and tumor retention times could possibly also be improved by targeting of two non-overlapping epitopes on the same receptor. This could be done with a biparatoptic Affibody molecule, comprising two different Affibody molecules separated with a flexible linker long enough to target two distinct epitopes on the same receptor. Improved tumor uptake has been reported for a biparatoptic Fab targeting two epitopes on CEA (Robert et al., 1999).

Having a good targeting agent, a natural question is whether it can be used for therapy. In systemic therapeutic applications longer half-lives in vivo is usually desired. In a recent paper, Tolmachev and co-workers have shown that the fast clearance and high renal accumulation of radiometal in Affibody targeting can be overcome by the genetic fusion of an albumin binding domain (ABD) in order to achieve a reversible interaction with human serum albumin (HSA) and prolong the circulation (Tolmachev et al., 2007b). The in vivo results demonstrated a prolonged half-life, a 25-fold decrease in kidney uptake and a high and specific uptake of the conjugate in HER2-expressing tumor, as well as complete prevention of tumor formation (Tolmachev et al., 2007b). The high expression of EGFR on some normal organs, like liver or skin, will however likely hamper systemic treatment. There is, however, a possible application for the EGFR-targeting Affibody molecules in locoregional treatment of glioma patients after surgery. Patients with glioblastomas
are often treated with surgery to remove the bulky part of the tumor. The area around the tumor cavity is then irradiated with external beam radiotherapy. Intracavitary radionuclide therapy has been proposed to be a promising modality for postoperative treatment of glioblastomas, reviewed by Carlsson and coworkers (Carlsson et al., 2006). The small EGFR-specific Affibody molecule could potentially be a good tracer for reaching migrating glioma cells outside the operation cavity and eradicate them. This would circumvent the problem of affecting normal tissues with high EGFR expression since intracavitary administration can be used. Another possible local treatment modality is the intravesical treatment of urinary bladder cancer (Bue et al., 2000).

Gene therapy has been extensively investigated as a potential treatment strategy for cancer (Edelstein et al., 2007; Flotte, 2007). It includes different approaches for killing the tumor cells, such as (i) stimulating the natural immunity to cancer cells with a cancer vaccine, (ii) kill and interfere with cancer cell growth by delivery of “suicide genes” that encode enzymes capable of converting pro-drugs into cytotoxic drugs, and (iii) replacement of abnormal tumor suppressor cell activity, e.g. by transfer of p53 (Edelstein et al., 2007). Although cancer is the major therapeutic indication for many nucleic acid-based therapeutics being explored, so far only one nucleic acid-based therapeutic has been approved for treatment of cancer. An engineered adenovirus delivering human wild-type p53 tumor suppressor gene for treatment of head and neck squamous cell carcinoma was approved in China in 2003 (Walsh, 2006). One important issue in cancer gene therapy is to enhance the specificity of the virus vector. This can be done by de-targeting of the virus vector’s natural receptors and re-targeting to cancer specific receptors. Recently, studies were published where successful re-targeting of adenoviruses to the HER2 receptor was performed with a HER2-binding Affibody molecule incorporated into the virus fiber (Magnusson et al., 2007; Belousova et al., 2008). This could in principle also be achieved for EGFR-specific Affibody molecules targeting an adenovirus to EGFR-expressing cells. Once at the tumor site the adenovirus could be delivering transgenes, such as prodrug activating enzymes, apoptosis inducers and toxins. The adenovirus could possibly also be delivering the gene encoding the EGFR-specific Affibody molecules for ER retention (as discussed above). For therapeutic applications, one needs to pay special attention to toxicity in organs expressing relatively high amounts of EGFR, like the liver, as has been discussed above.

For in vivo applications of the EGFR-binding Affibody tracer, like molecular imaging and possibly therapy, it is important to study the effects on downstream cellular signaling. Several possible effects of Affibody binding remains to be studied, such as receptor phosphorylation, phosphorylation of MAPK kinases (e.g.
Erk1/2 associated with proliferation), phosphorylation of PI3-kinases (e.g. Akt associated with apoptosis), growth proliferation, and the possibility to sensitize tumor cells for radiation (Ekerljung et al., 2006). Furthermore, it would be interesting to select Affibody molecules for other epitopes on EGFR with possibly higher affinity than the existing binder or that would affect the downstream cellular signaling differently.

The Z_{EGFR:1907} Affibody molecule, described in this thesis, has been a precursor to a modified Affibody molecule that is now in preclinical development at the biotechnology company Affibody AB. Therefore, to conclude, I hope that the initial studies presented here should contribute to the development of Affibody-based molecular imaging of EGFR-expressing tumors and metastases to facilitate patient management in the near future.
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