Rational and combinatorial protein engineering for vaccine delivery and drug targeting

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Cover: Gamma camera image showing mouse carrying SKOV-3 xenografted tumor, injected with radiolabeled His$_5$-(Z$_{HER2/neu}$)$_2$ affibody into its tail vein (paper VI).
Abstract

This thesis describes recombinant proteins that have been generated by rational and combinatorial protein engineering strategies for use in subunit vaccine delivery and tumor targeting.

In a first series of studies, recombinant methods for incorporating immunogens into an adjuvant formulation, e.g. immunostimulating complexes (iscoms), were evaluated. Protein immunogens, which are not typically immunogenic in themselves, are normally administered with an adjuvant to improve their immunogenicity. To accomplish iscom incorporation of a *Toxoplasma gondii* surface antigen through hydrophobic interaction, lipids were added either *in vivo* via *E. coli* expression, or *in vitro* via interaction of an introduced hexahistidyl (His₆) peptide and a chelating lipid. The possibility of exploiting the strong interaction between biotin and streptavidin was also explored, in order to couple a *Neospora caninum* surface antigen to iscom matrix, i.e. iscom particles without any antigen. Subsequent analyses confirmed that the immunogens were successfully incorporated into iscoms by the investigated strategies. In addition, immunization of mice with the recombinant Neospora antigen NcSRS2, associated with iscoms through the biotin-streptavidin interaction, induced specific antibodies to native NcSRS2 and reduced clinical symptoms following challenge infection. The systems described in this thesis might offer convenient and efficient methods for incorporating recombinant immunogens into adjuvant formulations that might be considered for the generation of future recombinant subunit vaccines.

In a second series of studies, Affibody® (affibody) ligands directed to the extracellular domain of human epidermal growth factor receptor 2 (HER2/neu), which is known to be overexpressed in ~ 20-30% of breast cancers, were isolated by phage display *in vitro* selection from a combinatorial protein library based on the 58 amino acid residue staphylococcal protein A-derived Z domain. Biosensor analyses demonstrated that one of the variants from the phage selection, denoted His₆-*Z*₂₆HER2/neu, selectively bound with nanomolar affinity (Kₐ ≈ 50 nM) to the extracellular domain of HER2/neu (HER2-ECD) at a different site than the monoclonal antibody trastuzumab. In order to exploit avidity effects, a bivalent affibody ligand was constructed by head-to-tail dimerization, resulting in a 15.6 kDa affibody ligand, termed His₆-*Z*(HER2/neu)₂, that was shown to have an improved apparent affinity to HER2-ECD (Kₐ ≈ 3 nM) compared to the monovalent affibody. Moreover, radiolabeled monovalent and bivalent affibody ligands showed specific binding *in vitro* to native HER2/neu molecules expressed in human cancer cells. Biodistribution studies in mice carrying SKOV-3 xenografted tumors revealed that significant amounts of radioactivity were specifically targeted to the tumors *in vivo*, and the tumors could easily be visualized with a gamma camera. These results suggest that affibody ligands would be interesting candidates for specific tumor targeting in clinical applications, such as *in vivo* imaging and radiotherapy.

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**Keywords:** affibody, affinity chromatography, biotin, combinatorial, delivery, phage display, HER2, ligand, lipid tagging, *in vivo* imaging, immunogen, iscom, NcSRS2, *Neospora caninum*, scaffold, streptavidin, radionuclide, recombinant protein, selection, subunit vaccine, targeting, *Toxoplasma gondii*, tumor

Till M&L
List of publications

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INTRODUCTION

1. Proteins

Proteins are responsible for an enormous range of essential cellular functions. Apart from being components of structural features of various cell types, e.g. muscle cells, they are frequently involved in various biomolecular interactions. For example, many proteins participate in the diverse signaling systems that regulate cellular growth and differentiation. Proteins are also involved in the transportation and storage of diverse substances within cells. Furthermore, our immune system comprises a variety of proteins that are responsible for the recognition and destruction of foreign substances, thereby protecting us from harmful pathogens. The most central proteins in this context are the highly selective antibodies. Further examples include the enzymatic proteins that contribute in the metabolic process of food digestion. Hence, proteins are extremely diverse biomolecules, of which enzymes, hormones, and antibodies are a few examples.

All the information required for the production of proteins is stored in the genetic material, called deoxyribonucleic acid (DNA). In humans, 46 chromosomes constitute the genome, of which one copy is present in each cell. Depending on the type of cell, different proteins are expressed from the genetic code, which is composed of four different nucleotides (organic bases), adenine (A), guanine (G), cytosine (C), and thymine (T). The nucleotides are organized into specific sequences, genes, which carry the information necessary for the construction of proteins. The diverse collection of human proteins consists of combinations of only 20 different amino acids, linked together by peptide bonds. A set of three nucleotides codes for one amino acid, and the synthesized polypeptide sequence
constitutes the primary structure of a protein. The linear polypeptide is locally arranged to form the secondary structure, which is subsequently folded into the tertiary structure. Some proteins comprise multiple polypeptide chains that interact to create a quaternary structure. More simplistically, the DNA provides the instructions for protein production, whereas the proteins are the molecules that perform most of the work in a cell.

Over the years, natural proteins have been employed for a broad range of applications. For example, many proteins are efficient agents for medical purposes, and various enzymes have important roles in the biotechnology and food industries. However, purification from natural sources has often been associated with high cost, limitation of available quantities, and a risk of hazardous contamination. A breakthrough in biotechnology research came in the 1970s, when the development of recombinant DNA technology made it possible to design and produce proteins in a well-defined manner (Glick and Pasternak, 1998). This approach involves the use of specific enzymes, i.e. restriction enzymes and ligases, for the precise cutting and ligation of DNA fragments. By this means, a gene coding for a certain protein can be inserted into a selected host organism that is non-pathogenic, safe, and can subsequently synthesize the protein of interest. Thus, recombinant technology enables relatively cheap large-scale production of safe proteins. Accordingly, most of the proteins generated by the pharmaceutical and biotechnology industries are today produced by recombinant DNA technology.

Foreign proteins can be expressed in a number of different host cells (Glick and Pasternak, 1998), of which the bacterium *Escherichia coli* is probably the most commonly used. The vast amount of research that has been carried out on its structure and function underlie the extensive use of *E. coli* as a host, which has several major advantages as a research organism since it is inexpensive, convenient to handle, and grows rapidly (with a minimum generation time of about 20 minutes), allowing it to be simply cultivated. Nevertheless, other organisms, prokaryotic as well as eukaryotic, are also employed as hosts. The yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* represent simple eukaryotic hosts that are easy to proliferate in simple culture media. In addition, certain insect, plant and mammalian cells are useful hosts since they can be grown in culture, even though more complex procedures are required. Proteins that originate from eukaryotic organisms, for example humans, sometimes require post-translational modifications, for example glycosylation, in order to be functional. The main advantage associated with the use of eukaryotic host cells is thus their biosynthetic ability to perform these adjustments, allowing the production of correctly modified proteins, an operation that cannot easily be implemented in prokaryotic cells.

Recombinant DNA technology provides a variety of tools that can be applied to design and generate proteins with desired characteristics. Rational gene fusion strategies are frequently used to furnish the recombinant protein with properties that
may facilitate their production, increase their solubility, enhance their proteolytic stability, or simplify their recovery. For example, a signal sequence can be fused N-terminally of the protein to enable secretion to the *E. coli* periplasm. The oxidative environment of the periplasm is particularly appropriate when producing proteins that contain disulphide bridges, whose formation is crucial for the correct folding of certain proteins. Furthermore, the recombinant protein can be ‘tagged’ by an affinity protein to allow efficient affinity purification of the target fusion protein (Terpe, 2003). In addition, combinatorial protein engineering strategies can be used to construct diverse libraries, followed by the selection of proteins with novel characteristics, e.g. enzymes or various binding proteins (Nygren and Skerra, 2004).

Recombinant proteins, in addition to various techniques used to alter their characteristics, were central to the work described in this thesis. Approaches for the development of recombinant proteins as tools to generate straightforward delivery systems for subunit vaccines (I, II, III), and as devices in specific drug targeting (IV, V, VI) were explored.
2. Vaccines

In 1798, Edward Jenner developed the first human vaccine, with which he successfully prevented smallpox infection in milkmaids. Over the years, numerous vaccines against a number of different diseases have been developed, and today vaccination is considered by the World Health Organization (WHO) to be the most cost-effective strategy for controlling infectious diseases (O’Hagan and Valiante, 2003). Smallpox and poliomyelitis are examples of diseases that have been totally eradicated throughout the world by means of vaccination. In addition, the prevalence of diphtheria, measles, mumps, pertussis (whooping cough), rubella (German measles), and tetanus has declined dramatically as vaccination has become more frequent (Goldsby et al., 2000). Nevertheless, there are still no effective vaccines against a number of insidious pathogens, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and malaria protozoa. These pathogens often establish chronic infections and the induction of cell-mediated immunity is probably required to combat them (O’Hagan et al., 2001).

2.1. Different types of vaccines

The aim of vaccination is to develop a protective immune response in the host against a certain disease by inducing an immunological memory, generally involving both the humoral and cell-mediated branches of the immune system. Traditionally, vaccines have been based on whole-cell organisms, such as live, attenuated or killed, inactivated bacteria or viruses. Live, attenuated vaccines are normally relatively effective in developing both humoral and cellular immunity, since cell-mediated responses can be induced due to their ability to replicate in the host. However, they can potentially revert to a virulent form and subsequently cause
disease. Killed, inactivated vaccines have the advantage of being safer than live, attenuated vaccines, but on the other hand they are less effective and can typically only produce humoral immunity (Goldsby et al., 2000). In addition, the whole-cell vaccines contain undefined substances, which have the potential to make them highly immunogenic, but can also give rise to several adverse effects. The typical characteristics of the different types of vaccines are summarized in Table 1.

Table 1. Different types of vaccines and some of their characteristics.

<table>
<thead>
<tr>
<th>Vaccine type</th>
<th>Characteristics</th>
<th>Selected examples of prevented diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole-cell vaccines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live, attenuated</td>
<td>+ Cellular and humoral immunity</td>
<td>Tuberculosis, typhoid (bacterial)</td>
</tr>
<tr>
<td>bacteria or viruses</td>
<td>– Risk of reversion to a virulent strain</td>
<td>Measles, mumps, polio, rubella, chickenpox, yellow fever (viral)</td>
</tr>
<tr>
<td></td>
<td>– Undefined composition</td>
<td></td>
</tr>
<tr>
<td>Killed, inactivated</td>
<td>+ No risk of infection</td>
<td>Anthrax, cholera, pertussis, plague (bacterial)</td>
</tr>
<tr>
<td>bacteria or viruses</td>
<td>– Less effective than live vaccines</td>
<td>Hepatitis A, influenza, polio, rabies (viral)</td>
</tr>
<tr>
<td></td>
<td>– Undefined composition</td>
<td></td>
</tr>
<tr>
<td><strong>Subunit vaccines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purified components from</td>
<td>+ Well-defined composition</td>
<td>Diphtheria, tetanus, pertussis (inactivated exotoxin)</td>
</tr>
<tr>
<td>pathogens</td>
<td>– Cultivation of pathogens required</td>
<td>Influenza, meningitis, pneumonia (bacterial polysaccharides)</td>
</tr>
<tr>
<td></td>
<td>– Poor immunogens, need of adjuvant</td>
<td></td>
</tr>
<tr>
<td>Synthetic peptides</td>
<td>+ Well-defined composition</td>
<td>No vaccine licensed</td>
</tr>
<tr>
<td></td>
<td>+ No risk of pathogenicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Poor immunogens, need of adjuvant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Short half-life</td>
<td></td>
</tr>
<tr>
<td>Recombinant proteins</td>
<td>+ Well-defined composition</td>
<td>Hepatitis B (see also Appendix I)</td>
</tr>
<tr>
<td></td>
<td>+ No risk of pathogenicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Cost-efficient production</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Poor immunogens, need of adjuvant</td>
<td></td>
</tr>
<tr>
<td>Nucleic acids: DNA</td>
<td>+ Cellular and humoral immunity</td>
<td>No vaccine licensed</td>
</tr>
<tr>
<td></td>
<td>+ Cost-efficient production</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Inefficient transfection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Risk of integration into host genome</td>
<td></td>
</tr>
<tr>
<td>Nucleic acids: RNA</td>
<td>+ No risk of integration into host genome</td>
<td>No vaccine licensed</td>
</tr>
<tr>
<td></td>
<td>+ Do not need to enter nucleus for translation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Unstable</td>
<td></td>
</tr>
<tr>
<td>Live vectors</td>
<td>+ Cellular and humoral immunity</td>
<td>No vaccine licensed</td>
</tr>
<tr>
<td>(bacterial, viral)</td>
<td>– Risk of reversion to virulent form</td>
<td></td>
</tr>
</tbody>
</table>
Although the whole-cell vaccines are associated with numerous side effects, they are still the dominant products on the market. However, in recent years research has been concentrated on the development of a new class of vaccines, i.e. subunit vaccines. Using defined subunits, often surface molecules of the pathogen, can obviously increase the safety of the vaccine and eliminate the risk of infection. In contrast to traditional whole-cell products, which contain all of the compounds necessary for activating an integrated immune response, subunit antigens are often poorly immunogenic and need to be co-administered with an adjuvant to enhance the antigen-specific immune response. Subunit molecules such as bacterial polysaccharides, detoxified toxins, and viral surface proteins, can be purified directly from cultures of the pathogen, but this appears to be associated with a certain risk. Instead, recombinant production procedures are currently some of the most common strategies for generating safe, subunit vaccine candidates, for example protein immunogens or nucleic acid molecules encoding immunogens. Since the first recombinant vaccine (against hepatitis B) was approved in 1986, several recombinant products have been licensed (Walsh, 2003; Appendix I), and numerous candidates are presently being evaluated in clinical trials.

2.2. Adjuvants

In modern vaccine research, the development of new or improved adjuvants has become increasingly important. Vaccine adjuvants are functionally defined as diverse components that are able to augment the antigen-specific immune response when administered together with a vaccine antigen. In other words, adjuvants activate the non-specific innate immune response, while the antigen enhances the antigen-specific adaptive immune response. The immune response to vaccine antigens can be improved in a number of ways, for example adjuvants can: (i) enhance the immunogenicity of weak antigens, (ii) increase the speed and duration of the immune response, (iii) alter the antibody isotype or subclass distribution, (iv) stimulate cell-mediated immunity, and/or (v) decrease the dose of antigen required in the vaccine, thereby reducing their cost and, potentially, side effects (O’Hagan et al, 2001).

2.2.1. Different types of adjuvants

Today, adjuvants can be divided into two main categories; delivery systems and immune potentiators, depending on their mode of action (O’Hagan and Valiante, 2003; Table 2). Delivery systems function by localizing antigens and/or immune potentiators and targeting them to antigen-presenting cells (APCs), whereas immune potentiators directly activate these and other immune cells through specific receptors and/or pathways (O’Hagan and Valiante, 2003). Immune potentiators can be favorably combined with delivery systems to obtain improved adjuvant systems.

Vaccine delivery systems are typically particulate adjuvants that have comparable dimensions to pathogens that the immune system normally encounters,
and are therefore normally efficiently taken up by APCs (O’Hagan and Valiante, 2003). A first type of delivery system, the aluminum-based mineral salts Al(OH)$_3$ and AlPO$_4$, are the only adjuvants that are used widely with human vaccines. They have excellent safety and adjuvanticity records, but the disadvantages of being ineffective for some antigens and unable to induce cell-mediated immune responses (Gupta et al., 1998). A second group of delivery systems consists of a variety of lipid particles. Various emulsion adjuvants have been developed, ranging from the extremely potent, but toxic, Freund’s adjuvant (a water-in-oil adjuvant which may contain killed mycobacteria) (Chang et al., 1998) to the safe and highly tolerated oil-in-water emulsion MF59 (Ott et al., 1995). The latter was first licensed as an influenza vaccine for elderly humans in Italy in 1997 (De Donato et al., 1999). Liposomes are spherical phospholipid bilayers of 50-1000 nm (Gregoriadis et al., 1999; Langner and Kral, 1999) that are often used in combination with immune potentiators, for example monophosphoryl lipid A (MPL) (O’Hagan et al., 2001). In contrast, the so-called virosomes are liposomal adjuvants based on viral membrane proteins without additional immunostimulators (Daemen et al., 2005). These have been widely investigated in clinical trials and are now approved as vaccines against hepatitis A and influenza (Ambrosch et al., 1997). Immunostimulating complexes (iscoms), will be described in detail later in this thesis (section 2.3). Polymeric microparticles comprise a third group of delivery systems, amongst which the main candidates for adjuvant development are the biodegradable and biocompatible polylactide-co-glycolides (PLGs) (O’Hagan et al., 1998). These systems can release entrapped antigens in a controlled fashion, and thus have potential for the development of single-dose vaccines. Furthermore, as a safer alternative to live viruses, virus-like particles (VLPs) have been evaluated in subunit vaccine formulations against rotavirus-induced diarrhea (Coste et al., 2000).

Immune potentiators typically interact with immune cells through specific receptors. Recently, many immunostimulatory adjuvants have been shown to activate innate immunity by reacting with specific toll-like receptors (TLRs) (O’Hagan and Valiante, 2003). Cytokines, for example interleukin 1 (IL-1), IL-2, interferon gamma (IFN-γ), IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF), are small regulatory proteins that can directly modify and redirect the immune response (Heath, 1995). Unfortunately, however, they tend to be toxic and costly (O’Hagan et al., 2001). Other immune potentiators can instead induce cytokine production through receptor interactions. Saponins are a heterogenous group of sterol or triterpene glycosides that are commonly found in plants (Barr et al., 1998). The bark of the Chilean soap bark tree *Quillaia saponaria* Molina has frequently been used as a source of triterpenoid saponins for adjuvant purposes. Saponin is also the primary constituent of iscoms (described in section 2.3), and its incorporation into iscoms has been suggested to diminish their toxicity (Barr et al., 1998). A third group of immunostimulatory adjuvants are the muramyl dipeptide (MDP) derivatives, which are known to stimulate production of a number
of different cytokines (Namba et al., 1997). Bacterial CpG oligonucleotides comprise a fourth group of immune potentiators (Klinman et al., 1999). In vertebrates, these sequences are under-represented and, in contrast to bacterial CpGs, they are methylated (Krieg et al., 1995). Unmethylated bacterial CpG motifs can be formulated either as plasmids or as synthetic oligonucleotides, and can be used in combination with both protein and DNA vaccines (Klinman et al., 1999). Finally, monophosphoryl lipid A (MPL), isolated and structurally derivatized from lipopolysaccharide (LPS) of the Gram-negative bacteria Salmonella minnesota, has been demonstrated to be a safe and efficient vaccine adjuvant (Baldridge and Crane, 1999).

Table 2. Selected examples of vaccine adjuvants that have been evaluated in clinical trials (modified from O’Hagan et al, 2001).

<table>
<thead>
<tr>
<th>Type of adjuvant</th>
<th>Examples</th>
<th>Illustrative reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Delivery systems</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineral salts</td>
<td>Aluminum hydroxide*</td>
<td>Gupta, 1998</td>
</tr>
<tr>
<td></td>
<td>Aluminum phosphate*</td>
<td>Gupta, 1998</td>
</tr>
<tr>
<td>Lipid particles</td>
<td>Emulsions, e.g. Freund’s, Syntex adjuvant formulation (SAF), MF59*</td>
<td>Chang et al., 1998; Hjorth et al., 1997; De Donato et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Liposomes</td>
<td>Gregoriadis et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Virosomes*</td>
<td>Daemen et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Immunostimulating complexes (iscoms)</td>
<td>Barr et al., 1998</td>
</tr>
<tr>
<td>Microparticulate adjuvants</td>
<td>PLG microparticles</td>
<td>O’Hagan et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Virus-like particles (VLP)</td>
<td>Coste et al., 2000</td>
</tr>
<tr>
<td>Mucosal adjuvants</td>
<td>Heat-labile enterotoxin (LT)</td>
<td>de Haan et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Cholera toxin (CT)</td>
<td>Matsuo et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Mutant toxins (e.g. LTK63, LTR72)</td>
<td>Pizza et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Microparticles</td>
<td>O’Hagan, 1998</td>
</tr>
<tr>
<td><strong>Immune potentiators</strong></td>
<td>Cytokines, e.g. IL-2, IL-12</td>
<td>Baca-Estrada et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Saponins, e.g. QS21</td>
<td>Barr et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Muramyl dipeptide (MDP) derivatives</td>
<td>Namba et al., 1997</td>
</tr>
<tr>
<td></td>
<td>CpG oligonucleotides</td>
<td>Klinman et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Monophosphoryl lipid A (MPL) and synthetic derivatives</td>
<td>Baldridge and Crane, 1999</td>
</tr>
</tbody>
</table>

* Adjuvants that are included in currently approved vaccine products for humans.

2.2.2. Mucosal immunization

Traditionally, most vaccines have been administered by intramuscular or subcutaneous injection. Nevertheless, there are several important advantages of using a mucosal, such as oral or intranasal, route of vaccination (Holmgren et al., 2003; O’Hagan and Valiante, 2003). Firstly, mucosal immunization induces local immunity at the sites where a majority of infections initially establish. Secondly,
mucosal vaccines are more easily administered, which may be particularly beneficial in developing countries where access to health care professionals is limited. Thirdly, mucosal vaccination reduces the risk of transmitting infections due to the re-use of needles. Finally, needle-free vaccination is likely to be more acceptable among the population, since it will better suit people with needle phobia and cause no sore arms etc. (Holmgren et al., 2003; O’Hagan and Valiante, 2003).

Despite the numerous advantages related to mucosal administration, there are still no mucosal subunit vaccines on the market. Immunization through the mucosal route has appeared to be problematic, particularly when using the oral route of administration, because proteins are easily degraded and damaged during passage through the acidic gut (O’Hagan and Valiante, 2003). However, many potent adjuvants have been developed, for example heat-labile enterotoxin (LT) (de Haan et al., 2001) and cholera toxin (CT) (Matsuo et al., 2000), which are secreted from *E. coli* and *Vibrio cholera*, respectively. These native toxic molecules have been modified to obtain mutants, such as LTK63 and LTR72 (Pizza et al., 2000), with retained adjuvant activity but diminished toxicity. In addition, the possibility of administering many other adjuvants, for instance PLG microparticles, VLPs, Cpgs, and iscoms, mucosally is under investigation.

### 2.2.3. Mechanisms of action

Ramon first described immunological adjuvants in 1924 (Ramon, 1924). However, despite extensive research and the evaluation of a large number of candidates since then, the mechanisms of action of adjuvants remain poorly understood, and the only adjuvants currently approved for human use are aluminum-based mineral salts (generically called alum) (Gupta, 1998; Kenney and Edelman, 2003), the microemulsion MF59 (Ott et al., 1995; De Donato et al., 1999), licensed in North America and Europe, respectively, and virosomes (Sesardic and Dobbelaer, 2004; Daemen et al., 2005).

During the past ten years, however, there has been a breakthrough in understanding the role of the innate immune system. The non-specific innate immune system acts as a first line of defense by detecting diverse pathogen-associated molecular patterns (PAMPs) that are highly conserved in pathogens, but absent in their multicellular host organisms (Medzhitov et al., 1998; Takeda et al., 2003). This early recognition is mediated by broad and conserved families of pattern-recognizing receptors (PRRs), among which the ten recently discovered TLRs are currently the most intensively investigated (O’Hagan and Valiante, 2003). In contrast, the adaptive immune system responds later, and provides high antigenic specificity, which is required for complete elimination of the pathogen, as well as generation of the immunological memory required for long-lived protective immunity. Thus, the innate immune response detains the pathogen by recruiting pro-inflammatory molecules etc., whilst the adaptive immune response matures towards cellular and humoral protection. Although the function of most adjuvants is still incompletely understood, knowledge about how activation of the innate immune system initiates, amplifies, and drives the antigen-specific immune response...
response will hopefully lead to an increased rate of development of improved, safe and effective adjuvants. It should be emphasized that adjuvants cannot be approved as separate products, because specific adjuvant-antigen combinations have unique effects. Therefore, each adjuvant must be evaluated together with every specific antigen with which it may be used in order to be licensed as combined products. Given that each adjuvant is associated with a certain type of immune response, the choice of adjuvant depends on the type of immune response that is needed for protective immunity. In summary, there is an urgent need for adjuvants that are both safe and potent, in order to develop novel, effective subunit vaccines.

2.3. Iscoms

Iscoms are typically rigid, spherical, cage-like particles of about 40 nm, consisting of saponin, cholesterol, phospholipid, and protein antigen. The key components, saponin and cholesterol, form 10-12 nm hexagonal micelles that are held together by the phospholipid, forming the characteristic spherical structure (Fig. 1). Iscoms function both as a delivery system, by localizing the antigen and mimicking pathogen dimensions, and as an immunostimulatory adjuvant, by means of the incorporated saponin (described in section 2.2.3). Traditionally, the semi-purified saponin Quil A from the bark of the mature Quillaja saponaria Molina tree has been used as a source of saponin. However, Quil A can be simply replaced by other more highly purified saponin fractions (Rönnberg et al., 1995; Sjölander et al., 1997). Quil A (Brenntag AS, Vedbaek, Denmark), Spikoside (Isconova AB, Uppsala, Sweden), and Iscoprep 703 (CSL Ltd, VIC, Australia) are commercially available Quillaja saponins that have been tested and selected for iscom-forming ability. When antigens are excluded from iscoms, the particulate complex is called iscom matrix. The iscom matrix structures have the same composition, shape, and appearance as iscoms.

![Fig. 1](image1.png)

**Fig. 1.** Typical iscom particles, visualized by negative staining electron microscopy analysis. Magnification 1:90 000.
2.3.1. Incorporation of antigens into iscoms

It has been demonstrated that antigens must be physically attached to iscoms for them to exert their immunostimulating capacity. Many methods have been described for incorporating antigens into iscoms. Insoluble membrane proteins or amphipathic proteins can be directly incorporated by means of hydrophobic interactions. Furthermore, positively charged molecules, such as nucleoproteins of human deficiency virus (HIV) or influenza virus, can attach to the negatively charged iscom matrix (Morein et al., 2004). However, many recombinant protein antigens are hydrophilic, and thus need to be hydrophobically modified or chemically coupled to the iscom in order to be efficiently incorporated. For example, access to hydrophobic regions within proteins can be facilitated through partial denaturation by the use of agents like urea and mercaptoethanol (Åkerblom et al., 1993), exposure to low pH (Morein et al., 1990), or high temperature (Höglund et al., 1989). To overcome the possible loss of conformation-dependent B cell epitopes associated with denaturation, fatty acids can be covalently attached to soluble proteins (Reid, 1992). In addition, chemical coupling methods can be employed to associate antigens with iscoms containing influenza envelope proteins (Sjölander et al., 1991), or pre-formed iscom matrix (Larsson et al., 1993).

In recent studies, the scope for using gene fusion strategies to develop generally applicable methods for iscom incorporation of recombinant immunogens has been investigated (reviewed in Andersson et al., 2002; Wikman et al., 2005). Such strategies have the potential advantage of generating more uniform antigen preparations, for use primarily in early stages of vaccine development. Generally, in these approaches, hydrophobic amino acid residues, being either designed sequences or selected transmembrane sequences of viral or bacterial origin, are fused to immunogens via recombinant production in E. coli. In an initial study, the peptide tags were positioned N-terminally of the fusion proteins, resulting in relatively low production levels (Andersson et al., 1999). In a second study, Andersson et al. (2000) managed to obtain higher yields by instead positioning the hydrophobic tags at the C-terminus of the fusion proteins. In both of these studies, iscom incorporation levels of a malarial immunogen that is hydrophilic in itself were increased, and antigen-specific antibodies were generated when mice were immunized by the resulting iscom preparations (Andersson et al., 1999; 2000). In the work underlying this thesis, novel systems for the iscom incorporation of immunogens, using recombinant technology, was explored (I, II, III).

2.3.2. Mechanisms of action

Since Morein and coworkers first described iscoms in 1984 (Morein et al., 1984), numerous reports have generated a fairly good understanding of their mechanisms of action. It has been suggested that the adjuvant activity of iscoms is mainly due to their increased localization to lymphoid organs together with improved antigen uptake by APCs, such as dendritic cells, macrophages, and B cells (Barr et al.,
In contrast to most other adjuvants, iscoms are able to induce CD8+ MHC class I-restricted cytotoxic T lymphocytes (CTL) to a number of antigens after immunization by many different routes of administration. The mechanism of this induction is not clearly understood, but it is thought that the iscoms first interact with the cell membrane, which then facilitates the entry of the iscom, including the antigen, into the cell cytosol. The antigen is subsequently processed into peptides, which are presented on major histocompatibility complex (MHC) class I on the cell surface. Iscoms can also induce production of cytokines like IL-2 and IFN-γ, which are known to be elicited by Th1 type T helper cells. The production of high levels of IgG2a antibodies after immunization with iscoms further supports the hypothesis that a Th1 type of immune response is generated (Barr et al., 1998). In addition, as most particulate adjuvants, iscoms can induce a Th2 type of response (Morein and Lövgren Bengtsson, 1999). Thus, immunization with iscoms results in long-lasting humoral and cellular responses.

2.3.3. Advantages of iscoms

Despite the drawback that antigens may require modifications in order to be incorporated in iscoms, and the fact that it might not even be possible to incorporate certain antigens, the use of iscoms as adjuvants has numerous advantages. Iscoms are versatile, flexible adjuvant systems that allow the different components, with the exception of cholesterol and the saponin used, to be exchanged or additional immunomodulators to be incorporated (Morein et al., 2004). Furthermore, protective immunity against intracellular pathogens like HIV could be induced by means of a combined Th1/Th2 type of immune response. Finally, iscoms have been shown to induce potent immune responses in newborns of different species, even in the presence of maternal antibodies (Morein et al., 2004), and protective immunity has been confirmed in mice after their mucosal administration (Lövgren et al., 1990).
3. Antibodies and other affinity proteins

As mentioned in chapter 1, many proteins are naturally involved in biomolecular interactions, and thus are capable of specific recognition. This feature has been exploited in many aspects of biotechnology research, where there is a broad range of applications that require specific biomolecular recognition. For example binding proteins have often been produced for use as affinity reagents in bioseparation and detection assays, and more recently in the expanding area of proteomics. The use of proteins with highly specific binding properties is also rapidly growing in diagnostic and therapeutic applications to enhance the delivery of diagnostic markers and drugs to defined target molecules.

Antibodies are by far the most intensively explored and employed class of affinity proteins (Borrebaeck, 1995). In recent years, however, other classes of proteins have been engineered to possess novel binding activities. The so-called protein scaffolds are characterized by a stably folded supporting structure that can harbor diverse binding specificities. Thus, they must tolerate multiple substitutions or insertions at the primary structure level without losing their ability to fold into native structure (Nygren and Skerra, 2004). Recombinant DNA technology has enabled large protein libraries to be created, by means of combinatorial protein engineering techniques, from which individual variants can be selected for binding to specific target molecules (see chapter 4). Peptides, linear and cyclic, have also been randomized to obtain peptide libraries for potential selection of variants with defined characteristics (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990; Smith and Petrenko, 1997). Examples of applications of peptides have been epitope mapping (Scott and Smith, 1990), agonistic agents (Wrighton et al., 1996), and in vivo phage display (Arap et al., 2002). However, peptides will not be further discussed in this thesis.
In this chapter, antibodies together with selected examples of antibody fragments and alternative protein scaffolds will be described.

3.1. Antibodies

Immunoglobulins (Ig), also known as antibodies, are key proteins of the specific immune system. When humans and other vertebrates encounter a foreign substance, a so-called antigen, antibodies are produced in response to it. During evolution, a broad repertoire of antigen-binding specificities has developed, making antibodies capable of binding to almost any antigen.

The general structure of antibody molecules consists of two identical heavy chains (H) and two identical light chains (L), forming a typical Y-like shape, that are kept together by covalent disulphide bridges (Fig. 2A). In humans, there are five immunoglobulin classes (IgG, IgM, IgA, IgE, and IgD), which differ in the composition of the constant part of their heavy chains (Goldsby et al., 2000). In this thesis, however, only the 150 kDa IgG isotype, constituting approximately 80% of the total serum immunoglobulin population (Goldsby et al., 2000), will be discussed and henceforth referred to as ‘antibodies’.

Antibodies are typically extremely specific, and thus, they are well suited as molecular recognition tools in numerous biological research applications. Traditionally, antibodies have been generated through immunization of different animal species. After the injection of a certain antigen, the immune serum produced will contain a cocktail of heterogeneous antibodies directed to different parts of the antigen, i.e. different epitopes. Such collections, containing antibodies with different amino acid sequences recognizing the same antigen, are termed polyclonal antibodies. Although a polyclonal antibody response increases the immune protection of the organism, monoclonal antibodies, i.e. antibodies with the same amino acid sequence and specificity for the same epitope, are often preferred in cases where defined reagents are needed, e.g. for research, diagnostic, and therapeutic purposes.

3.2. Monoclonal antibodies and antibody fragments

In 1975, Köhler and Milstein introduced a new technique for the production of monoclonal antibodies (Köhler and Milstein, 1975). In this hybridoma approach, an activated antibody-producing B cell is fused with a myeloma cell (a cancerous plasma cell), generating a hybrid cell that combines the B-cell ability of secreting a defined antibody and the myeloma-cell property of immortal growth. These hybridoma cells secrete large quantities of monoclonal antibodies, and following screening for the required antigenic specificity, the selected cells can be propagated to produce the desired monoclonal antibody. Despite the impressiveness of this technique, recognized by the award of a Nobel Prize in 1984, ethical issues
concerning the use of animals for antibody generation have led to the development of alternative strategies for monoclonal antibody production.

**Fig. 2.** A schematic overview of an IgG antibody molecule and different antibody fragments. (A) The general structure of antibody molecules consists of two identical heavy chains (H) and two identical light chains (L), forming a typical Y-like shape, that are kept together by covalent disulphide bridges. Each half of an IgG antibody consists of a constant region, including three domains of the heavy chain (C\textsubscript{H}1, C\textsubscript{H}2, C\textsubscript{H}3) and one domain of the light chain (C\textsubscript{L}), plus a variable region, including one domain of the heavy chain (V\textsubscript{H}) and one of the light chain (V\textsubscript{L}). The constant region is responsible for recruiting different effector functions, whereas the variable domains serve as the antigen-specific binding site. Each heavy and light variable domain provides three hypervariable loops, denoted the complementarity-determining regions (CDRs). They form the antigen-binding site of the antibody and are indicated as black fields. (B) Fab fragment. (C) Fv fragment. (D) scFv fragment. (E) Diabody. (F) Variable domain of either heavy or light chain. Light grey corresponds to constant domains and dark grey to variable domains. Disulphide bridges are indicated.
In addition to ethical issues, a further problem associated with traditional hybridoma techniques is that the production route involved results in antibodies of rodent origin. If administered in humans, for example in therapeutic applications (see chapter 5), the mouse monoclonal antibody would be recognized as foreign, and thus, an immune response against the monoclonal antibody would be induced, thereby compromising repeated treatments. To overcome this problem, humanized antibodies have been constructed, in which only the CDRs of a mouse antibody have been grafted into a human antibody framework. In addition, more or less humanized antibodies have been produced in transgenic mice (Green, 1999; Borrebaeck, 1999). Furthermore, phage display techniques and other in vitro selection systems (described in chapter 4) have provided opportunities to generate target-specific antibodies.

Even though monoclonal antibodies offer many attractive features as reagents in a variety of applications, e.g. bioseparation, detection assays, diagnostics, imaging, and biotherapy, their relatively large size and complex architecture complicate their recombinant production. Indeed, the recombinant production of soluble, full-length monoclonal antibodies in *E. coli* has only recently been reported (Simmons et al., 2002). Therefore, smaller antigen-binding subfragments, such as Fab (Fig. 2B), Fv (Fig. 2C), scFv (Fig. 2D), diabodies (Fig. 2E), and various single domain antibodies (Fig. 2F), have been developed and are nowadays well established (Nilsson et al., 2000), as they can be produced more quickly and cheaply than whole antibodies. Since most antibody fragments require the formation of disulphide bonds for correct folding, production processes in which the antibody is secreted into the non-reducing periplasm have clear advantages. Recombinant antibody fragments can be produced in a number of different host cells, including bacterial, yeast, insect, plant and mammalian expression systems (Conrad and Fiedler, 1998; Verma et al., 1998).

In 1988, the first reports on *E. coli*-based production of functional antibody fragments, with similar affinity constants to those of the corresponding whole antibodies, were published. Better and coworkers reported the production of a chimeric mouse-human Fab (fragment, antigen binding) protein (Better et al., 1988), consisting of the *C*<sub>L</sub> and *V*<sub>L</sub> domains of a light chain and the *C*<sub>H1</sub> and *V*<sub>H</sub> domains of a heavy chain, linked together by a disulphide bridge. The two units were expressed separately and thereafter assembled spontaneously in the bacterial periplasm. Furthermore, the variable *V*<sub>L</sub> and *V*<sub>H</sub> domains of an antibody can be expressed and subsequently associated in the periplasm, forming an Fv (fragment, variable) heterodimer (Skerra and Plückthun, 1988). However, the Fv fragment is only non-covalently assembled, and hence, exhibits limited stability. Conversely, in the single-chain Fv fragment (scFv), the two domains are covalently linked through a flexible peptide (Bird et al., 1988; Huston et al., 1988).

More recently, other types of antibody fragments have emerged, for example the so-called ‘diabodies’ described by Holliger et al. (1993). These authors introduced a short linker of maximum ten amino acids in the scFv protein, thereby preventing the domains from associating. This modification forces the scFv...
domains to homodimerize with the complementary domains of another chain, resulting in a bivalent construct with two antigen-binding sites oriented in opposite directions. These can be either homobivalent or heterobivalent, with the ability to bind to one or two specific antigens, respectively. The homobivalent diabody could benefit from possible avidity effects. Finally, one of the smallest known antigen-binding fragments of antibodies are the single domain antibodies (dAbs), i.e. monomeric structures of either the V_L or the V_H variable domains (Holt et al., 2003; Nygren and Skerra, 2004). Ward et al. first isolated functional lysozyme-binding dAbs from a repertoire of murine V_H domains (Ward et al., 1989). Naturally, V_H domains have been found in camels, where they have evolved to occur in a soluble state even though their hydrophobic surface is not protected by a V_L domain (Hamers-Casterman et al., 1993). Following sequence and structural analyses of the cameld V_H domains, mutated, ‘camelized’, human V_H domains have been generated (Davies and Riechmann, 1995). The structure of cameld V_H domains seems to make them particularly suitable as enzyme inhibitors (Martin et al., 1997; Lauwereys et al., 1998; Desmyter et al., 2002).

3.3. Scaffold proteins

As antibody fragments have certain undesired characteristics, particularly related to stability and solubility issues, much current effort is focused on developing other protein scaffolds for generating novel affinity proteins (Nygren and Uhlen, 1997; Skerra 2000a; Nygren and Skerra, 2004; Table 3). The scaffold of choice is highly dependent on the intended purpose for the new affinity protein. For example, immunogenicity and serum half-life are important issues to consider in therapeutic applications (see chapter 5). Moreover, different scaffolds have differing degrees of suitability, from a topological perspective, for generating proteins that bind to different target molecules. Protruding loops, for instance, may be preferred if the intention is to bind to active binding sites of enzymes, whereas a flat protein surface would probably not be suitable for such target molecules. In general, for a protein to be appropriate as a scaffold, three important features would be; (i) a highly stable architecture, allowing randomization at certain positions, (ii) a single polypeptide-chain composition, facilitating its production, and (iii) a small size, in order to minimize unspecific binding and maximize its diffusivity (Nygren and Skerra, 2004).

3.3.1. Affibody ligands

Affibody ligands are based on a 58 amino acid residue protein domain, derived from staphylococcal protein A (SPA) (Fig. 3). SPA is composed of an N-terminal signal sequence (Abrahmsén et al., 1985) followed by five highly homologous Ig-binding domains (E, D, A, B, and C), each of which is capable of binding to the constant Fc (fragment, crystallizable) region, consisting of the C_H2 and C_H3 domains of IgG (Moks et al., 1986). In addition, the five SPA domains have been demonstrated to bind to the Fab part of Ig molecules belonging to the human V_H III
family (Jansson et al., 1998). SPA is displayed on the *Staphylococcus aureus* bacterial surface through its C-terminal cell wall-anchoring XM domains (Guss et al., 1984; Uhlén et al., 1984). Since SPA can bind to Ig molecules of various classes and subclasses from a wide range of mammalian species (Langone, 1982), it has been commonly used for the purification and detection of antibodies, even process-scale recovery of monoclonal antibodies, and also as a gene fusion affinity tag for the IgG-mediated recovery of fusion proteins (Uhlén et al., 1983; 1992).

![Diagram of Ig-binding domains of SPA and the Z-domain scaffold](image)

**Fig. 3.** A schematic picture of the 58 amino acid residue α-helical Z-domain scaffold, derived from staphylococcal protein A. Affibody libraries have been constructed by randomization of thirteen solvent-exposed residues (yellow) on helices 1 and 2 (blue). Helix 3 is represented in red.

The native B domain of SPA has been engineered in the following manner to obtain the affibody scaffold, denoted the Z domain (Nilsson et al., 1987; Fig. 3). Firstly, the asparagine-glycine dipeptide sequence (Asn28-Gly29) was changed to asparagine-alanine (Asn28-Ala29) to ensure resistance to hydroxylamine cleavage, in addition to the B domain’s inherent resistance to cyanogen bromide treatment, conferred by the absence of methionine. Secondly, the first alanine residue (Ala1) was replaced by a valine (Val1) for cloning purposes (Nilsson et al., 1987). These mutations resulted in a more useful Ig-binding tag (Z) for fusion production and cleavage, with the same overall structure and function as the native B domain (Nilsson et al., 1987). However, one difference is that the Z domain seems to have a
significantly lower affinity to the Fab region than the native domain (Jansson et al., 1998).

The Z domain is a low molecular weight protein (7 kDa), consisting of three anti-parallel α-helices forming a simple, highly soluble, bundle structure. It has a number of attractive properties for a broad range of biotechnological applications (Nygren and Skerra, 2004). Apart from its small size and high solubility, it contains no cysteines and is suitable for both cytosolic and periplasmatic production. Moreover, it is easily expressed and folded in several different hosts, and has proven to be stable with respect to both temperature and proteolysis (Moks et al., 1986; Ståhl and Nygren, 1997). Furthermore, its relatively small size enables production by solid-phase synthesis (Nord et al., 2001; Engfeldt et al., 2005). Finally, the solvent-exposed termini of the Z domain allow independent folding of fused proteins, and hence, multimeric constructs can easily be constructed by head-to-tail genetic fusion.

Initially, the Z domain was primarily used as a protein fusion partner, in order to allow affinity-mediated purification *inter alia* (Nilsson et al., 1987; Ståhl and Nygren, 1997). However, recently its high potential utility as a protein scaffold has also been proposed and explored for the selection of novel affibody ligands (Nord et al., 1997, 2001; Hansson et al., 1999; Eklund et al., 2002; Rönnmark et al., 2002a) from phagemid libraries (see section 4.1) constructed by combinatorial protein engineering (Nord et al., 1995, 1997). Based on structural data regarding the binding between the B domain and the Fc part of IgG (Deisenhofer, 1981), thirteen surface-exposed amino acid residues (Q9, Q10, N11, F13, Y14, L17, H18, E24, E25, R27, N28, Q32, and K35), located in helices one and two, were subjected to randomization (Fig. 3). Consequently, as most of these residues are involved in the native binding interaction, the IgG-binding capability was lost. However, the three-helix bundle structure remained intact (Nord et al., 1995, 1997), and novel affibody ligands, selected against desired target molecules using phage display technology (described in section 4.1), have exhibited specificity to a broad assortment of target proteins, ranging from small proteins like insulin (Nord et al., 1997) to larger proteins such as IgA (Rönnmark et al., 2002a) and Taq DNA polymerase (Nord et al., 1997). The first generation of affibody ligands was selected from libraries of approximately 4 x 10^7 variants, resulting in typical affinities in the micromolar range. More recently, ligands with affinities similar to that of the native binding between Z and human Fc (10-60 nM) (Jendeberg et al., 1995) have been obtained via affinity maturation strategies (Gunneriusson et al., 1999; Nord et al., 2001) and from larger libraries (IV, V, VI; section 7.9).

Recently, the crystal structure of an affibody in complex with its target, the wild-type Z domain (its own ancestor molecule), was determined by both x-ray crystallography (Högboom et al., 2003) and nuclear magnetic resonance (NMR) (Wahlberg et al., 2003). These studies revealed an extended and complementary
binding interface that directly involved ten out of the thirteen mutated amino acid residues on the affibody.

In the last few years, affibody ligands have been reported for use in various applications. Documented efficacy has been shown in several bioseparation applications (Nord et al., 2000; 2001; Gräslund et al., 2002a; Rönnmark et al., 2002a), and affibody ligands have shown promising potential as detection reagents (Karlström and Nygren; 2001; Rönnmark et al., 2002b; 2003; Renberg et al., 2004; 2005; Engfeldt et al., 2005), to engineer adenoviral tropism (Henning et al., 2002; see also section 7.9), and to inhibit receptor interactions (Sandström et al., 2003). In the work underlying this thesis, the potential utility of affibody ligands as tumor targeting agents was explored (IV, V, VI).

3.3.2. Other examples of affinity proteins

In the 1990s, various modified protease inhibitors were reported as potential scaffold proteins. They have several common features, being small and stable, and possessing binding activity in an exposed peptide loop that is suitable for engineering new binding specificities. However, their folding depends on the formation of intramolecular disulphide bridges. For example, scaffolds like bovine (or basic) pancreatic trypsin inhibitor (BPTI) (Roberts et al., 1992a,b), Alzheimer’s amyloid β-protein precursor inhibitor (APPI) (Dennis and Lazarus, 1994), human pancreatic secretory trypsin inhibitor (PSTI) (Röttgen and Collins, 1995), and human lipoprotein-associated coagulation inhibitor (LACI-D1) (Markland et al., 1996a,b) have been utilized to generate Kunitz domains, which contain three disulphide bonds. Further examples include two microprotein scaffolds belonging to the ‘knottin’ family; the trypsin inhibitor EETI-II from Echallium elaterium seeds (Le Nguyen et al., 1990) and the C-terminal cellulose-binding domain (CBD) of the fungal enzyme cellulohydrolase I (Smith et al., 1998; Lehtio et al., 2000). The name ‘knottin’ refers to the characteristic knotted architecture of these proteins, critical components of which are two or three disulphide bonds that stabilize three antiparallel β-strands, which can be produced either by chemical synthesis (Le Nguyen et al., 1990) or biosynthetically in the E. coli periplasm (Christmann et al., 1999). Interestingly, the ‘knottin’ proteins seem to utilize different surfaces of the protein structure when interacting with different target molecules (Smith et al., 1998; Nygren and Skerra, 2004).

Another type of protein scaffold is based on the fibronectin type III domains (FN3), which are naturally involved in molecular recognition in an extensive variety of different proteins. They consist of seven β-strands arranged in a small single-domain β-sandwich, with three loops at one end of the protein. The FN3 domains structurally resemble the V\textsubscript{H} domain of an antibody, but in contrast to antibody fragments, they lack disulphide bonds. Koide and coworkers first described the preparation of a human FN3 library, mutated in two of the exposed loops, and subsequent selection of ubiquitin-binding clones (Koide et al., 1998). In addition, Xu and coworkers isolated high-affinity binders to tumor necrosis factor α
(TNF-α) by selection from a larger library, randomized in all three surface loops (Xu et al., 2002). Members of this engineered class of FN3 variants have been termed ‘monobodies’.

Lipocalins constitute another class of small proteins that have been investigated for use as protein scaffolds (Skerra, 2000b). The lipocalin family is characterized by a highly conserved β-barrel architecture, comprising eight antiparallel β-strands winding around a central axis, with an α-helix attached to it (Flower, 1996). At the open end of the resulting conical supersecondary structure, four extremely variable loops form the entry to a ligand-binding cavity, while the other end represents a closed hydrophobic core. In nature, there are several hundred members of the lipocalin family, which exhibit great functional diversity (Flower, 1996). In 1999, Beste et al. demonstrated mutagenesis of the binding site of the bilin-binding protein (BBP), a lipocalin from Pieris brassicae, for the selection of fluorescein-specific lipocalin variants (Beste et al., 1999). In addition, a BBP variant that binds to the hydrophilic cardiac steroid digoxigenin was selected from the same library (Schlehuber et al., 2000). In summary, these engineered monomeric receptor proteins, designated ‘anticalins’, show high affinity and specificity to haptens like fluorescein and digoxigenin. Nonetheless, the four loops can be engineered to possess ligand pockets with diverse shapes and surface properties, and thus the ability to bind to macromolecular antigens (Breustedt et al., 2005).

Recently, Plückthun and coworkers described a new class of affinity proteins, the so-called repeat-motif proteins, based on repetitive structural units, which randomly assemble into extended repeat domains (Forrer et al., 2003). One example of such a unit is the 33 amino acid ankyrin repeat (AR), consisting of a β-turn followed by two antiparallel α-helices, responsible for the binding, plus a loop extending to the turn of the next repeat. In general, four to six repeats, flanked by designed N- and C-terminal capping repeats, form the robust and soluble AR domains (Binz et al., 2003). More than 2000 natural AR proteins are known and they have been found intracellularly, extracellularly, and in membrane-bound forms, indicating their broad flexibility. Binz and coworkers have constructed combinatorial libraries of designed AR proteins, containing distinct numbers of randomized repeats (Binz et al., 2003). Furthermore, high-affinity binders have been generated through selection against maltose-binding protein (MBP) and two eukaryotic protein kinases (Binz et al., 2004). The same approach has been employed to generate libraries of the intracellular leucine-rich repeat (LRR) proteins from the mammalian ribonuclease inhibitor (RJ) family (Stumpp et al., 2003).
Table 3. Examples of protein scaffolds of non-immunoglobulin origin for the generation of novel affinity proteins (modified from Nygren and Skerra, 2004).

<table>
<thead>
<tr>
<th>Name</th>
<th>Scaffold</th>
<th>Size (aa)</th>
<th>Cross-links</th>
<th>Engineered elements</th>
<th>Illustrative reference</th>
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<tr>
<td>Affibody</td>
<td>Protein A</td>
<td>58</td>
<td>(—)</td>
<td>2 α-helices</td>
<td>Nord et al., 1997</td>
</tr>
<tr>
<td>Kunitz domain</td>
<td>BPTI</td>
<td>~ 58</td>
<td>3 S-S</td>
<td>1-2 loops</td>
<td>Roberts et al., 1992a,b</td>
</tr>
<tr>
<td></td>
<td>APPI</td>
<td></td>
<td></td>
<td></td>
<td>Dennis and Lazarus, 1994</td>
</tr>
<tr>
<td></td>
<td>PSTI</td>
<td></td>
<td></td>
<td></td>
<td>Röttgen and Collins, 1995</td>
</tr>
<tr>
<td></td>
<td>LACI-D1</td>
<td></td>
<td></td>
<td></td>
<td>Markland et al., 1996a,b</td>
</tr>
<tr>
<td>Knottin</td>
<td>EETI-II CBD</td>
<td>28</td>
<td>3 S-S</td>
<td>loops</td>
<td>Le Nguyen et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36</td>
<td>2 S-S</td>
<td>3 β-strands</td>
<td>Smith et al., 1998</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lehtiö et al., 2000</td>
</tr>
<tr>
<td>Aptamer</td>
<td>Thioredoxin</td>
<td>108</td>
<td>1 S-S</td>
<td>1 loop insert</td>
<td>Lu et al., 1995</td>
</tr>
<tr>
<td>(—) Zinc-finger</td>
<td></td>
<td>26</td>
<td>Bound Zn^{2+}</td>
<td>α-helix</td>
<td>Bianchi et al., 1995</td>
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<tr>
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<td>136</td>
<td>2 S-S</td>
<td>1 loop</td>
<td>Hufton et al., 2000</td>
</tr>
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<td>Monobody/FN3</td>
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<td>94</td>
<td>(—)</td>
<td>2-3 loops</td>
<td>Koide et al., 1998</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td></td>
<td></td>
<td></td>
<td>Xu et al., 2002</td>
</tr>
<tr>
<td>Anticalin</td>
<td>Lipocalin (BBP)</td>
<td>160-180</td>
<td>0-3 S-S</td>
<td>4 loops</td>
<td>Skerra, 2000b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(174)</td>
<td>(2 S-S)</td>
<td></td>
<td>(Beste et al., 1999)</td>
</tr>
<tr>
<td>Repeat-motif</td>
<td>AR</td>
<td>33</td>
<td>(—)</td>
<td>β-turn/2 α-helices</td>
<td>Binz et al., 2004</td>
</tr>
<tr>
<td>proteins</td>
<td>LRR</td>
<td>57</td>
<td></td>
<td>β-strand/α-helix</td>
<td>Stumpp et al., 2003</td>
</tr>
</tbody>
</table>
4. Protein library selection systems

Proteins with novel binding specificities can be generated through the construction of large libraries containing a variety of different protein variants and subsequent screening against a defined target molecule, using one of many existing methods of which some are described below (Table 4). A common feature associated with all protein selection systems is the linkage between genotype and phenotype required. In contrast to the selection of isolated DNA molecules, which can be directly identified by sequencing, it is not generally feasible, or at least practical, to sequence isolated proteins. Therefore, it is essential for the selected protein to be linked to the corresponding DNA sequence for indirect identification. After that, the protein can be produced recombinantly and further characterized. It should be emphasized that different selection systems are suitable for different protein scaffolds and or different types of target molecules.

The success of any selection procedure ultimately depends inter alia on the diversity and quality of the original library. In general, the chance of finding a particular protein with a desired activity within a library correlates to the size of the library. However, library construction will not be discussed in detail in this thesis. Moreover, even though this thesis only considers selection systems for the isolation of novel affinity proteins, they can be applied for the identification of other desired protein functions, e.g. catalysis, or as powerful tools in epitope mapping and proteomic applications. For example, compartmentalization systems have been developed that are highly suited for assays involving enzyme catalysis, although these will not be described in this thesis.

Many of the available selection systems are cell-dependent, i.e. the proteins are translated inside a cell and selected variants are amplified through cell cultivation. The in vivo step introduces considerable selection pressure against
certain proteins. Secreted modes of expression of the protein, as induced by various phage display and bacterial display methods, are appropriate if the protein requires intramolecular disulphide bonds for stability. A major drawback when using cell-dependent systems is that creating large libraries is difficult because of the reliance of transfection efficiency. As an alternative to phage display and other cell-dependent systems, in vitro selection systems offer several attractive features. For example, cell-free systems are not dependent on transfection efficiency, enabling the creation of large libraries with up to $10^{15}$ members (Roberts, 1999). Also, cell-free selection systems allow the possibility of including in vitro mutagenesis and other recombination techniques during the amplification step in every round of selection (Roberts, 1999).

In this chapter, phage display techniques and selected examples of other selection systems will be described.

### 4.1. Phage display

The filamentous bacteriophages are a group of bacterial viruses that have been modified and exploited for phage display. The most thoroughly investigated phage for this purpose is M13, belonging to the Ff class of the filamentous phages. It consists of a single-stranded circular DNA molecule enclosed by a protein capsid tube, forming a 930 nm long phage particle with a diameter of 6.5 nm (Fig. 4A). The genome encodes eleven proteins, three of which are involved in DNA replication (pII, pV, and pX), three in the assembly procedure (pI, pIV, and XI), and five are capsid proteins (pIII, pVI, pVII, pVIII, and pIX). The small pVIII protein is produced in thousands of copies, covering the entire length of the phage particle. At one end of the phage, approximately five copies each of pIII and pVI are displayed, and approximately five copies each of the small pVII and pIX are located at the other end (Webster, 2001).

The Ff phages specifically infect *E. coli* cells bearing pili by attaching the N-terminal part of pIII to the tip of the F conjugative pilus, a protein tube extending from the *E. coli* membrane. Upon cell entry, the capsid proteins are dissembled into the cell membrane and the DNA moves into the cytoplasm. The single-stranded DNA is replicated via a double-stranded intermediate, and the phage proteins are expressed. The capsid proteins are directed to the cytoplasmic membrane, where they are assembled around the DNA, generating complete virions that concomitantly depart from the cell through the membranes. The infection process does not kill the bacteria, which continue to grow and divide, albeit with a 50% longer generation time. In the first generation after infection, approximately 1000 phage particles are produced, but in later division cycles the number of phages decreases to 100-200 per generation (Webster, 2001).

Since Smith first described the use of phage particles as selectable entities in 1985 (Smith, 1985), phage display has become the most common system for creating protein libraries and selecting novel proteins. In order to display them on
Rational and combinatorial protein engineering for vaccine delivery and drug targeting.

the phage surface, foreign proteins can be fused to any of the five capsid proteins (Sidhu, 2000), by simply introducing the genes encoding the proteins into the phage genome. Although the major coat protein pVIII has been utilized in several studies, the 42 kDa minor coat protein pIII is by far the most frequently used coat protein for the display of foreign proteins and peptides. The foreign protein can be fused N-terminally, which is the most conventional way, or in some cases C-terminally of Fig. 4. Schematic illustrations outlining different phage display systems. (A) The wild-type M13 bacteriophage (modified from Webster, 2001). (B) The foreign protein to be displayed is genetically fused to a phage coat protein. In type 3 and type 8 systems, the foreign gene is introduced in the phage genome, which theoretically results in the display of the foreign protein to all copies of pIII or pVIII, respectively. In type 33 and type 88 systems, two copies of the gene encoding pIII or pVIII are present in the phage genome, one of 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 and type 8+8 systems, the foreign gene is fused to the pVIII gene in a phagemid vector that contains the phage ori of replication and packaging signal but lacks genes encoding other phage proteins. Helper phages provide all phage proteins and thus allow the assembly of complete phage particles that display a mixture of wild-type coat protein and the coat protein fused to the foreign protein.
the coat protein. Prior to assembly into virions, the capsid proteins are integrated in the cytoplasmic membrane, with the N-terminal part in the periplasm and the C-terminal part in the cytoplasm (Webster, 2001). Therefore, proteins that require an oxidizing milieu to fold correctly and hence need to be secreted into the periplasm, for instance antibody fragments dependent on disulphide bond formation, are suitable for N-terminal display. In contrast, C-terminal fusion results in proteins folding in the reducing cytoplasmic environment, and hence the display of intracellular proteins (Sidhu, 2000).

The phage coat is remarkably tolerant to the insertion of new proteins. However, the function of the coat proteins is somewhat impaired when fused to large polypeptides (Sidhu, 2000). Furthermore, the foreign protein will be displayed on all copies of the coat protein used, i.e. five for pIII and about 2700 for pVIII, and consequently, the pIII display will affect the infection capability of the phage. To overcome those problems associated with the ‘type 3’ and ‘type 8’ systems, respectively, phagemid systems have been developed (Bass et al., 1990; Fig. 4B). The phagemid plasmid contains a gene encoding the coat protein fused to the foreign protein together with functional elements, such as phage and bacterial origins of replication, packaging signal and a gene encoding antibiotic resistance. To obtain phagemid-containing phage particles, E. coli cells containing the phagemid must be infected with filamentous helper phages that serve as sources of phage proteins, including the native form of the coat protein already represented by the phagemid, allowing the packaging of complete phage particles. These types of systems are denoted 3+3 when using pIII as the display molecule and 8+8 when using pVIII (Smith, 1993; Fig. 4B). In addition to the restoring infection functions of the 3+3 system, this system has the advantage of displaying the foreign protein in a monovalent fashion, and thus, avidity effects could possibly be avoided during the selection procedure. Alternatively, an additional gene encoding the display coat protein can be incorporated in the viral genome, resulting in the expression of a mixture of wild-type coat protein and the coat protein fused to a foreign protein (Rodi and Makowski, 1999). These display vehicle types are designated 33 and 88, respectively (Smith, 1993; Fig. 4B).

In general, the selection procedure in phage display (Fig. 5), commonly referred to as biopanning, is initiated by the helper phage infection of E. coli cells that have been transformed with a phagemid library. Then, the produced phages are harvested, and subjected to selection against a target molecule. After washing, bound phages are eluted with a high salt solution, low pH solution, or competitively with a solution of the target molecule or another known binder. The selected phages, expressing different protein variants on their surface, are amplified after the infection of new E. coli cells. Thereafter, the obtained clones can be identified by DNA sequencing or used for further rounds of biopanning. Finally, after about four to five panning cycles, the binding characteristics of the selected proteins to the target molecule can be determined.
Rational and combinatorial protein engineering for vaccine delivery and drug targeting.

Fig. 5. A schematic overview of the phage display selection technology. Library protein members are genetically fused to a phage coat protein, and will thereby be displayed on the surface of phage particles. The phage display library is exposed to an immobilized target molecule, and after washing, for removal of non-specifically or weakly bound phages, the bound phages are eluted and re-infected into E. coli cells. Thereafter, the obtained clones can be identified by DNA sequencing or used for preparation of a new phage stock by superinfection of helper phages and subsequent cultivation. Following harvest, the phages can be subjected to further rounds of biopanning. After typically four to five rounds of selection, the selected clones are identified by DNA sequencing and the produced proteins are characterized with respect to their binding to the target protein.

Phage display technology has been extensively used for the display of a number of different affinity proteins. Phage peptide libraries were the first phage libraries to be constructed (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990), and a number of such libraries are now commercially available. More than a decade ago, antibody fragments were successfully expressed on the phage surface (McCafferty et al., 1990; Barbas III et al., 1991; Kang et al., 1991). These citations and numerous recent reports (Dall’Acqua and Carter, 1998; Griffiths and Duncan, 1998) suggest that the time-consuming and costly hybridoma techniques could be substituted by phage display. In fact, about 30% of all human antibodies used in clinical trials, have been developed through phage display (Kretzschmar and von Rüden, 2002). In addition, several non-immunoglobulin scaffolds have been produced and selected by phage display (Nygren and Skerra, 2004). In theory, all proteins that can translocate into the cell membrane, escape periplasmatic degradation, fold properly, and allow the packaging of virions, could be displayed on the surface of phage particles (Webster, 2001). In this thesis, the selection of novel affibody ligands by the use of phage display will be presented (IV).
4.2. Other examples of selection systems

Strategies for expressing foreign proteins on a cell surface have been broadly investigated for several different cell types (Ståhl and Uhlén, 1997), such as Gram-negative *E. coli* bacteria (Charbit et al., 1986; Freudl et al., 1986; Francisco et al., 1992) and Gram-positive *Staphylococcus xylosus* (Hansson et al., 1992), *Staphylococcus carnosus* (Samuelson et al., 1995; Löfblom et al., 2005), and *Streptococcus gordinii* (Pozzi et al., 1992) bacteria. Moreover, yeast surface display on *Saccharomyces cerevisiae* has been explored (Boder and Wittrup, 1997). Recently, for instance, Boder et al. managed to isolate a single-chain antibody fragment with femtomolar affinity to fluorescein by yeast surface display (Boder et al., 2000).

Cell-surface display systems utilize outer membrane proteins as anchor molecules to display libraries of peptides and antibody fragments in order to select proteins with novel binding specificities (Lu et al., 1995; Boder and Wittrup, 1997; Fig. 6A). The possibility of using fluorescence-activated cell sorting (FACS) for screening and sorting fluorescence-labeled cells is the major advantage of using cell-surface display.

![Fig. 6. Schematic pictures of different selection systems, as alternatives to phage display (modified from Cornish and Lin, 2002).](image)

In 1992, Cull and coworkers first demonstrated a method for displaying peptides on plasmids (Cull et al., 1992). They constructed a peptide library by cloning $10^8$ random oligonucleotide variants downstream of the *lacI* gene, allowing the peptides to be produced C-terminally of the DNA-binding *lac* repressor. The *lac* repressor binds strongly to the specific *lacO* sequence, present on the same plasmid as the gene encoding the *lacI* repressor fused to the peptide, creating a direct non-covalent link between the peptide and the plasmid DNA that encodes it (Fig. 6B). In contrast to the established phage display method, the repressor fusions are produced intracellularly, thus hindering the correct folding of proteins with essential disulphide bridges.

The ribosome display selection method was first described by Mattheakis *et al.* (1994). They managed to select a monoclonal antibody-specific peptide from a DNA library encoding $10^{12}$ different ten amino acid residue peptides by using an *in*
vitro polyribosome display system. Some years later, selection of native folded proteins using ribosome display was presented by Hanes and Plückthun (1997). In the ribosome display system, a library of DNA sequences devoid of stop codons is transcribed and translated in an in vitro E. coli S30 extract, and thereafter the mRNA-ribosome-protein complexes can be selected against an immobilized target molecule (Fig. 6C).

Table 4. Examples of protein library selection systems and their characteristics.

<table>
<thead>
<tr>
<th>Selection system</th>
<th>Characteristics</th>
<th>Illustrative references</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell-dependent systems</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phage display</td>
<td>Foreign proteins are displayed on the surface of bacteriophages via genetic fusion to a phage coat protein.</td>
<td>Smith and Petrenko, 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barbas III et al., 2001</td>
</tr>
<tr>
<td>Cell-surface display</td>
<td>Foreign proteins are displayed on the surface of cells via genetic fusion to an outer membrane protein.</td>
<td>Boder and Wittrup, 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Löfblom et al., 2005</td>
</tr>
<tr>
<td>Plasmid display</td>
<td>Foreign proteins are genetically fused to the lac repressor that can bind to the lacO sequence present on the same plasmid as the gene encoding the lac repressor fused to the foreign protein.</td>
<td>Cull et al., 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gates et al., 1996</td>
</tr>
<tr>
<td><strong>Cell-free systems</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosome display</td>
<td>DNA sequences devoid of stop codons are in vitro transcribed and translated, and the mRNA molecules are linked to the protein via the ribosome.</td>
<td>Hanes and Plückthun, 1997</td>
</tr>
<tr>
<td>mRNA display</td>
<td>DNA sequences are in vitro transcribed and translated, and the mRNA molecules are linked to the protein via puromycin.</td>
<td>Nemoto et al., 1997</td>
</tr>
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<td></td>
<td></td>
<td>Roberts and Szostak, 1997</td>
</tr>
</tbody>
</table>

The mRNA display system avoids instability problems associated with ribosome display by covalently linking the mRNA to the peptide through the antibiotic puromycin (Nemoto et al., 1997, Roberts and Szostak, 1997). Selection by the use of mRNA display starts with in vitro transcription of a DNA library into mRNA, followed by ligation to a DNA linker with puromycin at the 3’ end. Then, the mRNA is translated in vitro, whereupon the ribosome pauses, allowing the puromycin to enter the ribosome A site and attach to the C-terminus of the translated polypeptide chain. Thereafter, the covalent mRNA-puromycin-peptide complexes are, in optional order, subjected to reverse transcription and selection against an immobilized target molecule (Fig. 6D).
5. Affinity proteins in biotherapy

Today, small organic molecules constitute the dominating group of drugs. The early optimism concerning the potential of the development of small molecular drugs for preventing every disease has been somewhat hampered, for the simple reason that it has proven more difficult than expected to rapidly identify new drugs by high-throughput screening or to generate them by rational design. Thus, interest has been rekindled in protein-based drugs as first generation pharmaceuticals. Recombinant DNA technology has provided opportunities to produce new protein drugs, as well as target molecules in large quantities. In general, protein drugs often act in one of the following manners; (i) as up-regulating agents, agonists (e.g. cytokines and growth factors), through stimulation of cell-surface receptors, (ii) as down-regulating agents, antagonists (e.g. antibodies and soluble receptors), through inhibition of native protein interactions, and (iii) as targeting agents (e.g. antibodies) that bind to overexpressed molecules in diseased tissue (Rao et al., 2005). The latter will be discussed in this chapter.

Since the 1970s, monoclonal antibodies (section 3.2) have been explored as therapeutic agents in clinical medicine. Antibodies can target specific cell-surface receptors, and their interactions with their respective receptors can induce direct cytotoxic effects by a variety of mechanisms, e.g. blocking growth factor receptors, induction of apoptosis, or formation of anti-idiotypic antibodies. In other cases indirect effects are induced, such as antibody-dependent cellular cytotoxicity (ADCC) or complement-mediated cytotoxicity (Green et al., 2000). In order to enhance the cytotoxicity of antibodies, they can be coupled to various toxic substances, including radioactive isotopes (see section 5.1), toxins, or chemotherapeutic drugs (Green et al., 2000). At present, more than 20 monoclonal
antibody-based products, intended for diagnostic, imaging, and therapeutic purposes, are licensed for human use (Walsh, 2003; Appendix II).

Monoclonal antibodies have been particularly intensively investigated for use in cancer therapy. Cancer patients today are normally treated by surgery and external radiation to eradicate primary tumors and large metastases, and chemotherapy to damage disseminated tumor cells (Carlsson et al., 2003). However, chemotherapy treatments are only curative for lymphomas, testicular tumors and paediatric-related tumors. For disseminated tumor cells of cancers like breast, prostate, colorectal, lung, and ovarian types, the chemotherapeutic effect is only palliative and/or may prolong survival (Carlsson et al., 2003). In addition, conventional cancer treatments, using radiation from external sources and chemotherapy, have numerous side effects, as these methods attack a broad spectrum of both normal and tumor cells. Therefore, defined targeting of monoclonal antibodies, directed to cell-specific markers, would be preferable. To date, the most promising results in clinical studies with such approaches have been obtained for lymphomas and leukemias, while using them to treat solid tumors has had limited success (Carlsson et al., 2003). In 1997, the first monoclonal antibody for human cancer therapy (rituximab) was approved (Walsh, 2003; Appendix II). Rituximab is a chimeric antibody intended for use in Non-Hodgkin’s lymphoma treatment, since it binds to the B-cell specific surface molecule CD20, involved in cellular growth and differentiation.

Although monoclonal antibodies have demonstrated promising results for tumor targeting, they are unfortunately associated with various side effects and have limited efficacy in certain cases. For example in radiotherapy (see section 5.1), slow blood clearance and liver uptake cause unwanted side-effects and their relatively large size may also result in poor tissue penetration (Nilsson et al., 2000). To modulate half-lives and the tumor penetration ability, antibody fragments (section 3.2), such as Fab, scFv, and multivalent scFv, have been evaluated as targeting agents (Nilsson et al., 2000). They benefit from being smaller and humanized to a higher degree, since they are produced in vitro by recombinant DNA techniques, but on the other hand they are not able to recruit effector cells, because they lack the constant region present in whole antibodies. Even though antibody fragment approaches are promising, they seem to have limitations. Stability issues, in terms of both proteolysis and shelf-life, are obvious challenges (Röthlisberger et al., 2005). Thus, targeting agents based on alternative protein scaffolds (section 3.3) are under investigation in parallel in the hunt for an ultimate therapeutic agent. In particular, scaffolds based on human proteins, e.g. fibronectin III (Koide et al., 1998) and lipocalin (Skerra, 2000b), could potentially be suitable for human therapy. However, the human origin could potentially be associated with a certain risk of inducing autoimmune reactions. The small staphylococcal protein A-derived affibody ligands (section 3.3.1) might also serve as a potential drug targeting candidates, as will be discussed in chapter 7.
It should be emphasized that it would probably be better to develop a range of therapeutic candidates, with properties that could be matched to specific requirements, rather than seeking a single ‘optimal’ agent. Two main aspects that should be considered are size and affinity. For example, the optimal drug for treating solid tumors would probably have to be quite small in order to efficiently penetrate the poorly vascularized tissue. A drawback with small agents, however, is their rapid elimination from the circulatory system, which might lead to inefficient drug delivery, since only a small fraction of the injected dose will reach the tumor. Moreover, it is not obvious that the ideal affinity should be ‘as high as possible’ for agents targeting solid tumors. Exceptionally high affinities might lead to impaired tumor localization and penetration, due to diffusion limitations. Adams and coworkers have concluded that in their experimental model an affinity of $\sim 10^{-9}$ M would be optimal for scFv antibody molecules (Adams et al., 2001). Nevertheless, the optimal value would almost certainly vary between different targeting agents and tumor types (Rao et al., 2005).

### 5.1. Radionuclide therapy

As an alternative or complement to chemotherapy, radionuclide therapy has emerged for the treatment of disseminated tumor cells and small metastases. In preclinical trials these approaches have shown promising results for the curative treatment of both primary and disseminated tumors (Carlsson et al., 2003). In radionuclide therapy, monoclonal antibodies or other target-binding agents can serve as delivery vehicles for specifically targeting radionuclides (Fig. 7). Aspects

**Fig. 7.** Specific target-binding agents, such as antibodies or affibody molecules, can be useful in radionuclide-based medical imaging or radiotherapy applications, to monitor or treat patients with tumors that overexpress defined markers.
that need to be considered are pharmacokinetic parameters, as described above, and the possibility of radiolabeling the agents while retaining their specificity and affinity. Today, there are only two monoclonal antibodies that are approved for use in human radiotherapy, namely Zevalin \(^{90}\text{Y}\) and Bexxar \(^{131}\text{I}\). However, many additional radiolabeled molecules with therapeutic potential, including monoclonal antibodies as well as antibody fragments, have been described (Carlsson et al., 2003). In addition to therapeutic applications, radiolabeled tumor-specific agents might become powerful tools for \textit{in vivo} imaging, e.g. by positron emission tomography (PET) scanners. An excellent review describing the state of both radiotherapy and \textit{in vivo} imaging was recently published (Tolmachev et al., 2004).

There are four major groups of therapeutic radionuclides; \(\alpha\)-particle emitters, \(\beta\)-particle emitters, Auger electron cascade emitters, and low energy electron emitters. The choice of radionuclide depends on the intended application, taking into account several physical, chemical, biological, and economic factors, of which half-life and particle range are particularly critical. Currently, the most widely used radionuclide for therapy purposes is the low energy \(\beta\) emitter \(^{131}\text{I}\), which is suitable for treating tumor cell clusters. However, the \(\alpha\) emitter \(^{211}\text{At}\) probably has the greatest potential for treating single cells and small clusters of cells (Tolmachev et al., 2004).

In the work underlying this thesis, a first effort towards generating radiolabeled affibody ligands for tumor targeting was explored (IV, V, VI).
PRESENT INVESTIGATION

Objectives

• To evaluate novel methods for incorporating immunogens into vaccine delivery systems, e.g. iscoms.

• To generate novel affibody ligands that could be investigated as tumor targeting agents.

Recombinant production constitutes a major strategy for the generation of subunit vaccine candidates (Liljeqvist and Ståhl, 1999). Protein antigens, which are typically not immunogenic in themselves, are normally administered with an adjuvant, to improve their immunogenicity. In previous studies, gene fusion strategies for direct iscom incorporation of recombinant immunogens have been explored (Andersson et al., 1999; 2000). Upon recombinant production in *E. coli*, hydrophobic amino acid residues have been fused to immunogens, either N-terminally (Andersson et al., 1999) or C-terminally (Andersson et al., 2000) of the fusion protein, resulting in efficient incorporation into iscoms and subsequent generation of antigen-specific antibodies following the immunization of mice (Andersson et al., 1999; 2000).

In the studies underlying this thesis, two additional strategies for iscom incorporation of protein immunogens were explored. Firstly, recombinant methods for the *in vivo* or *in vitro* lipid tagging of immunogens in order to incorporate them into iscoms through hydrophobic interaction, were evaluated. Secondly, the strong interaction between biotin and streptavidin ($K_D \approx 10^{-15}$ M) (Savage, 1992) was explored as a means to couple recombinant immunogens to iscom matrix, i.e. iscom particles without any antigen, by two different approaches. Recombinant immunogens, produced by the different methods, were evaluated in iscom incorporation experiments, and certain selected iscom preparations were used in immunization experiments to determine their efficacy.
Fig. A. The life cycle of *Toxoplasma gondii* (from Dubey, 1986; with kind permission from Dr. J.P. Dubey).

*Toxoplasma gondii* is a protozoan parasite that can cause the disease toxoplasmosis. This single-celled parasite can infect all warm-blooded animals, including humans, and is widely distributed around the world. However, most infected individuals show no symptoms because a healthy person’s immune system usually prevents the parasite from causing illness. However, in immunocompromised patients, *T. gondii* can cause severe, life-threatening disease. Also, if acquired during pregnancy, the infection can result in miscarriage or damage to the brain or eyes of the child. In veterinary medicine, Toxoplasma infection is a well-known cause of abortion and neonatal mortality in sheep and goat.

*T. gondii* has a two-phase life cycle, comprising a sexual phase that occurs in cats or other felids, and an asexual multiplication phase that takes place in intermediate mammalian or avian hosts. After sexual development in the intestinal epithelium of an infected cat, oocysts (10-12 µm) are shed with the feces. Within 1-5 days, the oocysts become infective through a sporulation process. The sporulated oocysts can survive in the environment for prolonged times and represent sources of infection, particularly for herbivorous animals. Following oocyst ingestion by an intermediate host, the parasites invade the intestinal epithelium and start to divide asexually, and free parasites, now called tachyzoites, are released and spread throughout the body. When the rapid proliferation phase terminates, slowly dividing organisms, denoted bradyzoites, accumulate in tissue cysts. The cysts can remain for a long time in extra-intestinal tissues, primarily muscle tissue and the central nervous system. If a carnivore ingests meat or organs from an infected animal, the infection develops as after ingestion of sporulated oocysts by intermediate hosts. However, if the carnivore happens to be a definitive host, a felid, the sexual phase occurs simultaneously, resulting in completion of the life cycle.

Although humans can try to prevent Toxoplasma infection in a number of ways, for example by cooking all meat thoroughly, washing vegetables carefully, and washing hands after contact with soil, there is a great need for a vaccine against toxoplasmosis in humans. In animals, only one live attenuated vaccine for use in sheep has been approved so far (Buxton, 1993). The potential value of live attenuated, killed or lysed parasites, and a range of subunit vaccines, has been explored in various immunization studies (Bhopale, 2003). For example, Lundén and coworkers have investigated the use of surface antigens (TgSAG1, TgSAG2, and others) as subunit vaccine candidates by incorporating them into iscoms. They exhibited protective immunity in mice (Lundén et al., 1993), and humoral and cellular immune responses in sheep (Lundén, 1995). However, research continues for the potential development of safe and effective vaccines, both for human and animal use.
6.1. Lipid tagging of protein immunogens for iscom incorporation (I)

Two recombinant strategies were employed to develop general systems for producing lipid-tagged protein immunogens. Lipids were either added in vivo via E. coli expression, or in vitro via the interaction of a hexahistidyl (His₆) peptide and a chelating lipid (Fig. 8). A His₆-ABP dual affinity tag, consisting of a His₆ tag and a serum-albumin binding protein (ABP) derived from streptococcal protein G (Nygren et al., 1988; Larsson et al., 1996), was included in both systems. This allowed recovery of expressed fusion proteins through affinity purification by either immobilized metal ion affinity chromatography (IMAC) (Hochuli et al., 1988), or on human serum albumin (HSA)-Sepharose columns (Ståhl et al., 1999). As a model immunogen in this study, a 238 amino acid immunogen, ∆SAG1, derived from the central region (amino acid residues 61-298) of the native major surface antigen SAG1 (Burg et al., 1988; Harning et al., 1996) of Toxoplasma gondii (Fig. A), was used.

**A**  
**In vivo lipidation**

\[ \text{lpp|His}_6\text{|ABP|∆SAG1} + \text{cholesterol} \]  
\[ \text{phospholipids} \]  
\[ \text{Quil A} \]  
\[ \rightarrow \]  
\[ \text{Iscom|lpp|His}_6\text{|ABP|∆SAG1} \]

**B**  
**In vitro lipidation**

\[ \text{Cu}^{2+}-\text{loaded iscom matrix} \]  
\[ \text{His}_6\text{|ABP|∆SAG1} \]

Fig. 8. Schematic illustration of iscom incorporation of recombinant immunogens by in vivo or in vitro lipidation strategies. (A) The in vivo-lipidated fusion protein lpp-His₆-ABP-∆SAG1 was incorporated into iscoms simply by mixing the fusion protein with iscom constituents (cholesterol, phosphatidyl choline and Quil A), followed by dialysis. (B) The fusion protein His₆-ABP-∆SAG1 was associated with pre-formed iscom matrix via the interaction of the His₆ tag and a Cu²⁺-chelating lipid, which was pre-incorporated into the iscom matrix.

6.1.1. **In vivo lipidation (I)**

To enable in vivo lipidation of recombinant immunogens, a novel general expression vector, pMLHABP, was constructed (Fig. 9A). This vector encodes the 20 amino acid signal peptide (S) and the nine N-terminal amino acid residues (lpp) of the major lipoprotein of E. coli, followed by a His₆-ABP dual affinity tag. The short lpp sequence has been found to be sufficient for lipidation by the well-
characterized *E. coli* lipidation machinery (Ghreyeb and Inouye, 1984; Laukkanen *et al*., 1993). The expression is under the control of the tac promoter (Amann *et al*., 1988). A gene fragment encoding the model immunogen ∆SAG1 was introduced in the vector.

**Fig. 9.** The general expression vectors (A) pMLHABP and (B) pAff8c, intended for *in vivo* or *in vitro* lipidation of recombinant immunogens. The vectors differ in that the pMLHABP encodes the 20 amino acid signal peptide (S) and the nine N-terminal amino acid residues (lpp) that enable *in vivo* lipidation upon expression in *E. coli*. Multiple cloning sites (mcs), suitable for insertion of immunogen-encoding genes, have been introduced downstream of the dual affinity tag His$_{6}$-ABP, common for both vectors. The expression is under the control of the tac or T7 promoters, respectively. The vectors also encode the lac repressor (LacI) responsible for tight repression of transcription prior to the induction with IPTG, and β-lactamase (bla) or Kanamycin resistance (Km) for antibiotic selection. Their encoded fusion proteins lpp-His$_{6}$-ABP-∆SAG1 and His$_{6}$-ABP-∆SAG1, in which ∆SAG1 has been included as a model immunogen, are schematically presented. Note, the signal peptide encoded by the pMLHABP vector is processed upon translocation.

The encoded fusion protein lpp-His$_{6}$-ABP-∆SAG1 (Fig. 9A), designed for *in vivo* lipidation, was produced in *E. coli* cells with expression levels of approximately 5 mg per litre of cell culture. After sonication of the bacteria, the protein was predominantly found in the insoluble protein and cell debris fractions, potentially indicating membrane association. Following purification by ABP-mediated affinity chromatography on HSA-Sepharose (Stål *et al*., 1989; 1999), pure protein of the expected molecular weight (42.4 kDa) was obtained. Its identity was confirmed in Western-blotting experiments using SAG1-reactive polyclonal antibodies. However, the monoclonal antibody DG52 (Kim *et al*., 1993), known to bind natively folded SAG1, did not recognize the fusion proteins, under either
reducing or non-reducing conditions. Nevertheless, the lpp-His$_6$-ABP-ΔSAG1 fusion protein would be potentially valuable for evaluation of in vivo lipidation in iscom incorporation experiments.

6.1.2. In vitro lipidation (I)
The in vitro lipidation strategy does not depend on E. coli lipidation determinants, but instead utilizes a metal-chelating lipid, which could thus be linked to a His$_6$ peptide. To enable production of recombinant immunogens suitable for in vitro lipidation, the general expression vector pAff8c (Fig. 9B) (Larsson et al., 2000) was used. This vector encodes a His$_6$-ABP dual affinity tag, under the control of the T7 promoter (Studier et al., 1990), preceding a multiple cloning site, and thus offers the same options for affinity chromatography as pMLHABP (Fig. 9A). Moreover, a gene fragment encoding the model immunogen ΔSAG1 was included in the vector.

The encoded fusion protein His$_6$-ABP-ΔSAG1 (Fig. 9B), intended for in vitro lipidation, was produced intracellularly in E. coli cells and recovered by affinity chromatography on HSA-Sepharose. As expected, this fusion protein was mainly soluble, and was expressed at high levels of approximately 100 mg/l. In addition, the fusion protein was found to be proteolytically stable and of correct size (41.6 kDa). In Western-blotting experiments, the His$_6$-ABP-ΔSAG1 protein could be detected, in the same way as lpp-His$_6$-ABP-ΔSAG1, with polyclonal antibodies but not with the conformation-dependent monoclonal antibody DG52. Hence, the His$_6$-ABP-ΔSAG1 fusion protein would be a candidate for evaluation of in vitro lipidation in iscom incorporation experiments.

6.1.3. Iscom incorporation experiments (I)
For the in vivo-lipidated fusion protein lpp-His$_6$-ABP-ΔSAG1, iscoms were prepared by mixing the affinity-purified fusion protein with cholesterol, phosphatidyl choline and Quil A, followed by dialysis (Fig. 8A). The fusion protein His$_6$-ABP-ΔSAG1, which was to be associated through the interaction of the His$_6$ tag with a Cu$^{2+}$-chelating lipid, was mixed with a pre-formed iscom matrix containing the chelating lipid (Fig. 8B). For evaluation, the iscom preparations were subjected to sucrose gradient ultracentrifugation, and collected fractions were analyzed for the presence of cholesterol and protein (Fig. 10). The cholesterol-rich fractions in the sucrose gradient, capable of recognition through addition of trace amounts of $^3$H-cholesterol during iscom preparations, can be considered indicators of iscom formation. These fractions are marked with horizontal bars in Fig. 10A-C. Based on earlier observations (Andersson et al., 1999, 2000), the co-migration of protein and cholesterol in the sucrose gradient was regarded as being indicative of successful iscom incorporation.

Significant proportions of the lipidated immunogens, both the in vivo lipidated lpp-His$_6$-ABP-ΔSAG1 (Fig. 10A) and the His$_6$-ABP-ΔSAG1 (Fig. 10B), the latter associated via in vitro lipidation through the interaction with the chelating lipid, were found in the cholesterol-rich fractions of the sucrose gradient, indicating iscom incorporation/association. In contrast, for a non-lipidated His$_6$-ABP-ΔSAG1
mixed with the iscom matrix (without the chelating peptide), only a small fraction of protein was detected in the cholesterol-rich fractions (Fig. 10C), indicating that \textit{in vivo} or \textit{in vitro} lipidation was indeed required for iscom incorporation or association with the iscom matrix. In a second control experiment, the two affinity-purified proteins, lpp-His\textsubscript{6}-ABP-\Delta SAG1 and His\textsubscript{6}-ABP-\Delta SAG1, were subjected to sucrose gradient centrifugation without the iscom constituents. As expected, it was found that the proteins alone migrated in fractions 11-13, not in fractions 6-8 (typical for iscom-incorporated proteins) or fractions 3-8 (typical for aggregated proteins). In addition, electron microscopic inspection of the iscom preparations verified the presence of the typical iscom structures, providing further evidence that iscoms had been successfully formed.

\textbf{Fig. 10.} Analysis of the protein content in the fractions collected from sucrose gradient centrifugation of the iscom preparations produced by the lipidation strategies. The incorporation of proteins was analyzed by measuring total protein content according to Bradford (Bradford, 1976), in fractions from the iscom incorporation experiments (A) \textit{in vivo}-lipidated lpp-His\textsubscript{6}-ABP-\Delta SAG1 mixed with iscom constituents (cholesterol, phosphatidyl choline and Quil A) (B) His\textsubscript{6}-ABP-\Delta SAG1 added to pre-formed iscom matrix containing a chelating lipid, and (C) His\textsubscript{6}-ABP-\Delta SAG1 added to iscom matrix constituents, excluding the chelating lipid. The horizontal bars indicate the cholesterol-rich fractions.
These results indicate that both the in vivo and in vitro strategies resulted in iscom incorporation/association of the fusion proteins. The in vivo lipidation strategy has the advantage that only the typical iscom constituents are needed, while the in vitro strategy requires a chelating lipid. Nevertheless, the expression vector connected with the in vitro strategy seems to offer much higher expression levels and, in addition, a straightforward method for iscom association, since iscom matrix with pre-incorporated chelating lipid can be prepared in bulk.

6.1.4. Immunization of mice (I)

Mice immunized subcutaneously at the base of their tails with the two different iscom preparations, generated by either in vivo or in vitro lipidation of the immunogen, responded to a first immunization by producing serum IgG antibodies that were reactive to His₆-ABP-ΔSAG1 with titers of approximately 1:1000. A booster immunization resulted in a 100-fold increase in the serum IgG responses. Interestingly, a control group receiving His₆-ABP-ΔSAG1 admixed with iscom matrix lacking the chelating lipid gave antibody responses of the same order of magnitude. This was very surprising since only a small fraction of fusion protein was found in the cholesterol-rich iscom fractions after sucrose gradient ultracentrifugation (Fig. 10C). Furthermore, the His₆-ABP-ΔSAG1 fusion protein was found to be immunogenic even in PBS alone, with end point titers exceeding 1:1000 after a booster immunization. This is likely due to the ΔSAG1 immunogen having potent inherent immunogenicity, since poorly immunogenic antigens normally fail to elicit antibody responses when supplied either admixed with iscom matrix or with PBS alone (Andersson et al., 2000). In addition, certain partially hydrophobic antigens have previously been found to elicit significant antibody responses when administered admixed with an iscom matrix (Andersson et al., 2000). The model immunogen ΔSAG1 contains a sequence of mostly hydrophobic amino acids (amino acids 140-185 in the native SAG1 (Burg et al., 1988)), which thus could yield certain aggregate structures. A drawback with a strategy involving the use of uncontrolled aggregation for the formulation of immunogens intended for vaccination trials would be that the well-defined iscom structures would not be formed.

In an attempt to verify that antibodies with activity against the parental immunogen were produced, a T. gondii iscom preparation that reportedly contains the native SAG1 antigen (Lundén et al., 1993) was used as a coating antigen in an enzyme-linked immunosorbent assay (ELISA). In this assay, statistically significant levels of SAG1-reactive antibodies were only detected in the immunization group receiving His₆-ABP-ΔSAG1 associated with iscoms through in vitro lipidation. The poor reactivity to the native antigen was consistent with the failure of recognition by the monoclonal antibody DG52 in Western blotting experiments, indicating that ΔSAG1 was suboptimally designed or presented. This was not entirely surprising since earlier attempts to produce correctly folded recombinant SAG1 in bacterial expression systems have met considerable difficulties (Kim et al., 1993; Biemans et al., 1998). The slightly better antibody responses obtained for the iscom preparation
with *in vitro*-lipidated His$_6$-ABP-ΔSAG1 suggest that the immunogen may be better presented this way. Following analysis, the induced SAG1-specific antibodies demonstrated balanced IgG1 and IgG2a reactivities, i.e., an IgG subclass profile often seen after immunization with iscoms (Lövgren, 1988; Sjölander *et al.*, 1996).

In conclusion, these results indicate that significant antibody responses can be elicited to target antigens incorporated into iscoms through lipidation strategies. Although the general applicability of the presented strategies need to be further evaluated for various immunogens, the described systems might offer convenient alternatives for efficient adjuvant incorporation of recombinant immunogens that normally incorporate poorly into adjuvants, such as iscoms and liposomes. The presented systems should thus be of interest to evaluate for adjuvant formulation of future recombinant subunit vaccines.

### 6.2. Biotin-streptavidin-based iscom association of immunogens (II, III)

Hydrophobic interaction is still not an ideal basis for iscom incorporation, since a highly hydrophobic immunogen might be difficult to incorporate in a directed manner. Therefore, the strongest bio-specific interaction known in nature, namely the interaction between biotin and streptavidin, was also used in two different approaches to achieve iscom association of recombinant immunogens (Fig. 11). In this study, a 232 amino acid segment (SRS2$'$), derived from the central region (amino acid residues P97 to K328) of the major surface antigen NcSRS2 of the protozoan parasite *Neospora caninum* (Fig. B), and a 53 amino acid malaria peptide (M5), derived from the central region of the *Plasmodium falciparum* blood-stage antigen Pf155/RESA, were used as model immunogens.

#### 6.2.1. Biotin-streptavidin approaches for iscom association (II, III)

In the first approach, a His$_6$-tagged streptavidin fusion protein (His$_6$-SA) was bound to a metal-chelating iscom matrix loaded with Ni$^{2+}$ ions (Ni$^{2+}$ matrix), and biotinylated immunogens were then associated with the streptavidin-coated iscoms (Fig. 11A). The immunogens were biotinylated, either *in vivo* via *E. coli* expression or both *in vivo* and *in vitro*. To achieve site-specific *in vivo* biotinylation, a 21 amino acid residue tag was utilized (Schatz, 1993), which is biotinylated upon expression at a specific lysine residue by the *E. coli* biotinylation machinery (Nilsson *et al.*, 1997). This short tag was selected from a combinatorial peptide library as it was found to be capable of mimicking the natural substrate of *E. coli* biotin ligase (Schatz, 1993; Nilsson *et al.*, 1997). In the second approach, the recombinant immunogens were expressed as streptavidin fusion proteins, which were directly bound to a biotinylated iscom matrix (Bio-matrix) (Fig. 11B).
Indirect biotin-streptavidin coupling

\[ \text{His}_6 \text{SA} \rightarrow \text{BioHis}_{6n} \text{ABP SRS2}^{-} \]

Direct biotin-streptavidin coupling

\[ \text{His}_6 \text{SA SRS2}^{-} \]

Fig. 11. Schematic illustration of iscom association of recombinant immunogens by the biotin-streptavidin interaction. (A) Iscom association by indirect biotin-streptavidin coupling between either of the two in vivo biotinylated fusion proteins Bio-His\(_6\)-ABP-SRS2^{-} and Bio-His\(_6\)-ABP-M5, and a His\(_6\)-streptavidin (SA) fusion protein pre-bound to a metal-chelating iscom matrix loaded with Ni\(^{2+}\) ions. (B) Iscom association by direct biotin-streptavidin coupling between either of the fusion proteins His\(_6\)-SA-SRS2^{-} and His\(_6\)-SA-M5, and biotinylated iscom matrix (Bio-matrix).

6.2.2. Fusion proteins used in biotin-streptavidin strategies (II)
To evaluate iscom association via the first biotin-streptavidin approach (Fig. 11A), a novel expression vector, pAff10cSA (Fig. 12A), was constructed from the pAff10c plasmid (Gräslund et al., 2002b). This plasmid encodes a His\(_6\)-SA fusion protein (Fig. 12A), under the control of the T7 promoter, consisting of an N-terminal His\(_6\) peptide fused to a streptavidin fragment (SA) (amino acid residues A13 to S139). The His\(_6\) tag allows affinity purification of the fusion protein by IMAC and iscom association via a Ni\(^{2+}\)-chelating lipid. To enable E. coli production of in vivo-biotinylated immunogens, the expression vector pAff2c (Fig. 12B) (Nilsson et al., 1996) was used, encoding a tripartite fusion tag, consisting of a 21 amino acid in vivo biotinylation sequence (containing a lysine residue, K13, for site-specific biotinylation) fused to a His\(_6\)-ABP dual affinity tag. The expression is under the control of the T7 promoter, and a multiple cloning site located C-terminally of the ABP allows insertion of target genes to be expressed. Gene
Neospora caninum is a recently recognized protozoan parasite, which closely resembles Toxoplasma gondii (Fig. A). In fact, until 1988 it was misidentified as T. gondii. The intracellular parasite N. caninum was originally described as a cause of neurological disease in dogs (Dubey et al., 1988). Shortly thereafter, it became evident that N. caninum is a major cause of reproductive failure in cattle due to abortion, stillbirths, and congenital disease (Anderson et al., 1991; Barr et al., 1991). Neospora infection of non-pregnant adult cattle is asymptomatic. On the other hand, abortions in pregnant cows may occur irrespective of whether the infection is recent, chronic, or congenital. Currently, evidence of human exposure to N. caninum infection has only been found in one study (Tranas et al., 1999), and thus, more research is obviously needed to prove whether this parasite is zoonotic or not.

The tissue-cyst forming coccidian parasite N. caninum has a two-host life cycle that is similar to that of T. gondii (Fig. A). For N. caninum, however, the sexual reproduction phase occurs in dogs (McAllister et al., 1998), the definitive host, and a number of mammalian species, such as cattle, sheep, goats, deer, and horses, act as intermediate hosts for the asexual phase.

Neosporosis causes the global farming industry substantial economic losses, and currently there is no effective method to control the disease. Vaccination has been suggested as a means to prevent neosporosis in cattle, and various approaches to develop an effective vaccine have been investigated (Innes et al., 2002; Dubey, 2003). A major problem is to elicit protective immunity against abortion in cattle that already harbor a latent infection. Several N. caninum antigens, including the major immunodominant surface antigens NcSAG1 and NcSRS2 (Hemphill et al., 1997; Howe et al., 1998; Sonda et al., 1998), have been described and suggested as potential vaccine candidates. NcSAG1 is a tachyzoite-specific antigen, while NcSRS2 is found on both tachyzoites and bradyzoites (Fuchs et al., 1998). However, research continues for the potential development of safe and effective vaccines.
fragments encoding the model immunogens SRS2’ and M5 were introduced into pAff2c, resulting in the vectors pAff2cSRS2’ and pAff2cM5, encoding the fusion proteins Bio-His6-ABP-SRS2’ and Bio-His6-ABP-M5, respectively (Fig. 12B).

The pAff10cSA plasmid was also used as a general expression vector in the second iscom association approach, in which a streptavidin-fused immunogen was to be directly bound to Bio-matrix (Fig. 11B). For this purpose, gene fragments encoding SRS2’ and M5 were inserted into a multiple cloning site C-terminally of the streptavidin gene, resulting in the vectors pAff10cSASRS2’ and pAff10cSAM5, encoding the fusion proteins His6-SA-SRS2’ and His6-SA-M5, respectively (Fig. 12A).

Fig. 12. The plasmid vectors and their encoded fusion proteins used in the biotin-streptavidin approaches. Numbers indicate the iscom association approach in which each protein was used. (A) The general expression vector pAff10cSA encodes a hexahistidyl tag (His6) and a streptavidin protein (SA). A multiple cloning site (mcs), suitable for insertion of immunogen-encoding genes, has been introduced downstream of the SA gene. The transcription is under the control of the T7 promoter. The vector also encodes the lac repressor (LacI), responsible for tight repression of transcription prior to the induction with IPTG, and the gene conferring resistance to kanamycin (Km). The encoded fusion proteins His6-SA, His6-SA-SRS2’, and His6-SA-M5, in which SRS2’ or M5 has been included as model immunogens in the latter two, are presented schematically. (B) The general expression vector pAff2c encodes an in vivo biotinylation sequence (Bio), a His6 tag, and an albumin binding protein (ABP). The transcription is under the control of the T7 promoter, and the vector encodes the lac repressor (LacI) for tight repression and β-lactamase (bla) for antibiotic selection. The encoded fusion proteins Bio-His6-ABP-SRS2’ and Bio-His6-ABP-M5, in which SRS2’ and M5 have been included as model immunogens, are presented schematically.
The fusion proteins His₆-SA, His₆-SA-SRS2´, His₆-SA-M5, Bio-His₆-ABP-SRS2´, and Bio-His₆-ABP-M5 were intracellularly produced in *E. coli* cells. High expression levels were demonstrated for His₆-SA, His₆-SA-SRS2´, and His₆-SA-M5 (approximately 12 mg/l, 84 ml/l, and 62 mg/l, respectively). In contrast, the expression levels for Bio-His₆-ABP-SRS2´ and Bio-His₆-ABP-M5 were significantly lower (approximately 4 mg/l and 2 mg/l, respectively). After sonication, the His₆-SA, His₆-SA-SRS2´, and His₆-SA-M5 fusion proteins were recovered by His₆-tag-mediated IMAC on Co²⁺-charged affinity resin columns under denaturing conditions, whereas the Bio-His₆-ABP-SRS2´ and Bio-His₆-ABP-M5 fusion proteins were purified by ABP-mediated affinity chromatography on HSA-Sepharose. All of the proteins were found to be of the correct size, i.e. 18.7 kDa, 41.8 kDa, 24.2 kDa, 41.8 kDa, and 24.2 kDa for His₆-SA, His₆-SA-SRS2´, His₆-SA-M5, Bio-His₆-ABP-SRS2´, and Bio-His₆-ABP-M5, respectively, and the presence of expected regions within the fusion proteins, including the added biotin, was confirmed in Western-blotting analyses.

6.2.3. Iscom association experiments (II)

Iscoms were prepared by mixing fusion protein with pre-formed Ni²⁺ matrix or Bio-matrix, followed by dialysis. In the first approach (Fig. 11A), pre-formed Ni²⁺ matrix was incubated with the His₆-SA protein before addition of the *in vivo*-biotinylated Bio-His₆-ABP-SRS2´ or Bio-His₆-ABP-M5 fusion proteins. The *in vivo*-biotinylated immunogens were expressed in fusion with a His₆ tag to allow efficient recovery even of proteins of low solubility. It could, of course, be argued that the Bio-His₆-ABP-SRS2´ or Bio-His₆-ABP-M5 fusion proteins could be directly associated with the Ni²⁺ matrix, to a certain extent, via the His₆-Ni²⁺ linkage instead of the intended biotin-streptavidin binding between Bio-His₆-ABP-SRS2´/Bio-His₆-ABP-M5 and His₆-SA, complicating interpretation of the results. However, since the biotin-streptavidin binding is at least a hundred times stronger (*Kₐ ≈ 10⁻¹⁵ M*) than the His₆-Ni²⁺ binding (*Kₒ ≈ 10⁻¹³ M*) (INDIA™HRP method; Pierce, Rockford, IL, USA), direct iscom association of the Bio-His₆-ABP-SRS2´ or Bio-His₆-ABP-M5 fusion proteins to the Ni²⁺ matrix would probably not influence the results significantly. Iscom association of the Bio-His₆-ABP-SRS2´ or Bio-His₆-ABP-M5 fusion proteins to the His₆-SA-coated Ni²⁺ matrix should thus mainly be due to the biotin-streptavidin binding. Nevertheless, to demonstrate that the Bio-His₆-ABP-SRS2´ or Bio-His₆-ABP-M5 fusion protein was not essentially binding to available Ni²⁺ positions on the His₆-SA iscoms, the biotinylation level of the *in vivo*-biotinylated Bio-His₆-ABP-SRS2´ was increased by *in vitro* biotinylation, using NHS chemistry, resulting in the double-biotinylated Bio-Bio-His₆-ABP-SRS2´. In the second approach (Fig. 11B), the His₆-SA-SRS2´ and His₆-SA-M5 fusion proteins were separately mixed with Bio-matrix to allow direct association through the biotin-streptavidin interaction. The different iscom preparations were subjected to sucrose density gradient centrifugation and collected fractions were analyzed for the presence of Quil A and protein. The Quil A-rich fractions, detected by its absorbance at 214 nm, are marked with horizontal bars in Figs. 13A, C, and
D, and the co-migration of fusion protein and Quil A was regarded as being indicative of successful iscom association.

To evaluate the first approach for iscom association (Fig. 11A), His$_6$-SA and Bio-His$_6$-ABP-SRS2$^-$ fusion proteins were mixed with pre-formed Ni$^{2+}$ matrix. After ultracentrifugation, large proportions of both fusion proteins were observed,

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

**Fig. 13.** Analysis of the protein content in the fractions collected from sucrose gradient centrifugation of the iscom preparations produced by the biotin-streptavidin approaches. The association of proteins was analyzed by ELISA with SA- (filled circles), NcSRS2- (open squares), and M5- (grey diamonds) specific antibodies in fractions from the iscom association experiments (A) His$_6$-SA + Bio-His$_6$-ABP-SRS2$^-$ admixed with preformed Ni$^{2+}$ matrix, (B) His$_6$-SA + Bio-His$_6$-ABP-SRS2$^-$, (C) His$_6$-SA + Bio-Bio-His$_6$-ABP-SRS2$^-$ admixed with preformed Ni$^{2+}$ matrix, (D) His$_6$-SA-M5 admixed with preformed biotinylated iscom matrix (Bio-matrix), and (E) His$_6$-SA-M5. The horizontal bars indicate the Quil A-rich fractions, detected by absorbance measurements at 214 nm.
as expected, in the Quil A-rich fractions of the sucrose gradient (Fig. 13A), indicating successful iscom association. In a negative control experiment, where the two fusion proteins were subjected to sucrose gradient centrifugation without the Ni\textsuperscript{2+} matrix, a majority of the proteins was found, again as expected, in the upper fractions (Fig. 13B), typical of soluble proteins. However, when mixing His\textsubscript{6}-SA and Bio-His\textsubscript{6}-ABP-SRS2\textsuperscript{´} with regular matrix, a significant proportion of the His\textsubscript{6}-SA, together with a small proportion of the Bio-His\textsubscript{6}-ABP-SRS2\textsuperscript{´}, could still be observed in the Quil A-rich fractions, probably due to unspecific binding of the His\textsubscript{6}-SA protein. For comparison, Ni\textsuperscript{2+} matrix was also mixed with His\textsubscript{6}-SA and the double-biotinylated Bio-Bio-His\textsubscript{6}-ABP-SRS2\textsuperscript{´}. The vast majority of both fusion proteins in this experiment were found in the Quil A-rich fractions (Fig. 13C), demonstrating successful iscom association. The fact that this association was stronger than the association obtained with \textit{in vivo}-biotinylated Bio-His\textsubscript{6}-ABP-SRS2\textsuperscript{´} alone, for which a significant proportion of the protein was found to be un-associated (Fig. 13A), suggests that the \textit{in vivo} biotinylation, through \textit{E. coli} expression using the pAff2c vector, might not be sufficient for quantitative association. The almost quantitative association of Bio-Bio-His\textsubscript{6}-ABP-SRS2\textsuperscript{´} indicates, as expected, that the biotin-streptavidin binding was the major factor in the iscom association. In a negative control experiment, in which the Bio-Bio-His\textsubscript{6}-ABP-SRS2\textsuperscript{´} fusion protein alone was subjected to sucrose gradient centrifugation, the majority of the fusion protein was observed in the upper gradient fractions, as expected, but a significant amount was unexpectedly also seen in the typical Quil A fractions. Positive association results were also obtained in an evaluation of Bio-His\textsubscript{6}-ABP-M5 according to the first approach for iscom association.

To evaluate the second approach for iscom association (Fig. 11B), the His\textsubscript{6}-SA-M5 fusion protein was allowed to interact with pre-formed Bio-matrix. Upon analysis of the sucrose gradient fractions, a majority of the fusion protein was seen in the Quil A-rich fractions (Fig. 13D), thus indicating successful iscom association. In negative control experiments, including His\textsubscript{6}-SA-M5 admixed with matrix and His\textsubscript{6}-SA-M5 alone (Fig. 13E), the fusion protein was, as expected, mainly found in the upper fractions. Positive association results were also obtained in an evaluation of His\textsubscript{6}-SA-SRS2\textsuperscript{´} according to the second approach for iscom association.

These results show that both of the biotin-streptavidin approaches resulted in iscom association of recombinant immunogens, but they do not indicate that one of the approaches would be clearly superior. In the first approach a general fusion protein is used to create streptavidin iscoms and the streptavidin can thus be prepared in bulk. Biotinylation of the immunogens can be performed using either \textit{in vivo} or \textit{in vitro} methods, of which the \textit{in vivo} methods give directed but perhaps incomplete biotinylation. The degree of biotinylation seems to influence association efficiency. In addition, the \textit{in vivo} biotinylation strategy requires cloning of each immunogen into the specific \textit{in vivo} biotinylation vector, and the expression levels from this vector do not seem to be impressive. It would be interesting to evaluate directed \textit{in vitro} biotinylation, which could be obtained using the \textit{E. coli} biotin ligase (Saviranta \textit{et al.}, 1998). The immunogens associated with the Ni\textsuperscript{2+} matrix in
the first approach are only bound to the Ni\textsuperscript{2+} matrix as strongly as by the binding provided by the Ni\textsuperscript{2+}-His\textsubscript{6} interaction, while the iscoms formed by the second approach bind the immunogens to the Bio-matrix directly through the biotin-streptavidin interaction. The second approach is obviously much simpler, involving only one fusion protein that seems to be expressed at high levels. However, an inconvenient feature of the second approach is that each immunogen has to be produced as a fusion to streptavidin.

6.2.4. Immunization of mice with subsequent neosporosis challenge (II, III)
To evaluate if the formed iscoms were capable of inducing antibodies recognizing the target immunogens NcSRS2 and M5, two iscom preparations were selected for immunization experiments; Ni\textsuperscript{2+} matrix + His\textsubscript{6}-SA + Bio-His\textsubscript{6}-ABP-SRS2\textsuperscript{'} (group 1) and Ni\textsuperscript{2+} matrix + His\textsubscript{6}-SA + Bio-His\textsubscript{6}-ABP-M5 (group 2). As shown in Fig. 14, mice from group 1 produced antibodies that were reactive to native *N. caninum* antigens with a mean titer of 1:269 after the first immunization. A booster immunization resulted in a 40-fold increase in the serum antibody titers (1:10784), which was only about two titre steps lower than positive control sera from mice infected with *N. caninum*. Antibodies reacting to M5 were detected in sera from group 2, with mean titers of 1:251 and 1:452 after the first and second immunization, respectively. As expected, no *N. caninum*-specific antibodies were detected in sera from group 2 after the first immunization (titre < 1:30), and only very low titers were detected after the booster immunization (1:85) (Fig. 14). To

![Fig. 14. Antibody titres to *N. caninum*-antigens in sera from mice immunized twice (on days 0 and 28) with Ni\textsuperscript{2+} matrix + His\textsubscript{6}-SA + Bio-His\textsubscript{6}-ABP-SRS2\textsuperscript{'} (group 1) or Ni\textsuperscript{2+} matrix + His\textsubscript{6}-SA + Bio-His\textsubscript{6}-ABP-M5 (group 2), 21 days after each immunization. Data are presented as geometric means ± SEM. Pooled sera from mice infected with *N. caninum* were used as positive control and normal mouse sera as negative control. Since the sera collected at days 21 and 49 were analysed at two separate occasions, the diagram shows the titres of the controls at each test occasion. For simplicity, antibody titers from group 3 mice have not been included in this figure.](image-url)
evaluate whether iscoms with immunogens associated by an unspecific interaction were capable of inducing antibody responses, control mice (group 3) were immunized with regular iscom matrix + His$_6$-SA + Bio-His$_6$-ABP-SRS2$. In subsequent sera analyses, $N$. caninum-specific antibodies were detected after the first and second immunizations with mean titers of 1:184 and 1:14355, respectively; similar to titers for mice from group 2, indicating that the unspecific matrix association of the His$_6$-SA fusion protein is sufficient for inducing high antibody titers to Bio-His$_6$-ABP-SRS2$. This study does not reveal the generality of this unspecific association phenomenon, but the directed association approaches, utilizing the highly specific interactions presented in Fig. 11, would most likely be preferable.

After challenge infection with $N$. caninum tachyzoites, 69 days after the initial immunization, mice from group 1, vaccinated with Ni$^{2+}$ matrix + His$_6$-SA + Bio-His$_6$-ABP-SRS2$, showed only mild and transient symptoms, whereas the non-vaccinated mice from group 2 showed clinical symptoms. Some of them exhibited typical neurological symptoms of murine neosporosis, including hind limb paresis, circling and walking disorders. In addition, mice from this group showed reductions in weight. No clinical signs of neosporosis were detected in mice from an uninfected control group. However, there were considerable variations between individual mice, especially in group 2, and the differences between the groups were not clearly significant ($P = 0.051$). Moreover, a semi-quantitative competitive PCR assay detecting Nc5-repeats was applied to evaluate the level of parasite DNA in the brain. The amount of Nc5-repeats in mice from group 1 was significantly lower than in mice from group 2.

In conclusion, it was found that immunization with recombinant NcSRS2, associated with iscoms through the biotin-streptavidin interaction, can induce specific antibodies to native NcSRS2, as confirmed by immunoblotting analysis, and immunity sufficient to reduce the proliferation of $N$. caninum in the brain of immunized mice. Hence, both of the described approaches exploiting the strong interaction between biotin and streptavidin might offer straightforward methods to achieve efficient adjuvant association of recombinant immunogens for the generation of future recombinant subunit vaccines.

6.2.5. Ongoing studies
The positive results from the neosporosis challenge experiment (III) have encouraged further immunization studies, including immunizations with streptavidin-fused proteins associated with Bio-iscoms by the second approach. Mice have been immunized with recombinant NcSRS2 iscoms by both approaches, but the results from this experiment are not yet available.
7. Selection and characterization of HER2/neu-binding affibody ligands (IV, V, VI)

Conventional drugs used in cancer therapy are often toxic and have limited efficacy. In an attempt to improve cancer treatments, therapies based on targeting monoclonal antibodies to cell-specific markers have been explored since the 1970s. For example, the humanized monoclonal antibody trastuzumab (Herceptin®) has been used in breast cancer therapy in recent years. Trastuzumab binds to the human epidermal growth factor receptor 2 (HER2/neu) (Fig. C), which is often overexpressed in breast and ovarian cancers. Initial attempts to target HER2/neu with radiolabeled intact antibodies resulted in poor tumor-to-blood ratios (Xu et al., 1997; Tsai et al., 2000). However, better results have been obtained for antibody fragments (Willuda et al., 1999; Di Paolo et al., 2003), indicating that their smaller size is advantageous for radionuclide-based in vivo imaging and therapy.

In the work underlying this thesis, the potential utility of SPA-derived affibody ligands as tumor-targeting agents was explored. Novel affibody proteins were selected for binding to a recombinant extracellular domain of HER2/neu (HER2-ECD), and then characterized with respect to their binding to HER2-ECD in biosensor studies and, after radiolabeling, to native HER2/neu overexpressed in the breast cancer cell line SKBR-3. A bivalent affibody ligand was also constructed and similarly evaluated. In addition, the radiolabeled bivalent construct was investigated for binding to the ovary cancer cell line SKOV-3 and for targeting to tumor xenografts in mice.
Human epidermal growth factor receptor 2 (HER2; also called HER2/neu and ErbB2) is a member of the epidermal growth factor receptor (EGFR; also known as ErbB) family of receptor tyrosine kinases. Other members are HER1 (EGFR, ErbB1), HER3 (ErbB3), and HER4 (ErbB4). Normally, these transmembrane receptors function as important mediators of cell proliferation and differentiation in developing embryos and adult tissues (Yarden and Sliwkowski, 2001). However, increased activity is associated with enhanced proliferation and decreased apoptotic capacity. Overexpression of HER2/neu is found in various cancers, including breast and ovarian cancers (Wang and Hung, 2001), but it is expressed only to a low extent or not at all in many normal adult tissues (Natali et al., 1990; Press et al., 1990). Tumor cell-associated overexpression of HER2/neu, often with several million copies of the receptor per cell, has been found in approximately 20-30% of breast cancer patients (Slamon et al., 1987). In patients, overexpression is correlated with more aggressive tumors, including a higher chance of forming metastases, and a poorer prognosis, involving short disease-free time and decreased overall survival. It has also been shown that the overexpression is often preserved in metastases (Ganceberg et al., 2002; Carlsson et al., 2004).

The 185 kDa HER2/neu structure comprises three regions (Cho et al., 2003); (i) a 630 amino acid residue extracellular region, composed of four domains arranged as a tandem repeat of a two-domain unit, (ii) a 23 amino acid residue single transmembrane region, and (iii) a 260 amino acid residue intracellular tyrosine kinase domain, responsible for triggering a signaling cascade event following receptor activation (Yarden and Sliwkowski, 2001). Although ligands have been identified for the other EGF receptors, no natural ligand is known to bind to HER2/neu. Instead, HER2/neu seems to be activated through heterodimerization with other receptors in the family (Yarden and Sliwkowski, 2001).

Since 1998, the humanized monoclonal antibody trastuzumab (Herceptin®) has been used in metastatic breast cancer therapy (Walsh, 2003; Appendix II). It binds to the C-terminal portion of the extracellular domain IV of HER2/neu (Cho et al., 2003) with an apparent affinity of ~1 nM (Park et al., 2000). Trastuzumab is not able to block dimerization, instead its mechanism of action is thought to involve many other processes (Albanell et al., 2003). However, trastuzumab treatment is not curative, and there is only a 40-65% response rate in patients whose tumors have a high expression of HER2/neu, whether the antibody is given alone or in combination with chemotherapy. Therefore, there is an urgent need for more effective treatments. Recently, the promising monoclonal antibody pertuzumab (Omnitarg®) has been shown to bind near the center of the extracellular domain II of HER2/neu, thereby preventing dimerization between HER2/neu and the other EGF receptors (Franklin et al., 2004). In addition, the potential utility of antibodies and antibody fragments directed to HER2/neu in the development of novel drugs for tumor targeting in humans is being investigated in several ongoing studies.

**Fig. C.** The structure of the extracellular region of human HER2/neu in complex with the trastuzumab Fab fragment.
7.1. Selection of HER2/neu-binding affibody ligands from a phage display library (IV)

Phage display in vitro selection technology was used to isolate novel affibody ligands that bind to the extracellular domain of HER2/neu (HER2-ECD). The selection was performed on a portion (8.7 x 10^8 members) of the combinatorial affibody library Zlib2002 (3 x 10^9 members), based on the 58 amino acid residue SPA-derived Z domain. This library was previously constructed by combinatorial substitution mutagenesis of 13 positions located at the surface responsible for Fc binding activity, applying NN(G/T) codons essentially as described earlier (Nord et al., 1995). The different members of the library are monovalently displayed on protein III of M13 bacteriophage particles using a phagemid vector system (Nord et al., 1995, 1997).

Table 5. Panning data from the selection of HER2/neu-binding affibody ligands.

<table>
<thead>
<tr>
<th>Panning cycle no.</th>
<th>Amount of HER2-ECD (µg)</th>
<th>No. of washing steps</th>
<th>Inserted phages (pfu/ml)</th>
<th>Eluted phages (pfu/ml)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>1</td>
<td>8 x 10^{12}</td>
<td>2 x 10^{8}</td>
<td>2 x 10^{-3}</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>3</td>
<td>4 x 10^{13}</td>
<td>5 x 10^{7}</td>
<td>1 x 10^{-4}</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>6</td>
<td>3 x 10^{13}</td>
<td>7 x 10^{8}</td>
<td>2 x 10^{-3}</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>12</td>
<td>5 x 10^{13}</td>
<td>4 x 10^{10}</td>
<td>8 x 10^{-2}</td>
</tr>
</tbody>
</table>

Four rounds of phage display selection were performed using a 100 kDa biotinylated recombinant HER2-ECD as the target protein, immobilized on streptavidin-coated paramagnetic beads. To increase the selection stringency, decreasing amounts of target protein together with an increasing number of washing steps were employed in each round. Panning data from the selection procedure are summarized in Table 5. DNA sequencing performed on 49 randomly picked colonies from the fourth round of biopanning revealed seven different clones, one of which was represented 33 times, indicating significant convergence. Such convergence typically indicates better binding affibody molecules (Nord et al., 1997), but all sequences represented more than once (clones 2, 4, 7 and 8) were selected for further characterization. An alignment of the 13 randomized amino acid residues of the seven unique variants is presented in Fig. 15, showing sequence homology in certain positions.

DNA fragments encoding four different affibody variants (Z_{HER2/neu:2}, Z_{HER2/neu:4}, Z_{HER2/neu:7}, and Z_{HER2/neu:8}) (Fig. 15) were subcloned into the pAY81 vector, resulting in expression vectors encoding, under the control of the T7 promoter, the different affibody molecules fused to an N-terminal His_6 peptide tag, allowing recovery by IMAC. The His_6-Z_{HER2/neu} fusion proteins were intracellularly expressed at high
levels in *E. coli* cells. Subsequent purification on IMAC columns under denaturing conditions showed that pure and stable proteins of expected molecular weights (~8.7 kDa) had been produced (Fig. 16A).

Fig. 15. Amino acid sequence corresponding to the wild-type Z domain aligned to deduced amino acid sequences of different affibody variants selected against HER2-ECD. The 13 randomized amino acid residues (Q9, Q10, N11, F13, Y14, L17, H18, E24, E25, R27, N28, Q32, K35) are presented. Amino acid residues that occur at the same position in more than one of the variants are presented in bold. Note, an amber stop codon is included in the *Z*HER2/neu:25 variant. Horizontal bars indicate amino acid identities. Figures to the right represent the number of times each variant revealed upon DNA sequencing of 49 colonies. The three alpha-helices in the wild-type Z domain are boxed.

### 7.2. Construction of a bivalent affibody ligand (V)

In order to potentially benefit from avidity effects, a bivalent affibody ligand was generated by head-to-tail dimerization. A second gene fragment encoding the *Z*HER2/neu:4 affibody variant was introduced into the pAY81-*Z*HER2/neu:4 vector, resulting in an expression vector denoted pAY81-(*Z*HER2/neu:4)2. This vector encodes the bivalent affibody fused to an N-terminal His6 tag. The His6-(*Z*HER2/neu:4)2 fusion protein was intracellularly expressed with a high yield in *E. coli* cells, and thereafter recovered on an IMAC column under denaturing conditions. Subsequently, the protein was found to be expressed as a full-length protein (~15.6 kDa), and purified to a high degree with no obvious proteolytic degradation (Fig. 16A).

### 7.3. Biosensor binding analyses (IV, V)

Purified affibody ligands were analyzed for HER2-ECD binding by real-time biospecific interaction analysis (BIA) using a BIAcore® biosensor instrument. Three monovalent affibody variants (His6-*Z*HER2/neu:2, His6-*Z*HER2/neu:4, and His6-*Z*HER2/neu:7) were identified as HER2-ECD-binding proteins, although the binding of His6-
Z_{HER2/neu:4} and His\textsubscript{6}-Z_{HER2/neu:7} was considerably stronger than that of His\textsubscript{6}-Z_{HER2/neu:2}.

As expected, no significant binding was observed to any of three control proteins; IgG, HIV-1 gp120, and streptococcal protein BB. In addition, an unrelated control affibody showed no obvious binding to HER2-ECD. These results suggest that the monovalent His\textsubscript{6}-Z_{HER2/neu:4} and His\textsubscript{6}-Z_{HER2/neu:7} affibody variants bind selectively to the target protein HER2-ECD.

To further characterize the His\textsubscript{6}-Z_{HER2/neu:4} and His\textsubscript{6}-Z_{HER2/neu:7} affibody variants, they were subjected to a kinetic analysis in order to determine their kinetic binding constants. Prior to the kinetic analysis, the protein concentration was determined by amino acid analysis. The affibody molecules were then injected over a HER2-ECD flow-cell surface at different concentrations. Evaluation of the binding curves showed the dissociation equilibrium constants (K\textsubscript{D}) to be \( \sim 50 \text{ nM} \) for His\textsubscript{6}-Z_{HER2/neu:4} (Table 6) and \( \sim 140 \text{ nM} \) for His\textsubscript{6}-Z_{HER2/neu:7}, the latter estimated by steady-state determination. The reason for the difference in K\textsubscript{D} of these variants is most likely due to the marked difference in their dissociation rates. For His\textsubscript{6}-Z_{HER2/neu:4}, the association rate constant (k\textsubscript{a}) was calculated to be \( \sim 1.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \) and the dissociation rate constant (k\textsubscript{d}) \( \sim 9.9 \times 10^{-3} \text{ s}^{-1} \) (Table 6), while for His\textsubscript{6}-Z_{HER2/neu:7}, the k\textsubscript{a} and k\textsubscript{d} values were difficult to estimate, due to its fast association and dissociation kinetics (K\textsubscript{D} determined through estimation at steady state). Thus, the most abundant affibody variant, His\textsubscript{6}-Z_{HER2/neu:4}, showed, as expected, the strongest binding to its target and was consequently selected for further characterization.

To investigate if the His\textsubscript{6}-Z_{HER2/neu:4} affibody binds to the same HER2-ECD site as the monoclonal antibody trastuzumab, the affibody was injected over a HER2-ECD flow-cell surface, before and after saturation with trastuzumab. Both of the resulting His\textsubscript{6}-Z_{HER2/neu:4} binding curves had a similar appearance and approximately the same amount of affibody could be bound to the surface, irrespective of preceding trastuzumab saturation, thus indicating that the His\textsubscript{6}-Z_{HER2/neu:4} affibody binds to a different site on the HER2-ECD molecule.

When comparing the HER2-ECD binding of the monovalent His\textsubscript{6}-Z_{HER2/neu:4} and the bivalent His\textsubscript{6}-(Z\textsubscript{HER2/neu:4})\textsubscript{2} affibody proteins, a similar association rate (Fig. 16A; Table 6) was demonstrated for both proteins. However, a significantly slower dissociation rate was observed for the bivalent His\textsubscript{6}-(Z\textsubscript{HER2/neu:4})\textsubscript{2} (Fig. 16A; Table 6), indicating that the binding between HER2-ECD and His\textsubscript{6}-(Z\textsubscript{HER2/neu:4})\textsubscript{2} was stronger than the binding to the monovalent His\textsubscript{6}-Z_{HER2/neu:4}. Such improved apparent affinity, due to avidity effects of dimeric constructs, has been previously demonstrated for affibody ligands (Gunneriusson et al., 1999). In addition, kinetic Biacore analysis confirmed that the bivalent His\textsubscript{6}-(Z\textsubscript{HER2/neu:4})\textsubscript{2} affibody bound with higher affinity (K\textsubscript{D} \( \approx 3 \text{ nM} \)) to HER2-ECD (Fig. 16B; Table 6) compared to the monovalent ligand, due to a reduction in the dissociation rate constant (k\textsubscript{d}) (Table 6). The observed avidity effect might be due to the ability of a bivalent affibody ligand to simultaneously bind two receptor domains, thus increasing the possibility for a dissociated subunit to re-bind if the other subunit is still bound. Alternatively, and perhaps more likely, even in cases where the molecule is not capable of binding to two targets at the same time, the increased concentration of binding sites on the
targeting molecule is often sufficient to accelerate re-binding. Taken together, the bivalent affibody His$_6$-(Z$_{HER2:4}$)$_2$ demonstrated excellent binding characteristics to HER2-ECD.

Table 6. Molecular weight and affinity constants for the monovalent and bivalent affibody ligands.

<table>
<thead>
<tr>
<th>Affibody</th>
<th>M$_a$ (kDa)</th>
<th>K$_D$ (nM)</th>
<th>k$_a$ (M$^{-1}$s$^{-1}$)</th>
<th>k$_d$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>His$<em>6$-Z$</em>{HER2:4}$</td>
<td>8.7</td>
<td>~ 50</td>
<td>~ 1.8 x 10$^5$</td>
<td>~ 9.9 x 10$^{-3}$</td>
</tr>
<tr>
<td>His$<em>6$-(Z$</em>{HER2:4}$)$_2$</td>
<td>15.6</td>
<td>~ 3</td>
<td>~ 2.5 x 10$^5$</td>
<td>~ 7.6 x 10$^{-4}$</td>
</tr>
</tbody>
</table>

*Molecular weight, *Dissociation equilibrium constant, *Association rate constant, *Dissociation rate constant

Fig. 16. Biosensor binding studies. (A) Sensorgrams obtained after injection of the purified monovalent His$_6$-Z$_{HER2:4}$ (open squares) and bivalent His$_6$-(Z$_{HER2:4}$)$_2$ (filled squares) affibody ligands over a sensor chip flow-cell surface containing amine-coupled HER2-ECD. Note, the y values of the curves have been normalized between 0 and 100 Resonance Units (RU). The inserted SDS-PAGE gel (Tris-Glycine 16% homogenous gel, reducing conditions) shows the expressed and IMAC-purified affibody fusion proteins His$_6$-Z$_{HER2:4}$ (lane 1) and His$_6$-(Z$_{HER2:4}$)$_2$ (lane 2). Lane M, marker proteins with molecular masses in kilodaltons (kDa). Protein bands were visualized with Coomassie Brilliant Blue staining. (B) Sensorgrams obtained after injection of the bivalent affibody His$_6$-(Z$_{HER2:4}$)$_2$ over a HER2-ECD flow-cell surface at selected concentrations; 156.3 nM (filled circles), 78.2 nM (filled triangles), 39.1 nM (open squares), 19.6 nM (open diamonds), 9.8 nM (open circles), and 4.9 nM (open triangles). All samples were run at 25°C in duplicates in random order, and the response obtained from an activated and deactivated reference surface has been subtracted from all curves.

7.4. SKBR-3 cell-binding studies using radionuclides (IV, V)

The His$_6$-Z$_{HER2:4}$ and His$_6$-(Z$_{HER2:4}$)$_2$ affibody proteins were indirectly radiolabeled with $^{125}$I by amine coupling. A subsequent Biacore analysis verified that the labeling procedure did not impair the binding affinity to HER2-ECD. The monovalent His$_6$-Z$_{HER2:4}$ affibody showed specific binding to the human breast cancer cell line SKBR-3, which reportedly expresses approximately 2 x 10$^6$ HER2/neu molecules per cell. The binding of radiolabeled His$_6$-Z$_{HER2:4}$ affibody could be efficiently blocked by the addition of an excess of non-labeled affibody,
indicating that it binds specifically to native HER2/neu. Furthermore, the His\textsubscript{6}-Z\textsubscript{HER2/neu:4} affibody and the monoclonal antibody trastuzumab were found to bind HER2/neu in a non-competitive manner, since the other ligand was unable to block binding when added in excess. The results of this cellular binding assay indicate that the presence of an excess of trastuzumab monoclonal antibody may improve binding of the His\textsubscript{6}-Z\textsubscript{HER2/neu:4} affibody molecule to HER2/neu. This effect could be due to trastuzumab binding stabilizing the extracellular domain of HER2/neu, but more experimental evidence would be needed to confirm this possibility. These data corroborate the Biacore results and suggest that there are separate binding sites on HER2/neu for the His\textsubscript{6}-Z\textsubscript{HER2/neu:4} affibody molecule and trastuzumab.

The cellular uptake of \textsuperscript{125}I after delivery with the monovalent His\textsubscript{6}-Z\textsubscript{HER2/neu:4} and bivalent His\textsubscript{6}-(Z\textsubscript{HER2/neu:4})\textsubscript{2} affibody molecules was studied as a function of time. The cellular association of \textsuperscript{125}I was slightly slower for the bivalent affibody, with maximal uptake occurring after 4.5 hours as compared to 2.5 hours for the monovalent affibody. However, the level of association was considerably higher for the bivalent affibody. About nine times more of the added radioactivity was cell-associated at the time of maximal uptake when using the bivalent affibody compared to the monovalent affibody. The unspecific binding of the bivalent affibody, as determined by addition of a 500-fold excess of unlabeled bivalent affibody to minimize specific binding of radiolabeled bivalent affibody, was equivalent to only about 5% of the cell-associated radioactivity. Furthermore, about 30% of the cell-associated radioactivity was internalized after delivery with the monovalent affibody, whereas delivery with the bivalent affibody gave about 50% internalization at all measured time points. Confocal microscopy confirmed that the bivalent His\textsubscript{6}-(Z\textsubscript{HER2/neu:4})\textsubscript{2} affibody was internalized. As shown in Fig. 17, the TexasRed staining was equally distributed throughout the cytoplasm in the 0.18 \( \mu \)m thick confocal section located in the middle of the cells. In control experiments without biotinylated bivalent affibody or without streptavidin-TexasRed conjugate no TexasRed staining appeared.

![Confocal microscopy image of SKBR-3 cells exposed to biotinylated His\textsubscript{6}-(Z\textsubscript{HER2/neu:4})\textsubscript{3} affibody for four hours at 37\(^\circ\)C. Detection was made with streptavidin-conjugated TexasRed. The cell nuclei were stained blue with DAPI. The section is 0.18 \( \mu \)m thick and located in the middle of the cell.](image-url)
The cellular retention of $^{125}$I was studied as a function of time, comparing the monovalent and bivalent affibody molecules as delivery vehicles. It was found that the membrane-associated radioactivity decreased much faster when delivered with the monovalent ($T_{1/2} \approx 3$ min) as compared to the bivalent ($T_{1/2} \approx 2.2$ hours) affibody. The clear difference in retention between the monovalent and bivalent affibody molecules is most likely a result of the avidity effect of the latter. It might even indicate that both binding sites of the bivalent affibody could be involved simultaneously in interaction with HER2/neu molecules that might be close enough on the cell surface. Taken together, the bivalent affibody exhibited profound improvements in both cellular retention and internalization as compared to its monovalent parental molecule.

7.5. SKOV-3 cell-binding studies using radionuclides (VI)

The cellular binding of the bivalent His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ affibody and the monoclonal antibody trastuzumab in the human ovary cancer cell line SKOV-3 was studied as a function of time. The accumulation of $^{125}$I after administration of His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ was somewhat faster than after administration of trastuzumab. $^{125}$I accumulation was maximal about six hours after administration of His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ and about ten hours after administration of trastuzumab. This fast binding of His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ to SKOV-3 cells was similar to its binding to SKBR-3 cells. Unspecific uptake accounted for only 1-2% of the total uptake for both His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ and trastuzumab.

The cellular retention of $^{125}$I delivered by both His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ and trastuzumab was studied as a function of time. Removal of $^{125}$I from the cells was faster after administration of His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ than after administration of trastuzumab ($T_{1/2} \approx 6$ hours for His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ compared to $T_{1/2} \approx 17$ hours for trastuzumab). After 24 hours, however, 30% of the initial $^{125}$I remained, irrespective of whether it was administered via His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ or trastuzumab.

7.6. Biodistribution in tumor-bearing mice (VI)

In biodistribution studies with nude mice carrying SKOV-3 xenografted tumors, the radioactivity distribution following injection with $^{125}$I-labeled His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ was followed as a function of time for up to 24 hours. As shown in Fig. 18, the clearance of $^{125}$I from the blood system was quite rapid, resulting in impressive tumor-to-blood ratios. After eight hours, 1.6 percent of the injected dose per gram tissue (%ID/g) remained in the tumor; ten times more than in the blood. In addition, high levels of radioactivity were found in the kidneys, as a consequence of kidney clearance; a common fate for most proteins of this size (~15.6 kDa).

To study the specificity of the HER2/neu binding in vivo, a 1000-fold excess of unlabeled His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$ was administered 45 minutes before the injection of $^{125}$I-labeled His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$. Since only tumor cells strongly express HER2/neu, only the tumors were expected to show reduced uptake due to the excess of unlabeled His$_6^\text{a}$(Z$_{\text{HER2/neu:a}}$)$_2$. The mice were euthanized four hours later and the
Rational and combinatorial protein engineering for vaccine delivery and drug targeting.

tumor, blood and organ distribution of $^{125}$I was determined. Only the tumor uptake of $^{125}$I was significantly lower ($P < 0.01$) in the group that received high levels of unlabeled His$_6$-(ZHER2/neu:4)$_2$. The tumor-associated radioactivity in this group was only about 25% of the tumor-associated radioactivity in the non-blocked group, indicating that the tumor uptake was specific. In blood and all analyzed normal organs there were no significant changes due to the delivery of unlabeled His$_6$-(ZHER2/neu:4)$_2$. In another specificity study, the biodistribution of an unrelated affibody was compared to the biodistribution of His$_6$-(ZHER2/neu:4)$_2$. Four hours after administration, the tumor uptake of $^{125}$I delivered with the unrelated affibody was significantly lower and only about 15% of the $^{125}$I uptake after delivery with His$_6$-(ZHER2/neu:4)$_2$ ($P < 0.01$). These findings provide further indications that HER2/neu-dependent $^{125}$I uptake occurs in the tumors when radiolabeled His$_6$-(ZHER2/neu:4)$_2$ is applied. In conclusion, significant amounts of radioactivity were specifically targeted to xenografted tumors in mice, and the radiolabeled His$_6$-(ZHER2/neu:4)$_2$ affibody was primarily excreted through the kidneys.

**Fig. 18.** Biodistribution data 1, 4, 8, and 24 hours post injection with $^{125}$I-labeled His$_6$-(ZHER2/neu:4)$_2$ affibody. Columns represent mean values of injected dose per gram tissue (%ID/g) from three to four mice. Error bars are standard deviations.

### 7.7. Gamma camera imaging (VI)

The biodistribution data indicated that gamma tumor images could be obtained about eight hours after injection of $^{125}$I-labeled His$_6$-(ZHER2/neu:4)$_2$. Therefore, animals were euthanized six or eight hours after injection of His$_6$-(ZHER2/neu:4)$_2$ and gamma camera images were acquired using a low energy collimator. Good tumor images were obtained at both the six and eight hour time points (Fig. 19). However, after eight hours, the background radioactivity was lower, and tumor, kidneys and
thyroid areas were better separated and more clearly visible (Fig. 19). As mice are very small, $^{125}$I could be used in these studies despite its low energy gamma emissions. The targeting achieved with $^{125}$I-labeled proteins can be readily extrapolated to other diagnostically and therapeutically interesting iodine-based radioisotopes, including $^{124}$I for PET and $^{131}$I for therapy.

Fig. 19. Gamma camera image. Nude mice carrying SKOV-3 xenografted tumors were injected with $^{125}$I-labeled His$_6$-(Z$\text{HER2/neu}$)$_2$ affibody into their tail vein. The mouse to the left was sacrificed eight hours after injection and the mouse to the right was sacrificed six hours after injection. The tumors had an average diameter of about 6 mm and were localized on the right hind leg.

7.8. Autoradiography/Immunohistochemistry (VI)

Tumors taken from the mice used for gamma camera imaging were analyzed for radioactivity uptake and HER2/neu expression using autoradiography and immunohistochemistry (IHC), respectively (Fig. 20). The IHC study showed uniform HER2/neu expression in the tumors, with clear membrane staining (Fig. 20B). The autoradiography (Fig. 20C) showed a granular distribution of the radioactivity within the tumors. There were no grains in the HER2/neu-negative normal tissue surrounding the tumor. As shown in Fig. 20C, the radioactivity in the tumor was spreading from the blood vessels into the surrounding tissue. There were no differences in the distribution of radioactivity related to tumor size or between the center and borders of the tumors.

The results of the intratumoral distribution study showed that after six hours the HER2/neu-specific delivery of radioactivity within the tumors was associated with the cell layers closest to the blood vessels. However, for radiotherapy applications, particularly those using short-range nuclides such as the $\alpha$-emitting nuclide $^{211}$At, deeper tumor penetration would be necessary. Nevertheless, for
medical imaging purposes, the perivascular localization, seen with the HER2/neu-binding bivalent affibody in this study, should be sufficient.

Fig. 20. Serial tumor sections, six hours post injection with $^{125}$I-labeled His$_6$-(Z$_{HER2/neu}$)$_2$ affibody. The arrows indicate blood vessels and the bars are 100 µm. (A) Hematoxylin staining of nuclei. (B) Immunohistochemistry showing the HER2/neu expression. (C) Autoradiography showing the intratumoral $^{125}$I distribution.
7.9. Ongoing studies

The promising results from the present studies (IV, V, VI) have encouraged further studies on the HER2/neu-binding affibody ligands. Even though the bivalent His₆-(Z₁HER2/neu-₄)₂ affibody variant showed a rather high affinity to HER2-ECD (Kᵥ ≈ 3 nM), stronger binders might be desirable for imaging and therapy purposes. In an attempt to improve the binding strength to HER2/neu, a second-generation affinity-maturated library has been constructed, based on the sequences of the seven clones selected in the work underlying this thesis (IV). Five of the 13 randomized positions were kept un-varied, whilst the remaining eight positions were subjected to further mutagenesis. Re-selection from the second-generation library resulted in the selection of several high-affinity affibody variants. The strongest binding second-generation affibody was demonstrated to bind to HER2-ECD with a Kᵥ of ∼ 22 pM. Furthermore, the selected variant showed specific binding to HER2/neu-expressing SKOV-3 cells, and to tumor xenografts in mice. Notably, the novel affibody variant was significantly improved in terms of its tumor uptake and tumor-to-blood ratio. Impressive tumor-to-blood ratios of 100:1 were obtained in tumor-bearing xenografted mice with the second-generation binder. In addition, a biodistribution analysis showed that uptake was considerably higher in the tumors than in the kidneys. This was further verified in an imaging experiment, in which distinct tumors could be observed in mice 24 hours after immunization with the improved radiolabeled affibody. These outstanding results support the use of affibody ligands as tumor targeting agents, and further studies are obviously proceeding.

In addition, the selected HER2/neu-binding affibody ligand from the present study, Z₁HER2/neu-₄, has been evaluated as a novel ligand in adenovirus-based gene therapy. The non-enveloped Adenovirus (Ad), generally known as the common cold virus, is a double-stranded DNA virus that has a typical icosahedral capsid with twelve homotrimeric fiber proteins protruding from apices on the viral particle. The fibers of Ad serotype 5 (Ad5) are responsible for cellular attachment, primarily via interaction with the coxsackie B virus and Ad receptor (CAR), present at the surface of most human cell types. To permit the therapeutic use of Ad5 vectors, Lindholm and coworkers have made pioneering developments towards re-targeting Ad5 by modifying the fiber protein (Magnusson et al., 2001). The wild-type fiber consists of three functionally and structurally different domains: (i) an N-terminal tail, containing the nuclear-localization signal, which anchors the fiber non-covalently to the penton base in the virion, (ii) a fiber shaft, comprising 22 repeats of an approximately 15 amino acid motif, and (iii) a C-terminal knob domain, containing the trimerization signal, responsible for the initial binding to cellular receptors. To re-target Ad5, the knob domain has been genetically removed, and subsequently replaced by the neck region peptide from human lung surfactant D, to function as a trimerization signal, followed by a new cellular ligand (Magnusson et al., 2001). It should be noted that in order to produce functional virions the introduced ligand must be compatible with virus assembly. Affibody ligands have been reported to be appropriate molecules in this context, since they are able to fold correctly and maintain their receptor-binding capacity in the reducing milieu of the
Rational and combinatorial protein engineering for vaccine delivery and drug targeting.

eukaryotic cell cytoplasm, where the virus proteins are synthesized (Henning et al., 2002). Accordingly, the $Z_{HER2/neu:4}$ ligand was genetically inserted at different positions in the Ad5 genome. First, it was incorporated, both as a monovalent and bivalent construct, into knobless fibers. However, previous studies have shown that recombinant Ad5, containing modified fiber proteins, is difficult to produce in wild-type titers and the fiber content of the de-knobbed virions has been low (Magnusson et al., 2001). For those reasons, the effects of altering other viral capsid sites were examined. The affibody ligand was either incorporated into the HI-loop of the fiber knob, or into the C-terminus of the pIX protein, a minor exposed protein of the Ad5 capsid. The recombinant fibers, both those in which the $Z_{HER2/neu:4}$ ligand replaced the knob and those in which it was inserted into the HI-loop of the knob, were demonstrated to bind to SKOV-3 cells. In addition, the de-knobbed $Z_{HER2/neu:4}$ fibers could be incorporated into viable Ad5 virions with retained specificity. However, Ad5 virions containing the $Z_{HER2/neu:4}$-liganded pIX protein showed no binding to HER2-ECD, probably due to steric hindrance. Nevertheless, these results represent an important step towards the development of genetically re-targeted adenoviruses for use as gene therapy vectors, and thus further studies are on-going.
8. Concluding remarks

In the first series of studies, novel methods were successfully developed for incorporating protein immunogens into iscoms. In many cases, subunit vaccine candidates are hydrophilic subdomains of proteins from the surface of bacteria, viruses, or eukaryotic parasites. Unfortunately, these protein antigens are both typically poorly immunogenic in themselves and difficult to incorporate into adjuvants, such as iscoms. The strategies presented in this thesis offer convenient methods to achieve efficient adjuvant incorporation of recombinant immunogens. Furthermore, these general methods could simplify the generation of well-defined and uniform vaccine preparations, which are important from regulatory perspectives. The described approaches are primarily intended for early stages of vaccine development, for example in screening antigens to find the most suitable immunogen or subdomain. Nevertheless, the presented strategies might also be useful in the design of final vaccines, especially in the veterinary field, but also potentially for human use.

In the second series of studies, HER2/neu-specific affibody ligands were selected and characterized. HER2/neu overexpression in breast cancer correlates with poor patient prognosis, and visualization of HER2/neu expression might provide valuable diagnostic information for case management. A bivalent version of the best affibody ligand bound HER2/neu with nanomolar affinity, similar to the affinity reported for the monoclonal antibody trastuzumab. However, an advantage of the bivalent affibody is that it has a ten-fold lower molecular weight than the antibody, which should improve its tissue and tumor penetration properties. In addition, it is highly stable and could be cost-efficiently produced in bacteria. The half-life of affibody molecules in mice, of the order of 15 minutes, seems to be quite suitable for imaging purposes, in which unnecessary exposure to radioactivity should be avoided. Full-sized antibodies with substantially longer circulation times give background staining and thus poor contrast. Furthermore, since this affibody ligand does not bind to the same epitope as trastuzumab, the affibody could potentially be used to monitor HER2/neu expression during Herceptin treatment. In biodistribution studies in mice, the His$_6$-(Z$_{HER2/neu}$:4$_2$) affibody demonstrated excellent tumor-to-non-tumor ratios which were further confirmed by gamma camera visualization. Thus, HER2/neu-binding affibody ligands might constitute promising targeting agents for future use in radionuclide-based medical imaging and treatment of breast, ovarian and other HER2/neu overexpressing tumors.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ABP</td>
<td>albumin binding protein</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>Bio-matrix</td>
<td>biotinylated iscom matrix</td>
</tr>
<tr>
<td>dAb</td>
<td>single domain antibody</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Fab</td>
<td>fragment, antigen binding</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>Fc</td>
<td>fragment, crystallizable; constant region of Ig</td>
</tr>
<tr>
<td>Fv</td>
<td>fragment, variable</td>
</tr>
<tr>
<td>HER2-ECD</td>
<td>extracellular domain of HER2/neu</td>
</tr>
<tr>
<td>HER2/neu</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>hexahistidyl</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal ion affinity chromatography</td>
</tr>
<tr>
<td>Iscom</td>
<td>immunostimulating complex</td>
</tr>
<tr>
<td>Iscom matrix</td>
<td>iscom particles without any antigen</td>
</tr>
<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>dissociation equilibrium constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>M5</td>
<td>malaria peptide from <em>Plasmodium falciparum</em></td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque-forming unit</td>
</tr>
<tr>
<td>Quil A</td>
<td>saponin derived from <em>Quillaja saponaria</em> Molina</td>
</tr>
<tr>
<td>SA</td>
<td>streptavidin</td>
</tr>
<tr>
<td>SAG1</td>
<td>surface antigen 1 of <em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>scFv</td>
<td>single-chain Fv fragment</td>
</tr>
<tr>
<td>SPA</td>
<td><em>Staphylococcus aureus</em> protein A</td>
</tr>
<tr>
<td>SRS2</td>
<td>surface antigen of <em>Neospora caninum</em></td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt;</td>
<td>variable domain of the Ig heavy chain</td>
</tr>
<tr>
<td>V&lt;sub&gt;L&lt;/sub&gt;</td>
<td>variable domain of the Ig light chain</td>
</tr>
<tr>
<td>Z&lt;sub&gt;HER2/neu&lt;/sub&gt;</td>
<td>HER2/neu-binding affibody</td>
</tr>
</tbody>
</table>
Acknowledgements

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References


toward mapping the human vasculature by phage display. Nat. Med. 8:121-127.


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Rational and combinatorial protein engineering for vaccine delivery and drug targeting.


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## Appendices

### Appendix I: Recombinant vaccines currently approved in the United States (US) or Europe (EU) (modified from Walsh, 2003).

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Description</th>
<th>Application</th>
<th>Company</th>
<th>Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hepatitis B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recombivax</td>
<td>rHBsAg produced in <em>Saccharomyces cerevisiae</em></td>
<td>Hepatitis B prevention</td>
<td>Merck</td>
<td>1986 (US)</td>
</tr>
<tr>
<td>Tritanrix-HB</td>
<td>combination vaccine, containing rHBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Vaccination against hepatitis B, diphtheria, tetanus, and pertussis</td>
<td>SmithKline Beecham</td>
<td>1996 (EU)</td>
</tr>
<tr>
<td>Comvax</td>
<td>combination vaccine, containing HBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Vaccination of infants against <em>Haemophilus influenzae</em> type B and hepatitis B</td>
<td>Merck</td>
<td>1996 (US)</td>
</tr>
<tr>
<td>Infanrix Hep B</td>
<td>combination vaccine, containing HBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Immunization against diphtheria, tetanus, pertussis, and hepatitis B</td>
<td>SmithKline Beecham</td>
<td>1997 (EU)</td>
</tr>
<tr>
<td>Primavax</td>
<td>combination vaccine, containing HBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Immunization against diphtheria, tetanus, and hepatitis B</td>
<td>Aventis Pasteur</td>
<td>1998 (EU)</td>
</tr>
<tr>
<td>Procomvax</td>
<td>combination vaccine, containing HBsAg as one component</td>
<td>Immunization against <em>H. influenzae</em> type B and hepatitis B</td>
<td>Aventis Pasteur</td>
<td>1999 (EU)</td>
</tr>
<tr>
<td>Hexavax</td>
<td>combination vaccine, containing HBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Immunization against diphtheria, tetanus, pertussis, hepatitis B, polio, and <em>H. influenzae</em> type B</td>
<td>Aventis Pasteur</td>
<td>2000 (EU)</td>
</tr>
<tr>
<td>Hepacare</td>
<td>rS, pre-S, and pre-S2 HBsAgs produced in a murine cell line</td>
<td>Immunization against hepatitis B</td>
<td>Medeva Pharma</td>
<td>2000 (EU)</td>
</tr>
<tr>
<td>Infanrix-Penta</td>
<td>combination vaccine, containing HBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Immunization against diphtheria, tetanus, pertussis, polio, and</td>
<td>SmithKline Beecham</td>
<td>2000 (EU)</td>
</tr>
</tbody>
</table>
Rational and combinatorial protein engineering for vaccine delivery and drug targeting.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Description</th>
<th>Application</th>
<th>Company</th>
<th>Approved</th>
</tr>
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<tbody>
<tr>
<td>Infanrix-Hexa</td>
<td>combination vaccine, containing rHBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Immunization against diphtheria, tetanus, pertussis, <em>H. influenzae</em> type B, hepatitis B, and polio</td>
<td>SmithKline Beecham</td>
<td>2000 (EU)</td>
</tr>
<tr>
<td>Twinrix</td>
<td>Adult and pediatric forms in EU, combination vaccine, containing rHBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Immunization against hepatitis A and B</td>
<td>SmithKline Beecham (EU) GlaxoSmithKline (US)</td>
<td>1996 (EU) (adult) 1997 (EU) (pediatric) 2001 (US)</td>
</tr>
<tr>
<td>HBVAXPRO</td>
<td>rHBsAg produced in <em>S. cerevisiae</em></td>
<td>Immunization of children and adolescents against hepatitis B</td>
<td>Aventis Pharma</td>
<td>2001 (EU)</td>
</tr>
<tr>
<td>Pediariix</td>
<td>combination vaccine, containing rHBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Immunization of children against various conditions inducing hepatitis B</td>
<td>SmithKline Beecham</td>
<td>2002 (US)</td>
</tr>
<tr>
<td>Ambirix</td>
<td>combination vaccine, containing rHBsAg produced in <em>S. cerevisiae</em> as one component</td>
<td>Immunization against hepatitis A and B</td>
<td>GlaxoSmithKline</td>
<td>2002 (EU)</td>
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**Other**

<table>
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<tbody>
<tr>
<td>Lymerix</td>
<td>rOspA, a surface lipoprotein of <em>Borrelia burgdorferi</em>, produced in <em>E. coli</em></td>
<td>Lyme disease vaccine</td>
<td>SmithKline Beecham</td>
<td>1998 (US)</td>
</tr>
<tr>
<td>Tricelluvax</td>
<td>Combination vaccine containing r modified pertussis toxin as one component</td>
<td>Immunization against diphtheria, tetanus, and pertussis</td>
<td>Chiron SpA</td>
<td>1999 (EU)</td>
</tr>
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</table>
### Appendix II: Monoclonal antibody-based products currently approved in the United States (US) or Europe (EU) (modified from Walsh, 2003).

<table>
<thead>
<tr>
<th>Trade name (Generic name)</th>
<th>Description</th>
<th>Application</th>
<th>Company</th>
<th>Approved</th>
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<tbody>
<tr>
<td>Orthoclone OKT3 (Muromomab CD3)</td>
<td>Murine mAb against the T-lymphocyte surface antigen CD3</td>
<td>Reversal of acute kidney transplant rejection</td>
<td>Ortho Biotech</td>
<td>1986 (US)</td>
</tr>
<tr>
<td>OncoScint CR/OV (Satumomab pendetide)</td>
<td>Murine mAb against TAG-72, a tumor-associated glycoprotein</td>
<td>Detection/staging/ follow-up of colorectal and ovarian cancers</td>
<td>Cytogen</td>
<td>1992 (US)</td>
</tr>
<tr>
<td>ReoPro (Abciximab)</td>
<td>Fab fragments derived from a chimeric mAb against the platelet surface receptor GPIIb/IIIa</td>
<td>Prevention of blood clots</td>
<td>Centocor</td>
<td>1994 (US)</td>
</tr>
<tr>
<td>Indimacis 125 (Igovomab)</td>
<td>Murine Fab2 against the tumor-associated antigen CA125</td>
<td>Diagnosis of ovarian adenocarcinoma</td>
<td>CIS Bio</td>
<td>1996 (US)</td>
</tr>
<tr>
<td>CEA-scan (Arcitumomab)</td>
<td>Murine Fab against human carcinoembryonic antigen CEA</td>
<td>Detection of recurrent/metastatic colorectal cancer</td>
<td>Immunomedics</td>
<td>1996 (US, EU)</td>
</tr>
<tr>
<td>MyoScint (Imiciromab-pentetate)</td>
<td>Murine mAb fragment against human cardiac myosin</td>
<td>Myocardial infarction imaging agent</td>
<td>Centocor</td>
<td>1996 (US)</td>
</tr>
<tr>
<td>ProstaScint (Capromab-pentetate)</td>
<td>Murine mAb against the tumor surface antigen PSMA</td>
<td>Detection/staging/ follow-up of prostate adenocarcinoma</td>
<td>Cytogen</td>
<td>1996 (US)</td>
</tr>
<tr>
<td>Tecnemab KI</td>
<td>Murine Fab/Fab2 mix against HMW-MAA</td>
<td>Diagnosis of cutaneous melanoma lesions</td>
<td>Sorin</td>
<td>1996 (US)</td>
</tr>
<tr>
<td>Verluma (Nofetumomab)</td>
<td>Murine Fab against carcinoma-associated antigen</td>
<td>Detection of small-cell lung cancer</td>
<td>Boehringer Ingelheim/NeoRx</td>
<td>1996 (US)</td>
</tr>
<tr>
<td>Rituxan (Rituximab)</td>
<td>Chimeric mAb against CD20 surface antigen of B lymphocytes</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>Genentech/ IDEC Pharmaceuticals</td>
<td>1997 (US)</td>
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<tr>
<td>LeukoScan (Sulesomab)</td>
<td>Murine Fab against NCA 90, a surface granulocyte nonspecific cross-</td>
<td>Diagnostic imaging for infection/inflammation in bone of</td>
<td>Immunomedics</td>
<td>1997 (EU)</td>
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</table>
Rational and combinatorial protein engineering for vaccine delivery and drug targeting.

<table>
<thead>
<tr>
<th>Trade name (Generic name)</th>
<th>Description</th>
<th>Application</th>
<th>Company</th>
<th>Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simulect (Basiliximab)</td>
<td>Reacting antigen</td>
<td>Prophylaxis of acute organ rejection in allogenic renal transplantation</td>
<td>Novartis</td>
<td>1998 (EU)</td>
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<td>Mabthera (Rituximab)</td>
<td>Chimeric mAb against the α-chain of the IL-2 receptor</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>Hoffman-La Roche</td>
<td>1998 (EU)</td>
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<tr>
<td>Humaspect (Votumumab)</td>
<td>Human mAb against cytokeratin tumor-associated antigen</td>
<td>Detection of carcinoma of the colon or rectum</td>
<td>Organon Teknika</td>
<td>1998 (EU)</td>
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<tr>
<td>Zenapax (Daclizumab)</td>
<td>Humanized mAb against the α-chain of the IL-2 receptor</td>
<td>Prevention of acute kidney transplant rejection</td>
<td>Hoffman-La Roche</td>
<td>1997 (US) 1999 (EU)</td>
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<tr>
<td>Synagis (Palivizumab)</td>
<td>Humanized mAb against an epitope on the surface of respiratory syncytial virus</td>
<td>Prophylaxis of lower tract respiratory disease caused by syncytial virus in pediatric patients</td>
<td>MedImmune (US) Abbott (EU)</td>
<td>1998 (US) 1999 (EU)</td>
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<td>Remicade (Infliximab)</td>
<td>Chimeric mAb against TNF-α</td>
<td>Treatment of Crohn’s disease</td>
<td>Centocor</td>
<td>1998 (US) 1999 (EU)</td>
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<td>Herceptin (Trastuzumab)</td>
<td>Humanized mAb against HER2</td>
<td>Treatment of metastatic breast cancer if tumor overexpresses HER2</td>
<td>Genentech (US) Roche Registration (EU)</td>
<td>1998 (US) 2000 (EU)</td>
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<tr>
<td>Mylotarg (Gemtuzumab zogamicin)</td>
<td>Humanized antibody-toxic antibiotic conjugate against CD33 antigen found on leukemic blast cells</td>
<td>Acute myeloid leukemia</td>
<td>Wyeth</td>
<td>2000 (US)</td>
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<tr>
<td>Campath (US) Mabcampath (EU) (Alemtuzumab)</td>
<td>Humanized mAb against CD52 surface antigen of B lymphocytes</td>
<td>Chronic lymphocytic leukemia</td>
<td>Berlex, ILEX &amp; Millennium Pharmaceuticals (US) Millennium &amp; ILEX (EU)</td>
<td>2001 (US, EU)</td>
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<td>Zevalin (Ibritumomab tiuxetan)</td>
<td>Murine mAb produced in a CHO cell line, against the CD20 antigen, a radiotherapy agent</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>IDEC Pharmaceuticals</td>
<td>2002 (US)</td>
</tr>
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<td>Humira (Adalimumab)</td>
<td>Recombinant human mAb (anti-TNF), created using phage display technology and produce in a mammalian cell line</td>
<td>Rheumatoid arthritis</td>
<td>Abbott Laboratories</td>
<td>2002 (US)</td>
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<td>Xolair (Omalizumab)</td>
<td>Recombinant IgG1kMab that binds IgE, produced in CHO cells</td>
<td>Asthma</td>
<td>Genentech</td>
<td>2003 (US)</td>
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<td>Bexxar (Tositumomab)</td>
<td>Radiolabeled mAb against CD20, produced in a mammalian cell line</td>
<td>Treatment of CD20 positive follicular non-Hodgkin’s lymphoma</td>
<td>Corixa/ GlaxoSmithKline</td>
<td>2003 (US)</td>
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</tbody>
</table>