Kaija Alfthan

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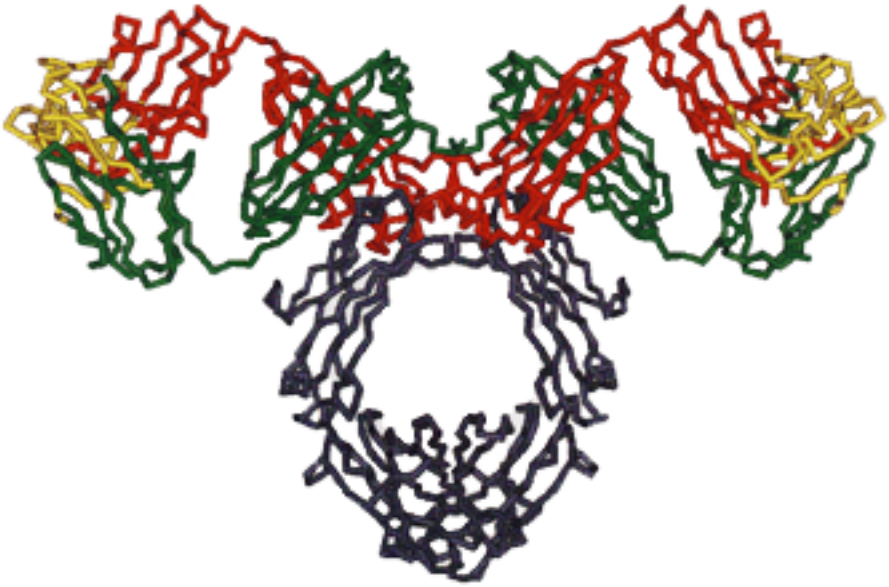

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Structural stability and binding properties of soluble and membrane-anchored recombinant antibodies

Kaija Alfthan
VTT Biotechnology

Department of Biosciences
Division of Biochemistry
Faculty of Science
University of Helsinki, Finland

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puh. vaihde (09) 4561, faksi (09) 456 4374

Statens tekniska forskningscentral (VTT), Bergsmansvägen 5, PB 2000, 02044 VTT
tel. växel (09) 4561, fax (09) 456 4374

Technical Research Centre of Finland (VTT), Vuorimiehentie 5, P.O.Box 2000, FIN–02044 VTT, Finland
phone internat. + 358 9 4561, fax + 358 9 456 4374

VTT Biotekniikka, Biomolekyylit, Tietotie 2, PL 1500, 02044 VTT
puh. vaihde (09) 4561, faksi (09) 455 2103

VTT Bioteknik, Biomolekyler, Datavägen 2, PB 1500, 02044 VTT
tel. växel (09) 4561, fax (09) 455 2103

VTT Biotechnology, Biomolecules, Tietotie 2, P.O.Box 1500, FIN–02044 VTT, Finland
phone internat. + 358 9 4561, fax + 358 9 455 2103

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Abstract

Antibody engineering and advances in microbial expression systems have enabled production of small, active antibody fragments. However, the functional expression yields of these recombinant antibodies vary widely. The results described in this thesis elucidate factors influencing the expression of stable and active antibody fragments in bacteria. The model antibody used throughout this study was a mouse monoclonal antibody binding to 2-phenyloxazolone (Ox). It was shown that the first constant domain of the heavy chain (C_H1) has a remarkable effect on secretion of functional and stable Fab fragments. Comparison of the production of the Ox IgG1 and Ox IgG3 subclass Fab fragments in bacteria demonstrated the superiority of the Ox IgG1 Fab compared to the Ox IgG3 Fab. In addition to its effect on secretion, the C_H1 domain contributes to the thermal stability of the antibody fragments. To study the effect of a linker peptide on both proteolytic stability and binding activity of single-chain antibodies, six different Ox scFv derivatives and an Ox Fv fragment with no joining peptide between the variable domains were constructed. It was shown that joining of the variable domains with a linker peptide improved hapten binding properties compared to the Ox Fv fragment, but may expose the fragment to proteolytic degradation. Truncation of the linker peptide to less than 12 amino acids induced formation of dimers or multimers. The binding affinities determined for the monomeric Ox scFv and Ox IgG1 Fab fragments using the BIAcore biosensor and fluorescence quenching methods were close to each other and comparable to that of the parental monoclonal antibody.

In addition to studies with soluble antibody fragments, this work was extended to cover characterization of antibody fragments displayed on liposomes and on the surface of baculovirus. Immunoliposomes have potential applications both in therapy and in immunodiagnostics. In this work liposomes displaying antibodies were generated by incorporation of purified lipid-tagged Ox scFvs expressed in

E. coli into phospholipid liposomes. It was demonstrated that the biosynthetically lipid-tagged Ox scFv molecules can be immobilized in a functional, stable and oriented manner onto liposomal membranes, resulting in immunoliposomes showing specific hapten binding. BIAcore analysis of the immunoliposomes revealed very slow dissociation from the Ox surface, which is in good agreement with the predicted multivalent nature of the immunoliposomes. The baculoviral display approach holds a promise as a candidate for a eukaryotic display system. In this work single-chain antibodies were used as a model to investigate functional expression of foreign proteins on the surface of baculovirus. Viral vectors displaying cell targeting moieties such as single-chain antibodies have aroused interest as gene transfer vehicles. Two Ox scFv derivatives and a human scFv specific for carcinoembryonic antigen (CEA) were fused to the major envelope protein (gp64) of the *Autographa californica* nuclear polyhedrosis virus (AcNPV). It was shown that the two single-chain antibodies which contained a $(GGGGS)_3$ linker peptide were incorporated into the budded virus particles and expressed as functional hapten/antigen binding fragments on the viral surface. In the case of the third scFv containing a natural linker peptide derived from a fungal cellulase, less efficient incorporation into the virus particles was observed.

Preface

This work was carried out at VTT Biotechnology (previuosly named VTT Biotechnology and Food Research). I express my sincere gratitude to Research Director, Professor Juha Ahvenainen, Professor Matti Linko, Research Professor Hans Söderlund and Research Manager Richard Fagerström for providing excellent working facilities and for their encouragement during this work. Professor Carl Gahmberg at the Department of Biosciences, Division of Biochemistry, University of Helsinki, is warmly acknowledged not only for his co-operation during the final stages of my thesis but also for his constant support throughout my studies. I am grateful to Docents Ari Hinkkanen and Harri Siitari, the official referees of my thesis, for providing constructive criticism and valuable comments, which clearly improved the content of my thesis.

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List of publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals (I–V). In addition some unpublished data is presented.

I Alfthan, K., Takkinen, K., Sizmann, D., Seppälä, I., Immonen, T., Vanne, L., Keränen, S., Kaartinen, M., Knowles, J.K.C. and Teeri, T.T. 1993. Efficient secretion of murine Fab fragments by *Escherichia coli* is determined by the first constant domain of the heavy chain. *Gene*, 128, 203–209.

II Laukkanen, M.-L., Alfthan, K. and Keinänen, K. 1994. Functional immunoliposomes harboring a biosynthetically lipid-tagged single-chain antibody. *Biochemistry*, 33, 11664–11670.

III Alfthan, K., Takkinen, K., Sizmann, D., Söderlund, H. and Teeri, T.T. 1995. Properties of a single-chain antibody containing different linker peptides. *Protein Eng.*, 8, 725–731.

IV Mottershead, D., Alfthan, K., Ojala, K., Takkinen, K. and Oker-Blom, C. 2000. Baculoviral display of functional scFv and synthetic IgG-binding domains. *Biochem. Biophys. Res. Commun*., 275, 84–90.

V Alfthan, K. 1998. Surface plasmon resonance biosensors as a tool in antibody engineering (a review). *Biosens. Bioelectron.*, 13, 653–663.

Contents

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/inf/pdf)

Abbreviations

aa	amino acid
BSA	bovine serum albumin
BV	budded virus
CBHI	cellobiohydrolase I of *Trichoderma reesei*
cDNA	complementary deoxyribonucleic acid
CDR	complementary determining region
CEA	carcinoembryonic antigen
C_H1	first constant domain of the Ig heavy chain
C_L	constant domain of the Ig light chain
ELISA	enzyme-linked immunosorbent assay
Fd	V_HC_H1 fragment
Ig	immunoglobulin
IMAC	immobilized metal affinity chromatography
K_A	affinity constant
k_{ass}	association rate constant
kDa	kilodalton
k_{diss}	dissociation rate constant
lpp	the major lipoprotein of *Escherichia coli*
mRNA	messenger ribonucleic acid
OG	*n*-octyl-β-D-glucopyranoside
Ox	2-phenyloxazolone
Ox_nBSA	BSA containing n molecules of Ox
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
scFv	single-chain antibody
SPR	surface plasmon resonance
V_H	variable domain of a heavy chain
V_L	variable domain of a light chain

1. Introduction

Vertebrates have different defense mechanisms to protect themselves against infective microorganisms and other foreign substances. Skin and mucous membranes form physiological barriers, while B and T lymphocytes together with antibodies provide a more specific defense mechanism to neutralize or destroy the foreign invaders. Foreign substances that can induce an immune response are called immunogens or antigens. Haptens are molecules which are too small to alone act as immunogens, but which can generate an immune response when coupled to larger macromolecules (carriers). Specific immune responses are divided into two classes, humoral and cell-mediated, depending on the components of the immune system which mediate the response. The humoral response is mediated by B lymphocytes and results in secretion of specific circulating antibodies that eliminate the antigen. In the cell-mediated response T lymphocytes perform helper functions as well as induce and promote destruction of cells infected by various types of viruses or intracellular bacteria.

1.1 Antibody structure

Antibodies or immunoglobulins (Igs) have a common Y-shaped core structure consisting of two identical light (L) chains (about 25 kDa) and two identical heavy (H) chains (50–75 kDa). One L chain is joined to each H chain and the two H chains are joined together by disulfide bonds (Fig. 1). Antibodies are divided into five main classes depending on the structure of the H chain. These classes are IgA, IgD, IgE, IgG and IgM and the corresponding H chains are designed by α, δ, ε, γ and μ, respectively. IgG antibodies can be further divided into four subclasses: IgG1–IgG4 in humans and IgG1, IgG2a, IgG2b and IgG3 in mouse. In a complete antibody molecule, each of the H chains is joined either to a kappa (κ) or a lambda (λ) L chain. In human serum, antibodies with κ and λ light chains are represented in about equal numbers whereas in mice, L_κ-containing antibodies are about ten times more frequent than L_λ-containing antibodies. IgG and IgE antibodies circulate in the blood as monomers, whereas IgA and IgM are found as dimers and pentamers, respectively. In addition to dimers, IgA antibodies can exist in monomeric form. IgD is rarely secreted and can be found as membrane-bound on the surface of B lymphocytes.

Both the L and H chains consist of several homologous structural polypeptide units, which fold independently forming a globular motif called an immunoglobulin domain (or fold) (Poljak *et al.*, 1973). In both chains the N-terminal domains have the greatest sequence variation between different antibody molecules and are therefore known as the variable (V) domains. These domains form the antigen binding site of the molecule. An intact antibody molecule is thus bivalent, containing two identical antigen binding sites (Fig. 1).

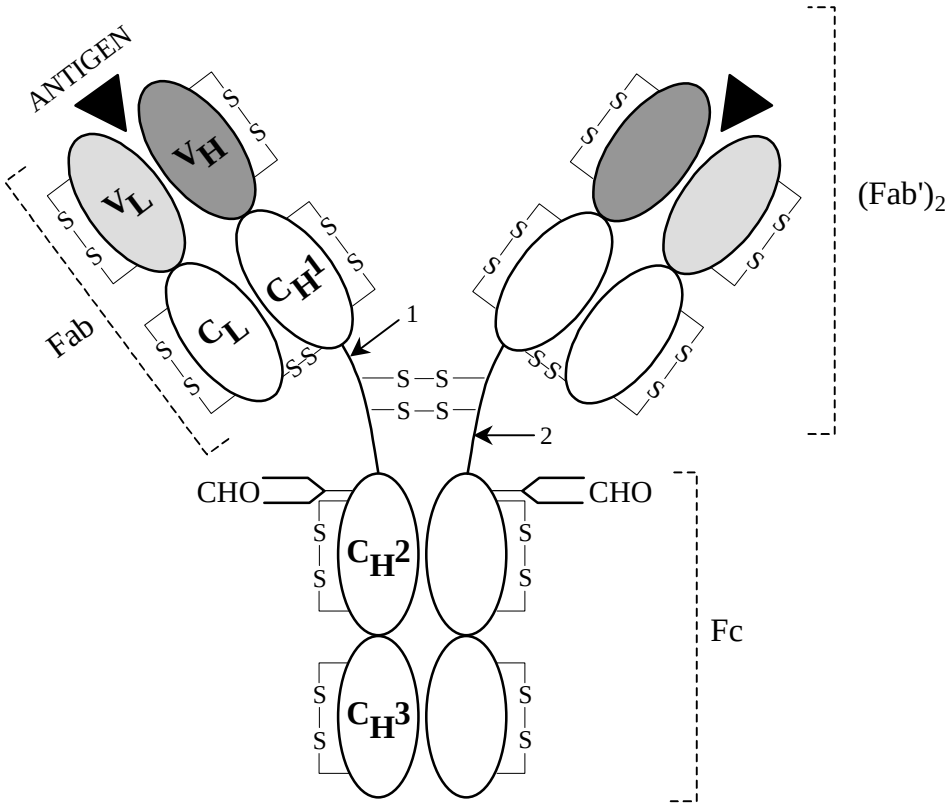


Figure 1. Structure of an IgG molecule. The heavy (H) and light (L) chain and the variable (V) and constant (C) domains are shown. The positions of the interchain and intrachain disulfide bonds (-S-S-) and the carbohydrate moieties (CHO) are marked. The proteolytic cleavage sites by papain (1) and pepsin (2) together with the resulting proteolytic fragments $(Fab')_2$, Fab and Fc are indicated.

The V regions of the H chain (V_H) and the L chain (V_L) contain four relatively conserved sequence stretches called framework regions (FR1–FR4). These structures form a scaffold for three hypervariable loops known as the complementary determing regions (CDR1–CDR3) which contain most of the antigen binding amino acid residues. Sequence and structure analysis of antibody molecules have shown that five of the six hypervariable regions occur in a limited set of main-chain conformations called canonial structures (Chothia and Lesk, 1987). The V domains are joined to more conserved constant (C) domains. There is one C domain in the L chain (C_L), whereas their number in H chains varies between three and four (C_H1–C_H4) depending on the Ig class. Further, the H chains are characteristically glycosylated at asparagine side chains located in most cases at the C_H2 domain (Fig. 1) (Sutton and Philips, 1983). Some antibody classes (IgM and IgA) are glycosylated at the C_H1 domain (Davies and Shulman, 1989; Young *et al*., 1990).

In the H chains there is a nonglobular helical sequence between the C_H1 and C_H2 domains specified as the hinge region. The hinge region varies in length between different antibody classes and it confers further flexibility to the antibody molecule. Proteolytic digestion of an IgG molecule with papain occurs at the hinge region producing two identical monovalent antigen binding Fab fragments (Fig. 1) each consisting of the entire L chain and the V_HC_H1 domains (Fd). Pepsin digests the IgG molecule on the C-terminal site of the disulfide bonds leaving the H chains joined. This enzyme produces thus a bivalent $F(ab')_2$ fragment (Fig. 1). The rest of the molecule, the Fc fragment, mediates effector functions of the antibody. Glycosylation of the H chain contributes to antibody stability and mediates effector functions such as complement binding and activation (Nose and Wigzell, 1983; Muraoka and Shulman, 1989; Miletic and Frank, 1995).

1.2 Recombinant antibodies

Antibodies can be obtained by immunizing animals with the antigen and collecting the sera which contain a heterogeneous mixture of antibodies. However, nowadays these polyclonal antibodies are in many cases replaced by monoclonal antibodies (Köhler and Milstein, 1975) which have a defined specificity, and can be produced in consistent quality. Although murine

antibodies have been widely used in diagnostic assays and for research purposes, their use in human therapy is limited due to immune responses against murine antibodies frequently observed in patients during clinical trials (Schroff *et al.* 1985; Shawler *et al.* 1985; for a review, see Colcher *et al*., 1998). Production of human monoclonal antibodies by conventional hybridoma technology has proved to be difficult due to the inefficiency of the fusion process and unstability of the human hybridoma cell lines (James and Bell, 1987). One strategy to circumvent this problem is to produce human antibodies in transgenic mice. In this system endogenous murine Ig genes have been replaced with human Ig genes so that the mice upon immunization with antigen produce human antibodies (Brüggemann *et al*., 1991; Jakobovits, 1995; Brüggemann and Taussig, 1997).

An alternative approach to decrease the immunogenicity of the murine antibodies is to redesign or engineer the antibodies using recombinant DNA technology. It is possible to create chimeric antibodies which contain mouse V domains joined to human C domains (Boulianne *et al.,* 1984; Morrison *et al.,* 1984). The immunogenicity can be further minimized by grafting the murine V region CDRs in to a human V region framework, resulting in a reshaped (humanized) antibody molecule, most of which is of human origin (Jones *et al.,* 1986; Morrison, 1992; Colcher *et al*., 1998).

Many applications involving the use of antibodies are based on their specific binding properties, whereas their other biological functions, such as complement activation mediated by the Fc fragment, are not necessary. The globular domain structure of an antibody molecule is convenient for genetic engineering and enables generation of tailor-made antibody fragments for a variety of purposes. One advantage of the new technology is that the size of the antibody molecule can be reduced while retaining the intact antigen binding site of the molecule. Compared with the complete antibody molecule these minimized antibodies have several advantages in clinical applications, including improved tissue penetration, rapid clearance from serum and reduced immunogenicity (Colcher *et al*., 1998). The small size also facilitates the expression of these functional fragments and their fusions in bacteria (see 1.2.1) as well as allows their display on a filamentous phage (see 1.4.1). Nowadays, human antibody fragments and other antibodies which are difficult to obtain by hybridoma technology can be isolated from phage-based libraries (Marks *et al*. 1991; Griffiths *et al.,* 1993*;*

Nissim *et al.*, 1994). Further, the small size of the recombinant antibody fragments together with the efficient microbial production systems allow production of a homogenous protein in amounts sufficient also for structural studies (see 1.3.3).

Since the advent of modern gene technology, a wide variety of genetically engineered minimized antibodies have been produced (reviewed in Hudson, 1998, 1999; Little *et al*., 2000). These include Fab fragments (Better *et al*., 1988), Fv fragments in which the V domains are held together by non-covalent forces (Skerra and Plückthun, 1988) and so called single-chain antibodies (scFvs) in which the V_H and V_L domains are connected either with a short flexible linker peptide (Bird *et al*., 1988; Huston *et al*., 1988) or with a disulfide bond (Glockshuber *et al.*, 1990) (Fig. 2).

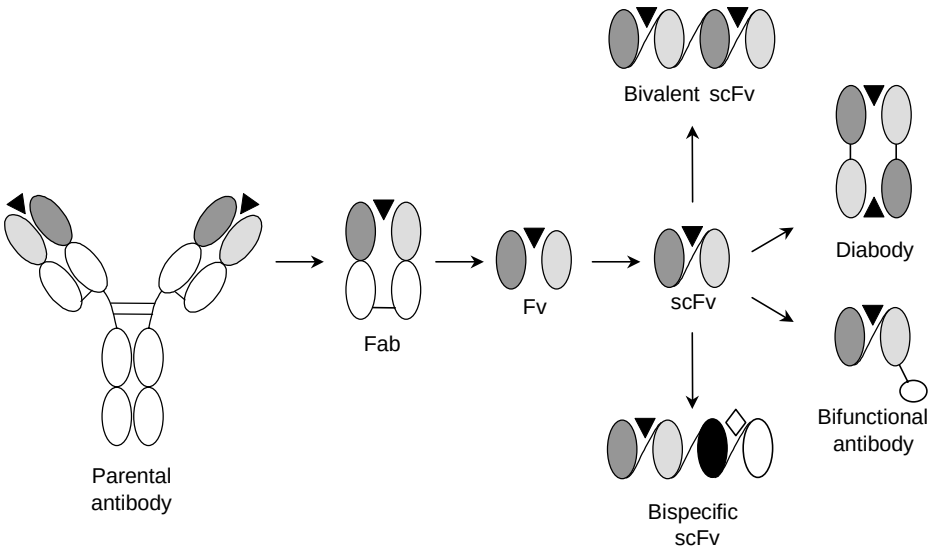


Figure 2. Recombinant antibody fragments. See text for details.

The minimized antibodies can further serve as building blocks for generation of novel recombinant proteins for different purposes. Antibody fragments of the same specificity have been fused to generate bivalent (for reviews, see Plückthun, 1992; Plückthun and Pack, 1997) or multivalent (Plückthun and Pack, 1997; Hudson and Kortt, 1999) antibodies, and two antibody fragments with different specificities have been fused to produce bispecific antibodies (Fig. 2) (Holliger *et al*., 1993; Mallender and Voss, 1994; Plückthun and Pack, 1997).

Diabodies are dimeric antibody molecules that can be designed for bivalent or bispecific interactions (Holliger *et al.*, 1993). Diabodies or even triabodies are formed spontaneously if an scFv has too short a linker peptide to allow pairing of the V domains within the same polypeptide chain but instead favours pairing between V domains in two adjacent chains (Fig. 2) (Iliades *et al*., 1997; Atwell *et al.* 1999). Bifunctional antibodies consist of antibody fragments fused to another functional partner, for example an enzyme (Wels *et al*., 1992; Carrier *et al.*, 1995) or a toxin (Chaudhary *et al*., 1989; Batra *et al*., 1992; Reiter and Pastan, 1998). A fusion to a lipoprotein (Fuchs *et al*., 1991) or introduction of a lipid tag (Laukkanen *et al*., 1993) converts a soluble antibody fragment into a membrane-bound molecule enabling the antibody fragments to anchor on the bacterial membranes during their production in *E. coli*.

1.2.1 Expression of antibodies in *Escherichia coli*

At present polymerase chain reaction (PCR) is commonly used to amplify the genes encoding monoclonal antibodies with desired specificity or even to produce the entire antibody gene repertoire of immunized or non-immunized animals for cloning purposes. By cloning the entire Ig V region gene repertoire it is possible to by pass hybridoma technology and the need for immunization and experimentation with animals. Furthermore, the hybridoma cell lines are sometimes unstable and in such cases it is advantageous to immortalize unique antibodies by cloning the corresponding genes.

Recombinant antibodies including Fab, Fv and scFv fragments have been produced as functional proteins in various expression hosts such as bacteria (Better *et al*., 1988; Bird *et al*, 1988; Huston *et al*., 1988; Skerra and Plückthun, 1988), yeast (Horwitz *et al*., 1988; Edqvist *et al*., 1991), filamentous fungi (Nyyssönen *et al*., 1993), insect cells (Bei *et al*., 1995), plants (Owen *et al*., 1992) and mammalian cells (Dorai *et al*., 1994). There are some general rules with regard to the design of vectors and expression systems used with the different hosts and each of these hosts has advantages and disadvantages for the production of functional antibody fragments (reviewed in Verma *et al*., 1998). However, among the large arsenal of different expression strategies available for heterologous protein production, the bacterial expression system is most often applied for the production of recombinant antibody fragments. Recent progress

in the understanding of both the genetics and biochemistry of *Escherichia coli* makes this organism a valuable tool as an expression host (reviewed in Baneyx, 1999). An advantage of this production system is that large quantities of homogenous protein preparation can be produced in a simple, rapid and cost-effective manner. Very high yields up to 1–3 g/l of functional antibody have been obtained with careful optimization of fermentation conditions (Carter *et al*., 1992; Horn *et al*., 1996). As the antigen binding domains of an antibody molecule are not usually glycosylated except in some rare cases (Sox and Hood, 1970; Wallic *et al*., 1988), the corresponding antibody fragments assume their natural conformation in bacterial cells and can thus be obtained in their active form.

There are several different strategies to express the recombinant antibody fragments in *E. coli* (for reviews, see Plückthun and Skerra, 1988; Plückthun, 1991). One possibility is to express antibody chains directly without a signal peptide in the cytoplasm of *E. coli* (Bird *et al*., 1988; Buchner and Rudolph, 1991). Overexpression of polypeptides expressed in the reducing environment of bacterial cytoplasm is often accompanied by formation of insoluble aggregates known as inclusion bodies. Therefore, to recover the correctly folded functional form with proper rearrangement of the disulfide bonds the polypeptides must be renaturated *in vitro* (Huston *et al.*, 1995). Another strategy, which circumvents this problem, is to use a signal peptide to direct secretion of the antibody chains into the periplasmic space (Better *et al*., 1988; Plückthun and Skerra, 1988; Skerra and Plückthun 1988). The periplasmic space lies between the inner and outer membranes of gram-negative bacteria (Fig. 3), and it is known to contain proteins such as chaperons and disulfide isomerases, which facilitate the correct folding of recombinant proteins (Baneyx, 1999). Production of Fab fragments in *E. coli* is usually based on the expression of a dicistronic operon unit, in which both the L and Fd gene are under the control of the same promoter. This allows synthesis of approximately the same amounts of both chains. The antibody polypeptides are expressed in the cytoplasm as precursors with N-terminal signal peptides. During translocation through the inner membrane into the oxidizing environment of the periplasm, the signal peptides are cleaved off, the chains fold and assemble, and both the intra- and interdomain disulfide bonds are formed (Fig.3). This secretory pathway of *E. coli* is similar to eukaryotic folding and assembly of proteins in which the polypeptide precursors are transported to the lumen of the endoplasmic reticulum (ER). The functional and soluble antibodies

can be easily purified from the periplasmic fraction of the bacteria (Skerra and Plückthun, 1988; Carter *et al*., 1992). During prolonged induction, the outer membrane of the bacteria often becomes leaky and the antibody fragments accumulate into the culture medium (Better *et al*., 1988; Takkinen *et al*., 1991).

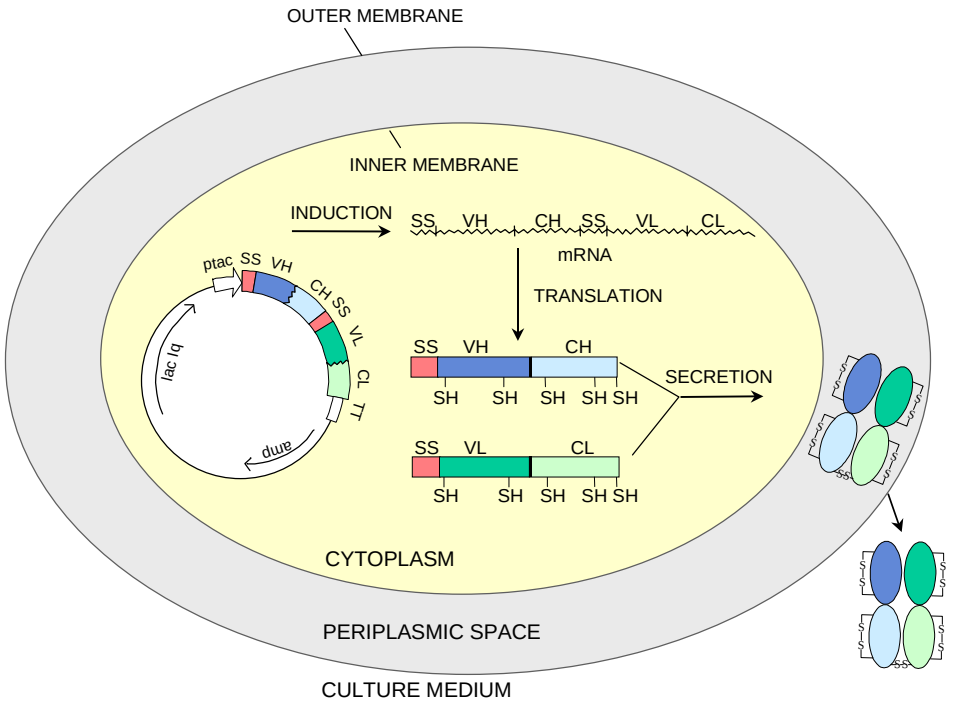


Figure 3. Expression of soluble Fab fragments in Escherichia coli. After induction dicistronic mRNA is synthesized and translated into preproteins in the cytoplasm of E. coli. Secretion of the H and L chains into the periplasm is achieved by the use of signal peptides which are removed during the translocation process across the bacterial inner membrane. The secreted polypeptide chains fold and assemble in the oxidizing environment of the periplasm and the formed Fab fragments leak into the culture medium.

Although periplasmic expression has been successfully used in the production of large variety of specific antibody fragments, the efficiency of folding appears to be dependent on the individual protein and in some cases antibodies are observed as insoluble and non-functional material in the periplasm (Malby *et al*., 1993; Smallshaw *et al*., 1999). Recently, thorough studies on factors improving both phage display and periplasmic folding of scFvs have led to the

identification of accessory proteins which decrease aggregation and facilitated folding of the antibody fragments (Bothman and Plückthun, 1998, 2000; Ramm and Plückthun, 2000). By optimizing those parts of an antibody fragment which are not directly involved in antigen binding it is possible to decrease the aggregation of the molecules and thus to improve the yield of a functional protein (Knappik and Plückthun, 1995; Wall and Plückthun, 1999). In the scFv format hydrophobic patches which are normally buried in a hydrophobic V/C domain interface are exposed to the solvent, which may promote aggregation during the folding. Substitution of the hydrophobic residues by hydrophilic ones has been shown to improve the *in vivo* folding properties of the protein (Nieba *et al*., 1997).

The disulfide bonds of antibodies make a significant contribution to the stability of the structure and are therefore conserved in evolution. In some cases, however, it is possible to replace the disulfide-forming cysteines with other amino acids and still obtain active and correctly folded antibody fragments (Wörn and Plückthun, 1998). Disulfide-free functional antibodies (intrabodies), which can be produced in the reducing environment of the cytoplasm, are of increasing interest because they can be targeted to bind and inactivate cellular components (for reviews, see Richardson and Marasco, 1995; Cattaneo and Biocca, 1999).

1.2.2 Fab fragments

Although functional Fab fragments can be produced proteolytically from the parental antibody, bacterial expression provides a convenient source of large amounts of relatively homogenous protein preparation. Bacterial expression systems have been applied to produce Fab fragments originating from different antibody classes including IgG (Better *et al*., 1988; Hemminki *et al*., 1995), IgM (Hay *et al*., 1992; Adib-Conquy *et al*., 1995; Jahn *et al*., 1995) and IgE (Steinberger *et al*., 1996). In the case of IgM Fab fragments the production levels have been relatively low, most probably due to problems observed in the production of the Fd chains (Abid-Conquy *et al*., 1995; Jahn, *et al*., 1995). It has also been reported that the constant domains of the Fd and L chains may have a significant effect on the amount of functional Fab fragments produced in *E. coli* (MacKenzie *et al*., 1994; Pajunen *et al*., 1997).

The antigen binding properties of recombinant Fab fragments have been reported to be comparable to (Better *et al*., 1988; Anand *et al*., 1991; Hemminki *et al*., 1998a) or in some cases slightly decreased (Pajunen *et al*., 1997) compared to the proteolytically produced corresponding Fab fragments. Compared to intact antibody molecules Fab fragments, which lack the Fc portion, may offer potential advantages in therapy trials due to their lower immunogenity and better pharmacokinetic properties (Yokota *et al*., 1992; Colcher *et al*., 1998). In addition the use of Fab fragments rather than the complete antibody in diagnostic immunoassays may decrease unwanted interferences sometimes observed in the assays (Kato *et al*., 1979; Vaidya and Beatty, 1992). Retaining of the C_H1 and C_L domains makes it possible to further engineer the fragment without affecting those parts directly involved in the antigen binding. For example, it is possible to introduce appropriate amino acid residues into the constant domains to improve labelling properties of the fragment for sensitive immunoassay applications (Hemminki *et al*., 1995).

1.2.3 Single-chain antibodies

The smallest functional antibody fragment, which maintains the complete antigen binding site, is the Fv fragment (Fig. 2). This fragment is a non-covalent heterodimer composed of the V_H and V_L domains (Skerra and Plückthun, 1988). Active Fv fragments have been produced by proteolytic digestion of the antibody molecule (Hochman *et al*., 1973). However, the proteolysis often does not result in a homogeneous population of Fv fragments but rather produces a number of partially digested protein molecules, which may hamper both purification and characterization. Therefore, the antibody engineering approach provides a more efficient and generally applicable method to produce Fv fragments. However, usefulness of the Fv fragments is limited by dissociation of the V domains at low protein concentrations and in the absence of antigen (Glockshuber *et al*., 1990; Jäger and Plückthun, 1999). To stabilize the Fv structure the V_H and V_L domains have been linked either chemically (Glockshuber *et al*., 1990) or by disulfide bonds (Glockshuber *et al*., 1990; Brinkmann *et al*., 1993; Reiter *et al*., 1996). An alternative method is to connect the V domains with a flexible linker peptide so that the antigen binding site is regenerated in a single-chain antibody molecule, scFv (Fig. 2) (Bird *et al*., 1988; Huston *et al*., 1988). The order of the domains can be either V_H-linker-V_L or V_L-

linker-V_H and both orientations have been applied (reviewed by Huston *et al*., 1991, 1995).

The length of the linker peptide used to join the V domains is crucial in the folding of the polypeptide chain. It has been estimated that the linker peptide must span at least 3.5 nm (35Å) between the C-terminus of one V domain and the N-terminus of the other V domain without affecting the ability of the domains to fold and form an intact antigen binding site (Huston et al., 1991). A further evaluation based on calculations using computer-generated scFv structures has shown that the distance between the V domains in the V_L-V_H orientation is always 5–10 Å greater than in the V_H-V_L orientation (Huston *et al*., 1995). Consequently, the use of the same linker may lead to functional differences between the two scFvs molecules with opposite V domain order. The length of the linker most commonly applied varies between 12 and 18 amino acids (for reviews, see Huston *et al*., 1991, 1995) but also up to 30 residues long linkers have been used (Desplancq *et al*., 1994; Nieba *et al*., 1997). Use of very short linker prevents pairing of the V_H domain to its attached V_L domain and instead forces pairing with counterpart domains of another molecule to form bivalent dimers (Holliger *et al*., 1993; Atwell *et al*., 1996) and higher multimers (Desplancq *et al*., 1994; Whitlow *et al*., 1994). Further, the monomeric and dimeric forms are in a concentration-dependent equilibrium; in dilute solutions only monomers are present (Desplancq *et al*., 1994; Whitlow *et al*., 1994). Atwell *et al*. (1999) showed that linkers of three amino acid residues in length are needed to form dimers, whereas a linker with two residues or less induces formation of trimers.

In addition to the length, the amino acid composition of the linker also has a crucial role in the design of a viable linker peptide. The sequence should be hydrophilic to prevent intercalation of the peptide within or between the V domains during protein folding. Nowadays, the most frequently used linkers consist primarily of repeats of glycine (G) and serine (S) residues (GGGGS), in which serine provides hydrophilicity and glycine confers flexibility to the peptide backbone (Huston *et al*., 1988; Huston *et al*., 1991). In addition to the linker peptides designed *de novo*, peptide sequences from known proteins have been applied (Bird *et al*., 1988; Takkinen *et al*., 1991; Smallshaw *et al*., 1999). Functional linkers can also be selected from a large population of peptide candidates. Stemmer *et al*. (1993) constructed a single-chain antibody gene

library with randomized linker sequences and could select an scFv showing antigen binding affinity comparable to that of the parental antibody. Recently, phage display library technology combined with random mutagenesis was shown to be a potential method to isolate optimized linker peptides for scFvs (Tang *et al*., 1996; Turner *et al*., 1997; Hennecke *et al*., 1998).

Single-chain antibodies produced in bacteria have often been shown to have antigen binding properties similar to those of the parental monoclonal antibody or the corresponding Fab fragment (Colcher *et al*., 1990; Takkinen *et al*., 1991; Smallshaw et al., 1999). It has also been observed that the proteolytic stability of an scFv fragment depends on the linker peptide sequence (Whitlow *et al*., 1993). Recently, Sieber *et al*. (1998) developed a phage display method, which links the protease resistance of a protein with the infectivity of the filamentous phage. In this strategy a proteolysis step is included in the selection allowing isolation of proteins with improved conformational stability. The stability of scFvs against thermal and pH denaturation has been demonstrated to be slightly reduced compared to that of the parental monoclonal antibody and/or the corresponding Fab fragment (Takkinen *et al*., 1991; Smallshaw et al., 1999). By using phage display under different selection pressures such as elevated temperatures or in the presence of denaturating compounds it is possible to create single-chain antibodies with improved stability (Jung *et al*., 1999).

1.2.4 Multivalent antibodies

Antibodies are multivalent molecules, which can bind simultaneously to two or more antigen molecules. This multivalent nature of antibodies provides a high degree of functional affinity (avidity) compared to monovalent antibody fragments and improves the performance of antibodies both *in vivo* and *in vitro*. This increase in functional affinity is seen in reduced dissociation rates, which result from multiple binding to two or more target antigen molecules. Recently, small bi- or multivalent antibody fragments have aroused much interest in tumor imaging and therapy (for reviews see, Adams and Schier, 1999; Hudson, 1999; Hudson and Kortt, 1999). The gain in the functional affinity through multivalent binding depends on a number of factors including the density, spatial arrangement and accessibility of the antigen/epitope on the surface (Plückthun and Pack, 1997). In addition, the antigen binding sites of the multivalent

fragment should be flexible enough to allow numerous binding or cross-linking geometries at the same time.

There are several possibilities for generation of multivalent antibodies, some of which are presented below (for reviews, see Hudson and Kortt, 1999; Little *et al*., 2000). The simplest way to produce bivalent antibodies is to utilize the tendency of scFvs to self-associate spontaneously. While relatively stabile non-covalent dimers can be obtained in some cases, this method usually produces a mixture of monomers and dimers and is limited to those scFvs having the capacity to form homodimers spontaneously. The dimerization process can be promoted by connecting the V domains with short linker peptides, which drive the transition from monomers to dimers (diabodies) (Holliger *et al*., 1993). It has been shown that using linkers of between three and five residues only diabodies are formed, whereas trimers (triabodies) and even tetramers (tetrabodies)(Fig. 4) can be produced with linkers containing less than three amino acids (Iliades *et al*., 1997; Kortt *et al*., 1997; Le Gall *et al*., 1999; Dolezal *et al*., 2000). These high-avidity antibodies can be produced as functional antigen binding molecules into the bacterial periplasm (Power and Hudson, 2000). The flexibility and orientation of the antigen binding sites in diabodies and triabodies have been confirmed by electron microscopy (Lawrence *et al*., 1998, Atwell *et al*., 1999).

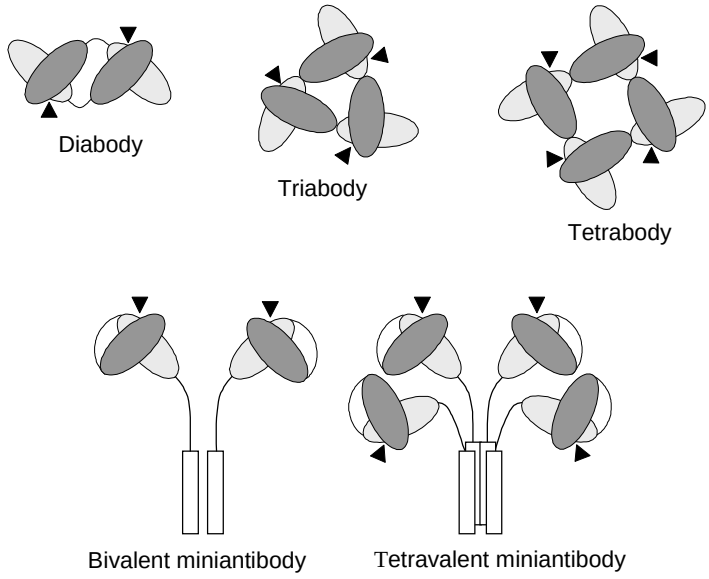


Figure 4. Multivalent recombinant antibody fragments. See text for details.

An alternative approach to increase the functional affinity is to fuse scFv fragments genetically with protein dimerizing motifs to produce "miniantibodies" (Fig. 4). For example, a sequence of an amphiphilic helix has been connected via a flexible hinge from mouse IgG3 to the C-terminus of an scFv molecule to produce bivalent scFv fragments (Pack and Plückthun, 1992; Pack *et al*., 1993). These bivalent miniantibodies were secreted as functional proteins to the periplasmic space of *E. coli* and produced in large scale using high-cell density fermentation (Pack *et al*., 1993). Assembly of functional bivalent miniantibodies was also demonstrated for an scFv fused to the human IgG1 C_H3 domain, which in an intact IgG1 molecule promotes and stabilizes dimerization of the H chains (Hu *et al*., 1996).

In addition to bivalent antibodies tetravalent miniantibodies (Fig. 4) with higher functional affinity have also attracted increasing interest in cancer therapy (Plückthun and Pack, 1997; Hudson, 1998, 1999). These antibody molecules have been generated by fusing the scFv to either tetramerizing helix (Pack *et al*., 1995), tetramerization domain of the human transcription factor p53 (Rheinnecker *et al*., 1996) or to core-streptavidin (Kipriyanov *et al*., 1996).

1.2.5 Bispecific antibodies

Bispecific antibodies incorporate the binding specificities of two different antibodies into a single molecule and, therefore, they are structurally bivalent but functionally univalent for each antigen-binding specificity (Fig. 2). These molecules have potential applications in cancer therapy and treatment of infectious disease (reviewed in Segal *et al*., 1999; van Spriel *et al*., 2000). For example, bispecific antibodies can be designed to bind both to the target cells (tumors or pathogens) and to the effector cells (e.g. T cells, natural killer cells and macrophages) as an approach to utilize cellular immune defense mechanisms to destroy the target cells (Holliger *et al*., 1996, 1999; Manzke *et al*., 1999). In addition to therapeutic applications, the dual binding specificity can also be employed in various immunochemical assays, including microtitre plate assays, immunoblotting and immunohistochemistry (Kontermann *et al*., 1997). One of the binding arms can be designed to bind to the target antigen and the other arm to the marker enzyme or another indicator molecule. This arrangement allows co-incubation of the antibody and the marker molecule and

thus simplifies the performance of the assay. As an approach to enhance the binding affinity of the antibody in immunoassays or to improve tumor targeting and retention, bispecific antibodies can also be designed to bind to two non-overlapping epitopes on the same target molecule (Cheong *et al*., 1990; Robert *et al*., 1999).

Bispecific antibodies have been produced by fusion of two hybridomas (Milstein and Cuello, 1983) or by chemical crosslinking of two IgGs with different specificities (Karpovsky *et al*., 1984) or Fab fragments (Cheong *et al*., 1990; Robert *et al*., 1999). Recently, a "knobs-into-holes" strategy was introduced to facilitate heterodimerization between two different antibody H chains co-expressed in mammalian cells (Ridgway *et al*., 1996). In this method knob structures were obtained by replacing at the C_H3 domain small amino acids with larger ones in one chain whereas on the other chain holes were created by replacing large residues with small ones.

Antibody engineering has led to the development of new bispecific antibody formats. Bispecific diabodies are recombinant constructs comprising two scFvs, V_HA-L-V_LB and V_HB-L-V_LA, derived from parent antibodies with specificities A and B, where L is a short linker peptide (Holliger *et al*., 1993; Whitlow *et al*., 1994; Atwell *et al*., 1996). Coexpression of these two polypeptides in a same bacterial cell results in their secretion and non-covalent association to form a functional molecule with both binding specificities. Further, it was demonstrated that good yields (up to 935 mg/l) of bispecific diabodies can be obtained by using high cell density fermentation (Zhu *et al*., 1996). Compared to IgG or antibody fragments in which the two binding sites are rather flexible, the structure of the diabody is more rigid and compact with the antigen binding sites at the opposite ends of the molecule (Perisic *et al*., 1994).

Alternatively, bispecific antibodies have been constructed by joining "in tandem" two different scFvs together by a polypeptide linker (Fig. 2) (Gruber *et al*., 1994; Mallender and Voss, 1994; de Jonge *et al*., 1995). These antibody fragments were secreted as inclusion bodies into the periplasmic space of *E. coli* and could after refolding be obtained as active antigen binding proteins. Müller *et al*. (1998a) constructed bispecific miniantibodies in which the C_H1 domain fused to the C-terminus of an scFv specific for epidermal growth factor receptor while the C_L (kappa) domain was fused to the C-terminus of a CD2 specific scFv.

Bispecific antibodies, heterodimerized via a disulfide bridge between the C domains, could be produced in *E. coli*. Further, by linking of two different scFvs with a helical dimerizing domain it is possible to produce miniantibodies with two valencies for each specificity (Müller *et al*., 1998b).

1.2.6 Bifunctional antibodies

Bifunctional antibodies are molecules in which whole antibody molecules or their antigen binding domains have been fused to other functional molecules such as drugs, toxins or enzymes (Fig. 2). For example, recombinant immunotoxins are targeted drugs in which a protein toxin or its effector fragment has been fused to an antibody (reviewed in Kreitman, 1999). Ideally an immunotoxin is capable of killing the target cell efficiently while causing little or no harm to other tissues. Functional recombinant immunotoxins recognizing tumor cells have been expressed in *E. coli* (Chaudhary *et al*., 1989, 1990; Chowdhury *et al*., 1998; Klimka *et al*., 1999). In these particular cases scFv molecules were fused to bacterial *Pseudomonas* exotoxin A or its truncated mutant, which inhibits protein synthesis and causes death of the target cell. Goyal and Batra (2000) constructed an immunotoxin by fusing the fungal toxin restrictocin to an scFv specific for human transferrin receptor and linking these molecules with a proteolytically cleavable peptide to enhance the cell-killing activity of the molecule. Immunotoxins in which the V_H-V_L heterodimer has been stabilized by disulfide bonds were shown in some cases to be more stable than scFvs (for reviews, see Reiter *et al*., 1996; Reiter and Pastan, 1998). Innovative improvements to the concept of using specific targeted immunotoxins in therapy include so-called antibody-directed enzyme prodrug therapy. In this strategy an enzyme linked to a tumor-specific antibody is first administered, followed by administration of a non-toxic prodrug (Bhatia *et al*., 2000). The prodrug is then converted at the tumor site into a cytotoxic drug by the pre-localized enzyme. Antibody fragments can also be armed with radionuclide chelating agents to provide tools for imaging and radioimmunotherapy (Wu and Yazaki, 1999; Wu *et al*., 2000).

Recombinant antibodies fused to enzymes such as alkaline phosphatase supply a reagent source for different applications including immunohistochemistry (Wels *et al*., 1992) and different immunoassays (Carrier *et al*., 1995). For purification

and detection purposes antibody fragments can be fused to the IgG binding domain of *Staphylococcus aureus* protein A (Tai *et al*., 1990; Gandecha *et al*., 1992) or the maltose-binding protein of *E. coli* (Brégégère *et al*., 1994). Further, when an scFv was fused to a cellulose-binding domain (CBD) of a fungal or bacterial cellulase the fusion protein obtained retained its hapten binding activity (Reinikainen *et al*., 1997). Linkage of an antibody fragment to a CBD allows immobilization to cheap and versatile cellulose matrices, which could be exploited to develop large scale industrial affinity purification schemes (Linder *et al*., 1998).

1.3 Applications of recombinant antibodies

Since the advent of hybridoma technology the exquisite specificity of monoclonal antibodies has been exploited for a number of applications in diagnostics, medicine and basic research. The development of antibody engineering technology together with the microbial expression systems now available provide tools to design and produce novel and tailored antibodies at affordable prices, even for large-scale applications. Furthermore, purification of antibody fragments can be greatly facilitated by linking tags or peptides to these molecules, which allows production of homogenous, well-defined and active preparations.

1.3.1 Immunodiagnostics

Current immunoassays mainly utilize monoclonal antibodies. In some cases, however, production of monoclonal antibodies with sufficient specificity and affinity by hybridoma technology has not been successful. During recent years, tailor-made recombinant antibody fragments produced in bacteria have become potential alternatives to these "conventional" reagents. Nowadays, antibody fragments for virtually any antigen can be isolated from large naive libraries, thus avoiding the necessity for immunization of animals (Marks *et al*., 1991; Hoogenboom *et al*., 1998).

The functionality of recombinant antibodies as immunological reagents has been demonstrated in a number of different assay formats (Kontermann *et al*., 1997;

Lindner *et al*., 1997; Emanuel *et al*., 2000). By using modern antibody engineering it is possible to manipulate and further improve the properties of these antibodies to meet the demands of the assay under consideration. It has been shown that the labelling efficiency of a recombinant Fab fragment by europium (Eu^{3+}) chelate derivative often applied in fluoroimmunoassays can be significantly improved by increasing the number of the lysine residues at the surface of the L chain constant domain (Hemminki *et al*., 1995). Further, Fab fragments have been labelled with biotin by fusing the Fd chain to a 13 amino acid acceptor peptide and performing the site-specific biotinylation enzymatically *in vitro* with *E. coli* biotin holoenzyme synthetase (BirA) (Saviranta *et al*., 1998). In another approach the Fd chain was fused with *E. coli* biotin carboxy carrier protein and biotinylation was accomplished *in vivo* by Bir A overexpressed in bacteria (Sibler *et al*., 1999). In addition to antibody labelling, modern engineering technology also provides excellent tools to improve or refine binding properties of antibodies. Especially it can provide a useful tool in those cases in which conventional methods have not provided antibodies with sufficiently high affinity and specificity. This has been demonstrated for testosterone specific Fab fragment, the specificity and affinity of which were significantly improved by random mutagenesis combined with phage display (Hemminki *et al*., 1998a and b). In another case a high affinity oestradiol specific scFv with simultaneously decreased cross-reactivity to related steroids could be isolated from a large repertoire of antibody fragments displayed on phage (Pope *et al*., 1996).

Recently, a sandwich-type enzyme-linked immunosorbent (ELISA) assay was developed based on scFv fusion proteins for both capture and detection of a plant pathogen (Kerschbaumer *et al*., 1997). The wells were coated with a miniantibody carrying a dimerizing motif and the antigen was detected by scFv-AFOS fusion. The assay was reported to be more sensitive than a conventional ELISA based on polyclonal antisera, partly due to the good coating properties of the miniantibody. In another study a dual-label time-resolved immunofluorometric assay based on recombinant Fab fragments was set up to measure both free and total prostate-specific antigen (Eriksson *et al*., 2000). The performance of this assay format was tested with clinical samples and was found to be comparable to a conventional assay based on monoclonal antibodies. Furthermore, a sample which gave a false positive result in the conventional assay probably due to interference caused by the Fc part, was undetectable in the Fab-based assay,

which demonstrates the potential of antibody engineering in establishing new immunoreagents.

1.3.2 Therapy

Mouse monoclonal antibodies, as well as chimerized and humanized derivatives of these, have shown therapeutic potential, and in recent years several of them have been approved for clinical use (reviewed in Glennie and Johnson, 2000). Although good performance has been observed in several cases, especially with humanized molecules, the widespread use of these antibodies as therapeutic agents has been limited by their immunogenicity. Over the past decade, molecular display technologies have been developed which allow generation of fully human antibodies (Marks *et al*., 1991; Vaughan *et al*., 1996, 1998). Further, scaling up of the microbial production system provides large amounts of antibody fragments, which can be efficiently purified even for clinical studies requiring particular precautions (Casey *et al*., 1995).

Small antigen binding molecules such as Fab and scFv fragments offer several advantages over a whole antibody molecule in therapeutic applications (for reviews, see Colcher *et al*., 1998; Adams and Schier, 1999; Hudson, 1999). Due to their reduced size these molecules penetrate more rapidly and evenly into tumors and other tissues compared to the whole antibodies (Yokota *et al*., 1992; Colcher *et al*., 1998). Since the antibody fragments have more rapid clearance from the blood, drugs and radionuclides can be coupled with reduced risk of toxic effects in normal tissues (Colcher *et al*., 1990; Wu and Yasaki, 1999). Comparison of mutant scFvs with different affinities has shown that increased affinity significantly improves tumor targeting and leads to longer retention of the fragment in tumors (Neri *et al*., 1997; Adams *et al*., 1998; Viti *et al*., 1999). On the other hand, extremely high-affinity antibodies designed to target solid tumors may be bound in an irreversible manner to the first target antigen encountered, which may hamper further penetration of the antibodies into the tumor tissue (Adams and Schier, 1999). One limitation of using the scFv and Fab formats for targeting *in vivo* is their monovalent binding to antigen. The efficiency of tumor targeting can be improved by using multivalent antibody fragments (Adams *et al*., 1993; Neri *et al*., 1997; Colcher *et al*., 1998; Viti *et al*., 1999).

Hitherto, therapeutic trials of bispecific antibodies composed of whole antibody molecules have been limited owing to their toxicity observed in initial trials as well as to the high cost of producing these molecules for clinical evaluation (reviewed in Segal *et al*., 1999; van Spriel *et al.*, 2000). Recently small bispecific antibodies produced in bacteria have been shown to be functional immunotherapeutic agents for tumor treatment *in vivo* (de Jonge *et al.*, 1998; Cochlovius *et al*., 2000) and may thus allow to overcome these problems in the future. Several different recombinant immunotoxins have also been produced and shown to be potent antitumor agents producing tumor regressions (Kreitman *et al*., 1994; Siegall, *et al*. 1994; Chowdhury *et al*., 1998). Although some recombinant immunotoxins are currently in clinical trials (Reiter and Pastan, 1998; Kreitman, 1999), there are still challenges to be met, including reduction of immunogenicity and toxicity of these molecules (Kreitman, 1999).

1.3.3 Structure analysis

Antibodies are large flexible glycoproteins, which can exist in a range of different conformations due to the intrinsic mobility of the individual domains. This causes difficulties to obtain high quality crystals for structure analysis and only a few crystal structures of intact antibodies have been solved (Harris *et al*., 1997, 1998). Reduction of antibody size by omitting the Fc part and the flexible hinge region increases the probability of obtaining crystals showing high resolution diffraction. Further, the smaller size of the antibody fragments makes it possible to crystallize them when complexed with antigens, and the detailed three dimensional structure obtained can be used to identify amino acid residues important for binding. A number of antibody fragments have been crystallized and structures determined with and/or without antigen, including Fab fragments (Whitlow *et al*., 1995), scFvs (Malby *et al*., 1993, 1998; Zdanow *et al*., 1994) and Fv fragments (Bhat *et al*., 1994; Fields *et al*., 1996). The crystal structure obtained for one diabody revealed that the molecule had a rigid structure with the antigen binding sites at opposite ends of the molecule (Perisic *et al*., 1994). Further, the distance between the antigen binding sites was measured to be 65Å, which is less than half the estimated distance in an IgG molecule. Determination of the crystal structure of a triabody revealed that in this trimeric antibody the three antigen binding sites were arranged in a cyclic head-to-tail manner (Pei *et al*., 1997). In addition to X-ray crystallography, NMR spectroscopy has also

been used in structural studies of antibodies (McManus and Riechmann, 1991; Freund *et al*., 1993).

A novel approach in determination of the crystal structures of membrane proteins is to use small antibody fragments in the co-crystallization of these macromolecules. Membrane proteins and their complexes are poorly soluble in aqueous environment and need detergents for solubilization. Most of the crystal lattice contacts are made by the polar extramembranous parts of the membrane protein. Binding of an antibody fragment to a membrane protein increases the polar area and thus increases the possibility to obtain well-ordered crystals for structure determination (Ostermeier *et al*., 1995). Fv fragment-mediated crystallization has successfully been applied for X-ray analysis of bacterial cytochrome c oxidase, which is a multisubunit complex (Iwata *et al*., 1995; Ostermeier *et al*., 1995, 1997). The cytochrome c oxidase complex was purified to homogeneity in one step procedure on an Fv fragment-based column and eluted directly as an Fv-cytochrome complex (Kleymann *et al*., 1995). Recently, the cytochrome bc_1 complex from the yeast *Saccharomyces cerevisiae* was crystallized together with an Fv fragment (Hunte *et al*., 2000).

1.4 Display technology

During recent years a number of different display technologies have been developed for enriching molecular diversity and producing novel types of proteins (for a review, see Li, 2000). Antibodies have been expressed on the surface of different microorganisms including bacteriophages (bacterial viruses), viruses, bacteria and yeast. All these systems share the principle of a linkage between genotype (the Ig genes) and phenotype (antigen-binding). This linkage allows isolation of a protein with desired binding properties from a large pool of non-binding antibodies, while the genetic material remains within the microorganism and is thus available for isolation and further engineering. In the future, recombinant antibodies and large antibody libraries containing binders virtually to any target antigen may become important tools in functional genomic and proteomic research (Borrebaeck, 1998; Holt *et al*., 2000).

1.4.1 Phage display

About fifteen years ago Smith (1985) first demonstrated that foreign DNA fragments can be fused to the gene III coding for pIII coat protein of a non-lytic filamentous phage and expressed as a fusion protein on the virion surface without destroying the infectivity of the phage. Thereafter McCafferty *et al.* (1990) demonstrated that an scFv fragment can be displayed as an active antigen binding domain on the surface of filamentous phage and that rare clones can be isolated from a large population by using antigen to select and screen the phage. Since these first applications, the technology has been extended to other antibody molecules including Fab fragments (Barbas *et al*., 1991; Hoogenboom *et al*., 1991; Kang *et al*., 1991), disulfide-stabilized Fv fragments (Brinkmann *et al*., 1995) and diabodies (McGuinness *et al*., 1996).

Antibody libraries are constructed by cloning PCR amplified antibody gene repertoires into a phage or phagemid vector, which allows display of the antibodies on the phage particle (for reviews, see Hoogenboom *et al*., 1992; Winter *et al*., 1994). The Ig gene repertoire can be cloned from stimulated B cells (high levels of mRNA) after immunization (Clackson *et al*., 1991; Persson *et al*., 1991) or viral infection (Burton *et al*., 1991). This approach results in higher frequency of binders specific for the antigen, and due to the *in vivo* affinity maturation of the antibodies the isolated binders usually have high affinities. Alternatively, antibody gene repertoires can be isolated from non-immunized individuals by PCR amplification of the heavy chain genes from IgM and the light chain genes from κ and λ cDNA (Marks *et al*., 1991). The IgM V_H repertoire is generally more diverse than the IgG V_H repertoire because it represents the entire B cell population, which has not undergone selection and amplification by the antigen. Basically, these non-immunized or "naive" antibody libraries can be repeatedly used for selection of antibodies with new specificities without the need to construct a new library for each antigen. The affinities of antibodies derived from such naive repertoires containing 10^7–10^8 individual clones are in the micromolar range (Marks *et al*., 1991; Griffiths *et al*., 1993). However, by constructing very large non-immunized libraries of about 10^{10} individual clones, antibodies with subnanomolar affinities have been isolated (Vaughan *et al*., 1996; Sheets *et al*., 1998; de Haard *et al*., 1999). A third approach is to use synthetically assembled variable regions to construct

synthetic antibody gene libraries, which provides high structural diversity (Barbas *et al*., 1992; Hoogenboom and Winter, 1992; Griffiths *et al*., 1994).

To express antibody fragments on the surface of filamentous bacteriophage the Ig genes can be fused either to the VIII gene (Kang *et al*., 1991) or the III gene (McCafferty *et al*., 1990) (Fig. 5). The major coat protein pVIII is present with

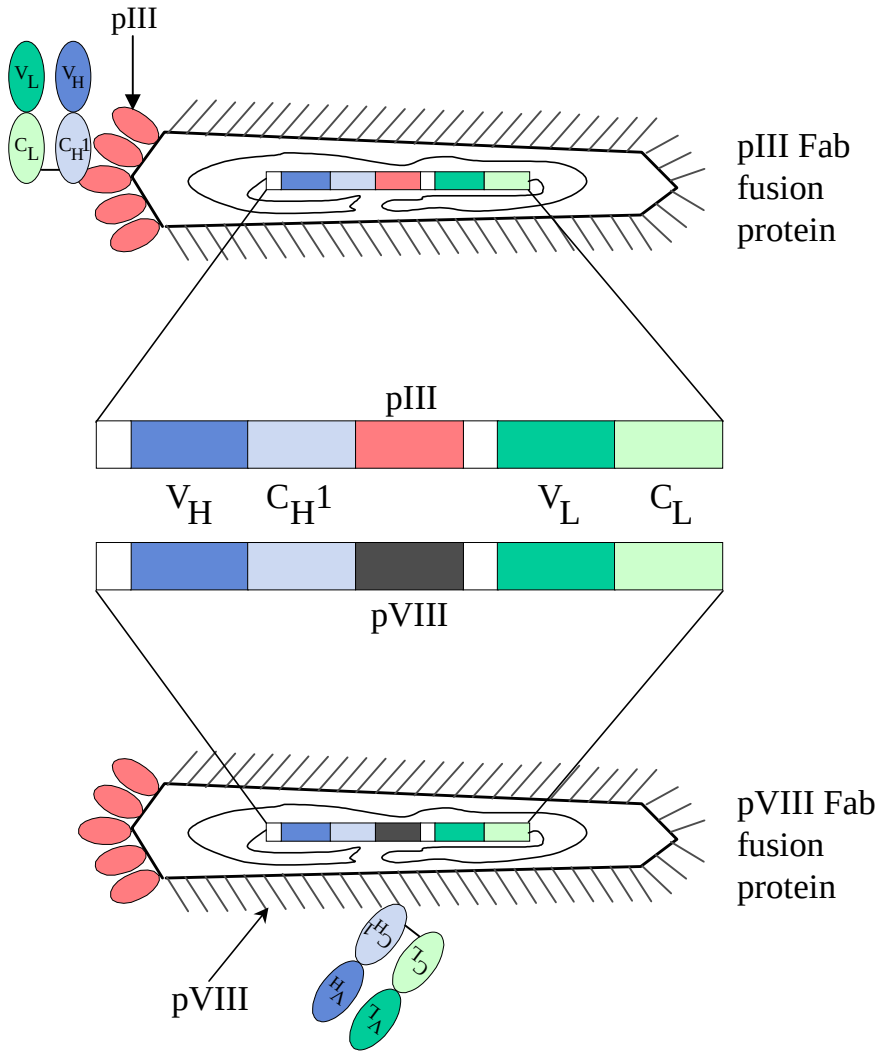


Figure 5. Schematic figure showing the display of Fab fragments as pIII and pVIII fusions on the surface of bacteriophage.

about 2700 copies on the phage particle and fusion to antibody results in multivalent display of the fragment. The minor coat protein pIII is present with between three and five copies at one tip of the phage and is needed for bacterial infectivity. By far the most extensively used display format is based on the use of phagemid vector and the pIII display, which allows high frequency of monovalent display and thus is preferred in selection for affinity (reviewed in Hoogenboom *et al*., 1998; Hoogenboom and Chames, 2000).

Selection of an antibody from the phage display library includes sequential enrichment of specific binding phage from a huge number of nonspecific phage particles. This is achieved by multiple rounds of phage binding to the antigen, washing to remove nonspecific phage and elution to rescue the specifically binding phages. After each round the eluted phages are amplified by infection of *E.coli* for the next round of selection. After the last round of the selection procedure, soluble antibodies are produced in bacteria and characterized. There are several different selection strategies that can be used to obtain specific antibodies (for reviews, see Hoogenboom *et al*., 1998; Hoogenboom and Chames *et al*., 2000). For most applications selection can be performed simply by incubating phage on antigen immobilized onto a solid support, such as immunotubes (Marks *et al*., 1991; Griffiths *et al*., 1993), microtitre plate wells (Burton *et al*., 1991; Hemminki *et al*., 1998 a and b), BIAcore sensor chips (Malmborg *et al*., 1996; Schier and Marks, 1996) or columns (McCafferty *et al*., 1990; Marks *et al*., 1991). Alternatively, the phage library can be incubated with the biotinylated antigen in solution, followed by capture of the antigen-phage complex on streptavidin surface (Hawkins *et al*., 1992; Griffiths *et al*., 1994). This approach can be used to select antibodies on the basis of affinity by using an antigen concentration significantly less than the desired dissociation constant Kd (Hawkins *et al*., 1992; Schier *et al*., 1996a; Hemminki *et al*., 1998b). Further, by choosing an appropriate design of the selection procedure, antibodies can also be selected on the basis of kinetic properties (Hawkins *et al*., 1992; Duenas *et al*., 1996), improved specificity (Hemminki *et al*., 1998b) or phage infectivity (Krebber *et al*., 1995; Hennecke *et al*., 1998). In cases in which pure antigen is not available (e.g. integral membrane protein) or the antigen is unknown (e.g. when searching for new markers on cells or tissues), specific antibodies must be isolated using more complex sources, such as whole cells (Marks *et al*., 1993; de Kruif *et al*., 1995; Klimka *et al*., 1999) or tissue fragments (van Ewijk *et al*., 1997).

The binding characteristics of antibodies isolated from phage libraries are often sufficient for their use in research applications, such as ELISA, Western blotting or immunofluorescence (Nissim *et al*., 1994; Lindner *et al*., 1997). However, the affinity or specificity of antibodies selected from the libraries may not be high enough for diagnostic or therapeutic applications and thus further improvement is required. The affinities of antibody fragments can be improved by targeting mutagenesis to one or more of the CDRs or only a part of them followed by selection of high affinity antibodies (Hawkins *et al*., 1992; Schier *et al*., 1996b; Thompson *et al*., 1996; Hemminki *et al*., 1998b; Chowdhury and Pastan, 1999). Further, mutagenesis of the CDRs and careful design of the selection methods have been used as a successful approach to increase the specificity of antibodies (Hemminki *et al*., 1998a). In addition to mutagenesis, pairing of a particular heavy chain with a library of light chains ("chain-shuffling"), or vice versa, has been used to increase the affinity of antibodies (Marks *et al*., 1992; Pope *et al*., 1996; Schier *et al*., 1996a). The binding affinities achieved *in vitro* have been in the nanomolar or picomolar range.

Phage display technology has revolutionized the way in which we can generate new specific antibodies without immunization, and thus enables production of antibodies which have been difficult or impossible to obtain by conventional methods based on immunization, such as human antibodies and antibodies against self-antigens (Griffiths *et al*., 1993; Vaughan *et al*., 1996; Hoogenboom and Chames, 2000). In addition to selection for binding properties, phage display system can be applied to isolate stable and well-folded proteins, catalysts or polypeptide substrates (reviewed in Forrer *et al*., 1999).

1.4.2 Baculovirus display

Although phage display systems have provided tools to generate specific tailor-made antibodies for several purposes, the technology has limitations imposed by the expression host. Phages are prokaryote viruses, and proteins intended for display must be suitable for secretion from a prokaryotic host such as *E. coli* and must not be dependent on eukaryotic post-translational modifications or folding requirements. Therefore, for such proteins a eukaryotic viral system capable of displaying complex eukaryotic proteins would be useful. Recombinant baculoviruses are nowadays widely used for high-level expression of

heterologous genes providing a eukaryotic processing environment. In addition, baculoviruses have proved to be powerful tools to investigate viral assembly processes and may provide a valuable approach in the development of new vaccines and gene deliver vehicles (for reviews, see Possee, 1997; Verma *et al*., 1998; Kost and Condreay, 1999).

Baculoviruses are a large family of double-stranded DNA viruses that infect only invertebrates, usually insects. During their infection cycle baculoviruses produce two structurally and functionally distinct virion phenotypes (Blissard and Rohrmann, 1990). One form is budded virus (BV), which is produced by budding from the surfaces of infected cells. The BV virions transmit cell-to-cell infection within the animal, and are highly infectious for tissues of the host and for cultured insect cells. The other phenotype, occlusion derived virus (ODV), is found within protective occlusion bodies in the host cell nucleus and is able to transmit infection from host to host by the oral route. Although the two different virus phenotypes (BV and ODV) share some common structural components, there are some components that are specific for each phenotype (reviewed in Rohrmann, 1992). One of these is the structural transmembrane protein gp64, which is the major envelope glycoprotein of the BV form and is used as a fusion partner to express proteins on the viral surface. The BV gp64 protein has been shown to be essential for the infectivity and it mediates the membrane fusion in a pH-dependent manner (Blissard and Wenz, 1992; Monsma *et al*., 1996). It is abundantly expressed on the plasma membrane of infected cells and is present on the virions as homo-trimers (Oomens *et al*., 1995). The gp64 protein is incorporated into newly formed BVs when nucleocapsids containing the viral DNA bud through the host plasma membrane. gp64 has two functional domains, the oligomerization domain and the fusion domain (Monsma and Blissard, 1995). The oligomerization domain within an amphipathic helix was thought to be responsible for oligomerization (or trimerization) and transport of the molecule (Fig. 6). The fusion domain containing a cluster of hydrophobic amino acids was suggested to be involved in the fusion of the virus into the plasma membrane.

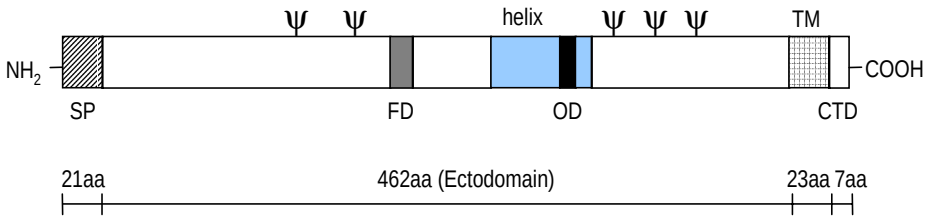


Figure 6. A schematic presentation of the predicted structure of AcNPV gp64 protein containing the signal peptide (SP), fusion domain (FD), oligomerization domain (OD), transmembrane domain (TM) and cytoplasmic tail domain (CTD). ψ denotes predicted N-linked glycosylation sites (modified from Monsma and Blissard, 1995; Oomens and Blissard, 1999).

Autographa californica nuclear polyhedrosis virus (AcNPV) has been used as an alternative for protein display in a eukaryotic system. Boublik *et al.* (1995) were the first to show that foreign proteins, glutatione-S-transferase and gp120 (the HIV major surface glycoprotein), could be fused to gp64 and displayed on the surface of a baculovirus particle. Further, they localized the position where insertions would be tolerated and demonstrated that a foreign sequence cloned in frame between the signal peptide and the sequence encoding the intact gp64 protein resulted in expression of a stable and functional protein on the virion surface. The same approach was taken by Mottershead *et al*. (1997), who demonstrated expression of green fluorescent protein and two rubella virus envelope proteins, E1 and E2, on the baculovirus surface. Grabherr *et al*. (1997) fused ectodomain of the glycosylated HIV-1 envelope protein gp41 both to the entire gp64 and its membrane anchor domain and found increased surface expression level of the foreign protein with the truncated gp64 fusion. A recent study by Ernst *et al*. (1998) demonstrated the applicability of baculovirus display technology for the construction and screening of eukaryotic expression libraries. They inserted derivatives of the HIV-1 gp41 epitope "ELDKWA" binding to a neutralizing human antibody into the antigenic site B of influenza virus hemagglutinin expressed on the surface of baculovirus-infected cells. By using fluorescence activated cell sorting (FACS) to screen the cells with a specific neutralizing antibody they were able to isolate a baculovirus clone with increased antibody binding activity. As an approach to modify the native gp64 protein without disturbing viral infectivity, short peptide coding sequences were inserted into the gp64 coding sequence utilizing a naturally occurring restriction

enzyme site (Ernst *et al*., 2000). Expression of these peptides as part of the gp64 envelope protein resulted in production of viable recombinant virus particles showing titers comparable to that of the wild-type virus. This approach allowed insertion of the peptide to every single copy of the gp64 protein resulting in high avidity display of the molecule. Lindley *et al*. (2000) fused the human nuclear receptors LXRβ and FXR to the N-terminus of the gp64 protein and used the baculovirus virions displaying the receptor proteins as immunogens to produce monoclonal antibodies. This strategy provided a novel alternative means to generate antibodies without the need for purification of the immunogen.

In addition to AcNPV, other viruses have also been investigated as a basis for a viral eukaryotic display system, including sindbis virus (London *et al*., 1992), poliovirus (Rose *et al*., 1994) and hepatitis B virus (Kratz *et al*., 1999). In addition, retroviruses have been employed for functional display of single-chain antibodies on the surface of virions, which may allow more specific targeting of viral vectors for therapeutic purposes (Russell *et al*., 1993; Konishi *et al*., 1998).

1.4.3 Other display systems

Display of heterologous proteins on the surface of cells provides an alternative or complement to phage- or virus-based systems. A number of different proteins have been expressed on the surface of both gram-negative and gram-positive bacteria or yeast by fusing the molecules to membrane proteins or cell wall components (for reviews, see Schreuder *et al*., 1996; Georgiou *et al*., 1997; Ståhl and Uhlén, 1997). These nonviral microorganisms displaying foreign sequences have several potential applications including presentation and screening of polypeptide libraries or they might be used as whole-cell vaccines, biocatalysts or cell-based reagents for diagnostic purposes (Schreuder *et al*., 1996; Georgiou *et al*., 1997; Ståhl and Uhlén, 1997). Libraries propagated in the cells are limited in size and in some cases factors such as steric interference with the native surface molecules, susceptibility to surface proteases or conformational constraints caused by the fusion to the carrier protein may affect the viability of the surface displayed proteins (Georgiou *et al*., 1997). On the other hand screening or sorting of the cell surface libraries by flow cytometry and FACS are powerful methods to enrich effectively positive clones (Francisco *et al*., 1993; Georgiou *et al*., 1997, Kieke *et al*., 1997). Fully functional antibody fragments

have been displayed on *E. coli* surface (Fuchs *et al*., 1991; Francisco *et al*., 1993) and, recently, antibodies with improved affinity were isolated by using affinity selection from mutant libraries displayed on bacterial surface (Daugherty *et al*., 1998, 1999) or yeast (Boder and Wittrup, 1997; Kieke *et al*., 1997).

Hanes and Plückthun (1997) applied an approach termed ribosome display for cell-free selection of antibody fragments from libraries. In this system large libraries ($\geq 10^{12}$ clones, Hanes *et al*., 1998) with virtually unlimited diversity can be constructed and the particular proteins are translated, displayed and selected completely *in vitro* on the ribosomes. Recently, the ribosome display technology was used to isolate antibodies with affinities in picomolar range (Hanes *et al*., 2000). Because the selection of proteins in the ribosome display system is performed *in vitro*, many drawbacks of the *in vivo* systems can be circumvented, which may render this approach important in the future (reviewed in Schaffitzel *et al*., 1999).

1.5 Immunoliposomes

Liposomes are artificial spherical vesicles which consist of a phospholipid bilayer surrounding an inner aqueous cavity. They have found increasing interest in different biomedical and biotechnical applications such as drug delivery, gene therapy and immunodiagnostics. Liposomes can be further equipped with target-specific ligands including receptors, antigens or antibodies (immunoliposomes), which act as homing or anchoring probes. Furthermore during or after the preparation of immunoliposomes it is possible to entrap almost any water-soluble molecule (drugs, DNA, enzymes, fluorophores, dyes etc.) within the internal cavity (Fig. 7).

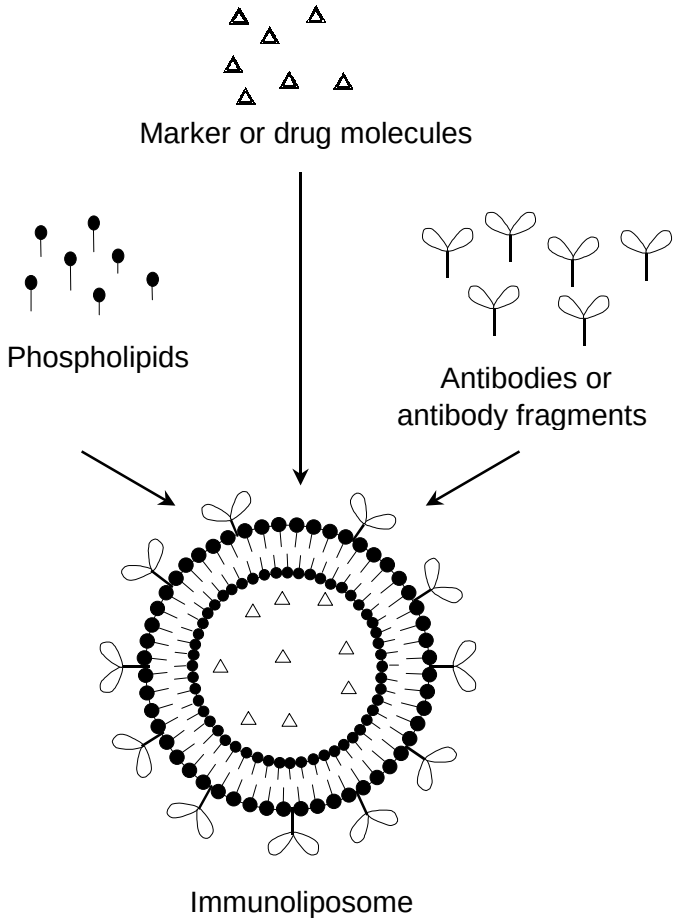


Figure 7. Schematic presentation of a marker or a drug molecule encapsulated in an immunoliposome.

1.5.1 Preparation

Liposomes form spontaneously when phospholipids are dispersed into water. In the lipid bilayer the hydrophilic polar heads of the phospholipids are exposed to the outer and inner aqueous environment, whereas the non-polar hydrocarbon tails are buried in the hydrophobic interior of the membrane layer (Fig. 7). The size of the liposomes varies, and the diameter can be between 25 nm and several micrometers. There are several well-established methods available for

preparation of liposomes, which differ from each other in the way in which the membrane components are dispersed in aqueous media (New, 1992).

Gregoriadis and Neerunjun (1975) were the first to describe specific targeting and cellular uptake of drug-loaded liposomes coated by nonspecific adsorption with rabbit polyclonal IgGs recognizing different types of cells both *in vitro* and *in vivo*. Since their work a number of methods have been developed to couple antibodies to liposomes. These methods include covalent coupling of the antibody via thiol or amino groups to membrane lipids (Leserman *et al*., 1980; Heath *et al*., 1981), or non-covalent methods applying the avidin (streptavidin)-biotin interaction (Loughrey *et al*., 1987; Urdal and Hakomori, 1980). In addition to intact antibodies Fab´ fragments have been covalently linked to lipid derivatives via the free thiol group in the Fd chain, which leaves the antigen-binding domains intact unlike in the other more random coupling methods (Martin *et al*., 1981; Shahinian and Silvius, 1995). Recently, a novel method was reported in which the DNA encoding the signal peptide and the aminoterminal part of the major lipoprotein of *E. coli* (lpp) was fused to the DNA encoding an scFv (Laukkanen *et al*., 1993; de Kruif *et al*., 1996). Expression of this fusion construct in bacteria led to *in vivo* fatty acylation of the scFv. These lipid-tagged scFvs produced in bacteria have been incorporated into lipid membranes to construct functional liposomes (Laukkanen *et al*., 1995; de Kruif *et al*., 1996; Kobatake *et al*., 1997). This approach avoids inactivation and low coupling efficiency often encountered with chemical modification and provides a whole arsenal of antibody engineering techniques, which enables preparation of tailor-made immunoliposomes for different application.

1.5.2 Applications

The successful use of liposomes for drug delivery *in vivo* requires a combination of prolonged presence in the circulation, specific targeting and efficient delivery of the liposomal content into the target cells. Although immunoliposomes are effective in specific binding to target cells *in vitro*, their utility for *in vivo* purposes has been limited by their rapid uptake by the reticuloendothelial system (RES) (reviewed in Gregoriadis, 1995). In earlier studies it was shown that by the coating liposome surface with ganglioside G_{M1} or with amphipathic poly(ethylene glycol) (PEG) the circulation half-life of liposomes was

significantly increased (Allen *et al*., 1989). These liposomes are less susceptible to uptake by RES, resulting in prolonged circulation time in the blood and enhanced tumor accumulation, and are referred to as "sterically stabilized" or "stealth" liposomes. Although the presence of PEG prolongs circulation time, binding of the antibody to the target cell may be reduced due to the steric hindrance caused by long PEG chains (Maryama *et al*., 1995). Coupling methods developed recently allow linkage of antibodies e.g. via thioether linkage to the terminus of a PEG chain to expose the antibody on the liposome surface (Allen *et al*., 1995). These sterically stabilized liposomes show more efficient target binding compared to those PEG-stabilized liposomes in which antibodies are coupled directly to membrane lipids. Recently, Ishida *et al*. (1999) showed a combinatorial approach to prepare a variety of immunoliposomal drugs. They prepared IgG-PEG-lipid micelles, which can be transferred into preformed drug-loaded liposomes in a one-step incubation. Ultimately, this combinatorial method might be applied to prepare drugs tailored for individual patient needs by selecting a defined antibody from a pool of antibodies and mixing it with an appropriate formulation of drug-containing liposomes.

There are several recent *in vitro* and *in vivo* studies in which immunoliposomes have been demonstrated to bind specifically to their targets and internalize into the cells for delivery of the encapsulated drugs (Park *et al*., 1995; de Menezes *et al*., 1998; Tseng *et al*., 1999). In addition to the targeted delivery of drugs, immunoliposomes may enable other biomedical applications, such as gene therapy. Liposomes intended for gene therapy may have different lipid compositions, but typically have synthetic cationic lipids on their surface. These cationic liposomes form complexes with negatively charged DNA via electrostatic interactions and can mediate gene transfer into different cells (Crystal, 1995; Park *et al*., 1997). Recently, Shi and Pardridge (2000) constructed neutral sterically stabilized immunoliposomes loaded with β-galactosidase or luciferase expression plasmid and carrying on their surface monoclonal antibodies specific for transferrin receptor (TfR). These liposomes were able to undergo endogenous TfR-mediated transport through the blood-brain barrier and expression of the exogenous genes in the brain was observed.

The use of liposomes as analytical reagents in immunoassays is usually based either on detection of the lysis of liposomes followed by release of the encapsulated marker molecules (for reviews, see Monroe, 1990; Rongen *et al*.,

1997) or on the detection of liposome-enhanced agglutination (Monroe, 1990). Liposome-based immunoassays closely resemble the formats used in standard immunoassays including homogeneous assays and competitive and non-competitive heterogeneous formats. The encapsulated marker molecules (e.g. fluorophors, chromophors or enzymes) serve as either indicators or amplifiers of the specific immune reaction measured in the assay. Therefore it is of outmost importance that the liposomes are stable and the leakage of the marker is kept at minimum. Furthermore, for analytical purposes both the detection sensitivity and a high signal-to-noise ratio are of crucial importance. For the signal measurement, the lysis of antibody-associated liposomes can be accomplished by adding detergent, complement, cytotoxin or certain divalent cations. Immunoliposomes with an encapsulated fluorescent label (Kobatake *et al*., 1997) or a lanthanide chelate (Laukkanen *et al*., 1995) have been demostrated to be potential multivalent immunoreagents providing both high specificity and sensitivity. Park and Durst (2000) developed a rapid field-portable colorimetric sandwich immunoassay for detection of a food pathogen, *E. coli* O157:H7. In this method a nitrocellulose strip with a zone of an immobilized pathogen-specific antibody is inserted into a test tube containing a mixture of the test sample and dye-loaded immunoliposomes carrying the specific antibody on their surface. Due to capillary migration the pathogen-immunoliposome complex reaches the antibody zone and the color density of the zone is directly proportional to the amount of *E. coli* O157:H7 present in the sample.

1.6 Real-time biospecific interaction analysis

In some cases the method chosen to characterize the binding properties of antibodies is straightforward and depends directly on the intended application. For example, the functionality of recombinant antibodies developed for reagents in immunoassays should be evaluated in the relevant assay format (Kontermann et al., 1997; Eriksson *et al*., 2000). Otherwise, several methods are available to study antigen-antibody interactions. Immunoassays, mainly ELISA, have often been employed as a first step in characterization of binding of the engineered antibodies and is currently the most common way to screen antibody phage libraries either by measuring binding of phages or/and the soluble antibodies (Marks *et al*., 1991). Beyond screening for positive and negative clones, ELISA may provide information on the relative binding activities of different antibodies

(Anand *et al*., 1991). Friquet *et al*. (1985) introduced an ELISA method allowing determination of the dissociation constant (K_D) of antigen-antibody equilibrium in solution, which has subsequently been applied to determine affinities of recombinant antibody fragments (Adib-Conquy *et al*., 1995; Thompson *et al*., 1996). Other methods employed to determine the affinity constant of engineered antibodies include fluorescence quenching (Takkinen *et al*., 1991; Nieba *et al*., 1997) and equilibrium dialysis (Pope *et al*., 1996). At present, however, increasing numbers of binding studies on recombinant antibody fragments rely on the use of surface plasmon resonance (SPR)-based biosensors, which enable a more detailed kinetic analysis.

SPR detectors are biosensors, which measure refractive index changes in solution close to the surface of a sensor. One of the interacting partners is immobilized on the sensor surface while the other component is introduced on the surface in solution. These instruments are capable of measuring the binding reactions in real time without the need for labelling any of the interacting partners. The first commercial SPR biosensor, BIAcore® (BIAcore AB, Uppsala, Sweden), was introduced in 1990 and thereafter five other SPR instruments have been been launched (for a review, see Rich and Myszka, 2000). The BIAcore biosensor can be used to characterize different properties of interacting biomolecules including specificity, concentration, affinity, kinetics and epitope specificity. A number of molecular interactions such as protein-protein, peptide-protein, DNA-protein, DNA-DNA, sugar-protein and ligand-protein interactions, can been studied by using this technology (Nagata and Handa, 2000). Recent developments in instrumentation, experimental design and software have significantly improved the performance of the technology (for reviews, see Malmqvist, 1999; Rich and Myszka, 2000). High sensitivity and reproducibility now make this technology suitable for characterization of combinatorial libraries as well as for secondary screening of small drug leads in the pharmaceutical industry (reviewed in Myszka and Rich, 2000). Furthermore, integration of the biospecific interaction analysis by BIAcore with mass spectrometry makes it possible to characterize and identify binding partners from crude samples, which may be an applicable approach for example in proteomics (Nelson *et al*., 1999; Williams and Addona, 2000).

The SPR technology as employed in the BIAcore instrumentation offers a number of different assay formats which can be chosen on the basis of the

information desired. It is possible to visualize the progress of antigen-antibody binding on a computer screen as a function of time and the strength of the binding can be expressed not only in terms of equilibrium affinity constants (K_a or K_d) but also in terms of association (k_{ass} or k_{on}) and dissociation (k_{diss} or k_{off}) rate constants. There are, however, several different factors that can influence the results and careful optimization of the experimental conditions is necessary to obtain reliable data (reviewed in Myszka, 1997). These factors include diffusion of the molecule in solution to and away from the sensor surface as well as rebinding to the immobilized molecule. This diffusion step can introduce a phenomenon called mass transport limitation, which results in k_{ass} and k_{diss} values that reflect diffusion rates of the molecule on and off the surface rather that the true rate constants (Glaser, 1993). High flow rates and low surface capacity are recommended means to avoid these problems (Myszka, 1997). Rebinding to the molecule immobilized on the sensor surface may have a remarkable effect on the affinity constants determined from the k_{ass} and k_{diss} constants compared to the affinities determined in solution, as demonstrated by Nieba *et al*. (1996). To eliminate the effects of rebinding they introduced a competition BIAcore method, which measures equilibrium binding in solution. This method is based on preincubation of the antibody with different concentrations of the antigen in solution followed by determination of the amount of the unbound antibody by BIAcore (Nieba *et al*., 1996; Hanes *et al*., 1998).

The BIAcore instrumentation has been employed to determine affinities and binding kinetics for recombinant antibody fragments derived from monoclonal antibodies (Hemminki *et al*., 1995; Wu *et al*., 1996) or isolated from phage display (Sheets *et al*., 1998; Chowdhury and Pastan, 1999), ribosome display (Hanes *et al*., 1998) and cell display libraries (Daugherty *et al*., 1998). Further, it has been used in studies of multivalent antibodies (MacKenzie and To, 1998) and in characterization of epitope specificies of antibodies (Mao *et al*., 1999; Viti *et al*., 1999). The biosensor also provides a means to confirm the capability of bispecific antibodies to bind to two different antigens simultaneously (Müller *et al*., 1998b; Robert *et al*., 1999). The versatility of this SPR technology in antibody engineering was recently demonstrated by de Haard *et al*. (1999) who, when isolating Fab fragments from a phage library, used BIAcore to screen the binding of phages during the selection steps, to screen the isolated soluble fragments produced in periplasmic fraction on the basis of off-rates and to

confirm their binding specificities. Further, they also used BIAcore to determine the concentration of Fab fragments present in the bacterial periplasm and to calculate the kinetic constants. The principle of BIAcore analysis and applications in antibody engineering are discussed more extensively in paper V.

1.7 Aims of the present study

The general aim of this work was to establish methods for the production and characterization of recombinant antibody fragments. In this technical approach the specific objectives were:

1. To study the influence of the heavy chain constant domain of a mouse Fab fragment on functional expression in *E. coli*.
2. To investigate the effect of the linker length and amino acid composition on the expression of soluble, active single-chain antibodies in *E. coli*.
3. To characterize binding properties of membrane-anchored, surface-displayed recombinant antibody fragments as a model for diagnostic applications.
4. To investigate functional antibody display on the surface of baculovirus as a model for a eukaryotic display system.

2. Materials and methods

All materials and methods used in this study are described in detail in papers I-IV and only a brief summary is given below.

2.1 The antibody expression constructions

To produce the soluble 2-phenyloxazolone (Ox) binding Fab, scFv and Fv fragments in *E. coli* (papers I, III) the corresponding expression units (Figure 8a) were cloned into the pKKtac vector (Takkinen *et al*., 1991), which consists of the *tac* promoter (*ptac*), the transcription terminator region (TT), *lac* repressor ($lacI^q$) from pMJR 1560 plasmid (Amersham) and the signal sequence coding region of pectate lyase (*PelB*) of *Erwinia carotovora* (Lei *et al*., 1987). For production of the membrane-anchored Ox scFv hexahistidyl tail fusion (Ox lpp-scFv-H6) (paper II), the signal sequence of the major lipoprotein (*lpp*) of *E. coli* and the nine N-terminal amino acids of the lpp were cloned into the pKKtac expression vector as described earlier (Laukkanen *et al*., 1993).

For baculovirus surface expression (paper IV) the antibody fragments were cloned under the polyhedrin promoter (*ppolh*) into the pFLAGgp64 vector, which contained the signal sequence of the GLUR-D glutamate receptor, the FLAG epitope and the gp64 gene (Kuusinen *et al*., 1995; Mottershead *et al*., 1997)(Figure 8b).

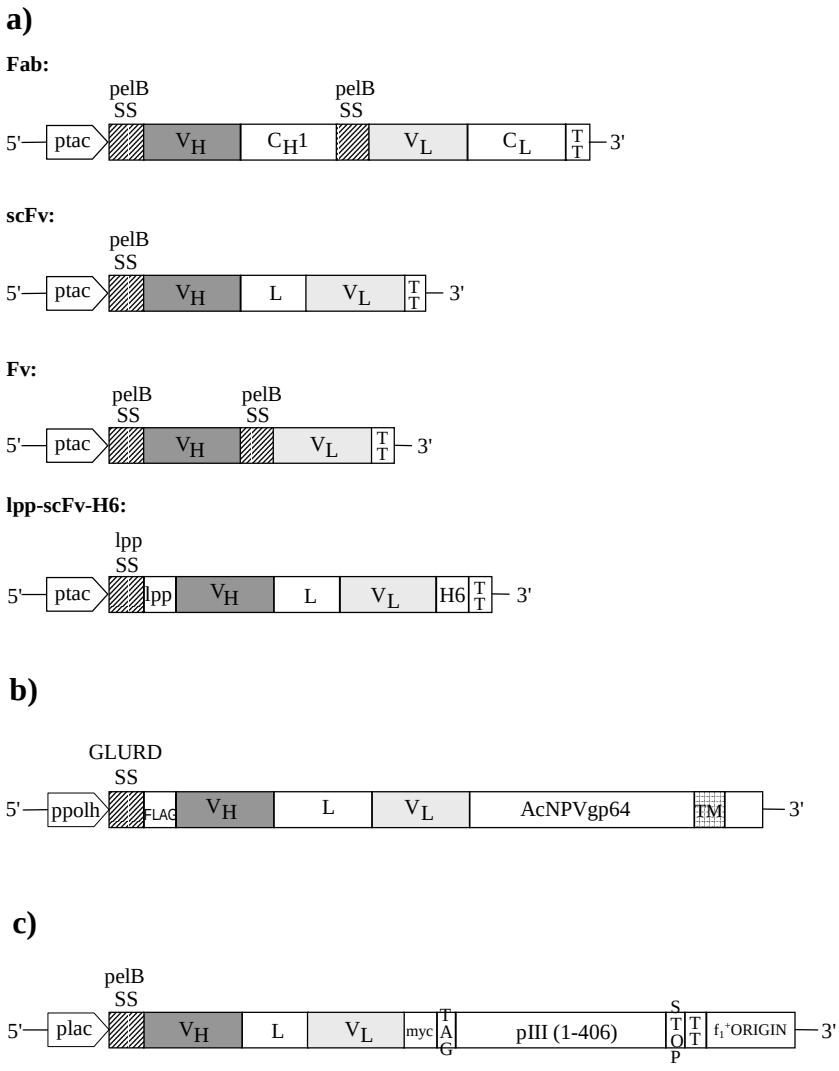


Figure 8. The expression units in (a) the pKKtac vector, (b) the pFLAGgp64 vector and (c) the phagemid vector used in this study contained DNAs encoding for: SS, signal sequence; L, linker peptide; lpp, the major lipoprotein of E. coli; H6, hexahistidinyl tail; FLAG, the epitope DYKDDDDK; AcNPVgp64, the major envelope protein gp64 of the AcNPV; TM, transmembrane domain; myc, the epitope EQKLISEEDLN and pIII protein (aa 1–406) of the filamentous bacteriophage M13. ptac, tac promoter; ppolh, polyhedrin promoter; plac, lac promoter; TT, transcription terminator; TAG, amber stop codon; f_1^+, phagemid origin of replication.

2.1.1 Construction of human scFv gene libraries

Construction of the human scFv/κ and scFv/λ gene libraries used in the paper IV will be described in detail elsewhere (our unpublished results). Briefly, to construct the scFv libraries the Ig genes were amplified and cloned from B lymphocytes of healthy human donors. The V_H chain repertoire was prepared by amplifying the IgM specific cDNA and the L chain repertoire by amplifying both V_κ and V_λ specific cDNAs (Marks *et al*., 1991). The PCR amplification of the H and L gene repertoires was achieved using primer sets published previously (Marks *et al*., 1991), and the gene repertoires were cloned into a phagemid vector modified from the pBluescript SK vector (Stratagene). The scFv/κ and scFv/λ libraries were estimated to contain 2.1 x 10^7 and 7.4 x 10^7 clones, respectively.

The phage display vector contained the *lac* promoter (*plac*), the pel B signal sequence, the transcription terminator region (TT) and the phagemid origin of replication (f_1^+) (Fig. 8c). The Ig V domains are joined by the $(GGGGS)_3$ linker peptide, the c-myc peptide tag permits detection of the scFv in immunoassays and fusion to the pIII protein (aa 1–406) allows incorporation of the scFv into the phage. The presence of the amber stop codon (TAG) between the myc tag and the gene III allows (Fig. 8c) to switch between antibody fragment displayed on the phage surface and soluble antibody fragment by use of either a suppressor (XL-1 Blue) or a non-suppressor (HB2151) *E. coli* strain without recloning the gene.

2.2 Expression and purification of the antibody fragments

The different pKKtac expression vectors (Fig. 8a) were transformed into the expression host *E. coli* RV308. For induction the cells were grown to OD_{600} ~ 1.5, after which isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the bacteria were grown overnight at 30 °C. The secreted soluble antibodies were purified from the culture supernatants in one step by affinity chromatography using an Ox_nBSA column (n = 19 or 22) (papers I, III). Protein concentrations of the purified soluble antibody fragments were determined from the A_{280} values using the molar extinction coefficients.

The membrane-bound lpp-scFv-H6 antibody was first solubilized in detergent from the isolated bacterial cell envelopes and then purified in two steps using immobilized metal affinity chromatography (IMAC) and affinity chromatography on Ox_{21}BSA column as described in paper II.

2.3 Preparation of immunoliposomes

Immunoliposomes were prepared by adding the purified Ox lpp-scFv-H6 to a mixture of pure egg yolk phospholipids (PC, PE) and cholesterol solubilized in 1% non-ionic detergent, *n*-octyl-β-D-glucopyranoside (OG). The detergent was removed by dialysis in dialysis bags or in a LIPOSOMAT dialyzer (Dianorm, Munich, Germany) and the immunoliposomes reconstituted were collected by ultracentrifugation and suspended in 10 mM HEPES, pH 7.4 (paper II). Liposomes prepared in the absence of the lipid-tagged single-chain antibody were used as controls in the measurements.

2.4 Isolation of scFvs from a phage library

The scFv library DNAs containing the IgM V_H sequences and either κ or λ V_L sequences cloned into the phagemid vector were transformed by electroporation into *E. coli* XL-1 Blue cells (Stratagene, USA). The phage particles were rescued by superinfection with the filamentous phage VCS-M13 (Stratagene, USA) and a mixture of the scFv/κ and scFv/λ phage pools (1:2) was selected for clones binding to human colon carcinoembryonic antigen (CEA) (Calbiochem) using antigen-coated microtitre plate wells as described in paper IV. To decrease non-specific binding the phage pool was preincubated in a blocked microtitre plate well containing no antigen before incubation in a CEA-coated well. One CEA-specific clone was isolated from the fifth round of panning. DNA encoding the CEA-binding scFv was isolated, sequenced, cloned into the pKKtac vector and transformed into RV308 cells for further characterization.

2.5 Characterization of antibody binding properties

In this work ELISA was used to monitor the binding activities and specificities of both the soluble and membrane-bound Ox- and CEA-binding antibodies as described in papers I–IV. Fluorescence quenching (Eisen, 1964) was employed to determine the affinity constants (K_A) of the soluble anti-Ox antibody fragments (papers I, III) as described previously (Takkinen *et al*., 1991). The BIAcore biosensor was used to analyse the binding kinetics of the Ox IgG1 Fab fragment (K. Alfthan, unpublished data), the different Ox scFv derivatives and the Ox Fv fragment as described in paper III as well as the Ox-binding activity of the immunoliposomes (paper II). BIAcore was also employed to test the binding of the soluble CEA scFv isolated from the antibody phage library (K. Alfthan, unpublished data). A periplasmic sample of an induced RV308/pKKtac culture was run over CEA, which was immobilized on a CM5 sensor chip (190 RU) using the EDC/NHS amino coupling chemistry according to the manufacturer´s instructions (BIAcore AB). Otherwise the conditions were those described in paper III. An untreated sensor chip surface was used as a control surface.

2.6 Other methods

Protein samples were analyzed by SDS-PAGE according to Laemmli (1970) and then transferred onto a nitrocellulose filter (Towbin *et al*., 1979). For immunostaining the filters were first incubated with specific antibodies recognizing either the Ox antibody fragments (papers I–III) or other epitopes fused to the proteins studied (paper IV), followed by incubation with an enzyme-labelled antibody before addition of the substrate. Preparation of the Ox_nBSA derivatives (n = 4–22) (papers I–IV) was performed basically as described by Mäkelä *et al.* (1978).

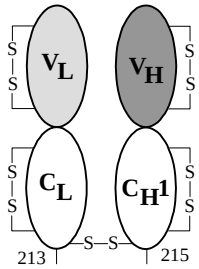
3. Results and discussion

Murine anti-2-phenyloxazolone antibody was chosen as a model antibody in this work (papers I–IV), because it has been extensively studied and much information was available on the maturation processes of the immune response to 2-phenyloxazolone (Ox) (Kaartinen *et al*., 1983; Berek *et al*., 1985). In addition, an anti-Ox antibody and its Ox-binding fragments have been used as model molecules for structural and functional studies of antibodies (Alzari *et al*., 1990; McManus and Riechmann, 1991). To extend the study to human antibodies recognizing human proteins, CEA was used as a model antigen to isolate single-chain antibody fragments from a phage library (paper IV).

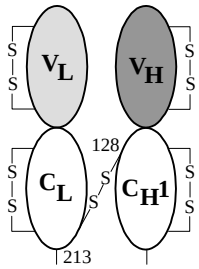
3.1 Characterization of soluble antibody fragments (I and III)

3.1.1 Stability of Fab fragments (I)

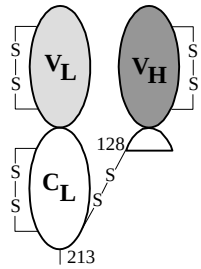
The Ox Fab fragment used in our first studies was constructed from an Ox IgG3 class antibody. The production levels of the active Ox IgG3 Fab fragments in the early experiments were consistently low, which prompted us to produce an Ox Fab fragment of another subclass, Ox IgG1. In paper I we compared the properties of these two different Fab fragments. The results showed that in the case of Ox IgG1 the Fd and L chains were able to assemble to form the Fab fragment while a minor amount of assembled Ox IgG3 Fab was detectable. The L chains in both Ox IgG3 and Ox IgG1 Fab are identical as are also the V_H domains except for five amino acid residues located in the D-J region. Thus, the main difference between the Ox IgG3 and Ox IgG1 Fab fragments is in the amino acid sequence of the C_H1 domain and in the interchain disulfide bond arrangement (Fig. 9). On the basis of this information we tested the effect of the C_H1 domain on the assembly of the chains. We therefore truncated most of the Fd chain while retaining the cysteine residue participating in the formation of the interchain disulfide bridge (Fig. 9). Deletion of most of the Fd chain from the C-terminus resulted in the assembly of a truncated, but fully functional Fab fragment, Ox IgG3ΔC_H (paper I, Fig. 2).



OxIgG1 Fab



OxIgG3 Fab



OxIgG3 ΔC_H

Figure 9. Schematic presentation of the interchain disulfide bond arrangement in the Ox Fab fragments studied.

The correct processing of the PelB signal sequence of the L and Fd chains was confirmed by N-terminal amino acid sequencing. In the case of the purified Ox IgG3 Fab only correctly processed L chain was observed and no intact Fd was detected. Several smaller molecular weight proteins were found, two of which were identified as C-terminal degradation products of the Fd chain (paper I, Fig. 4A). In the native, folded state proteins are normally much more resistant to cleavage by proteases compared to unfolded or misfolded molecules (Parsell and Sauer, 1989). In the case of the IgG3 Fd chain the observed instability may indicate problems in the folding of the Ox IgG3 Fab. A failure to produce functional proteins in *E. coli* may result either from the absence or limiting amount of appropriate molecular chaperones and/or disulfide isomerases necessary for folding and formation of disulfide bonds (Baneyx, 1999). Recent studies have led to the identification of new periplasmic catalysts, the

coexpression of which was shown to improve the folding of antibody fragments expressed in *E. coli* (Bothmann and Plückthun, 1998, 2000; Ramm and Plückthun, 2000). On the other hand, the IgG3 subclass antibodies have a tendency to aggregate and self-associate and at least in one case the site responsible for aggregation was reported to be located in the Fd fragment (Capra and Kunkel, 1970). Thus, precipitation in addition to the observed degradation of the Fd chain during synthesis in *E. coli* cannot be ruled out.

Similar observations of the Fd chain on the production of functional Fab fragments have been reported since the publication of paper I. It was shown recently that replacing the C_H1 domain of murine IgG2a and IgG2b antibodies by that of the IgG1 subclass antibody significantly increased the production level of immunoreactive Fab fragments (Pajunen *et al*., 1997). In addition, expression of IgM Fab fragments in *E. coli* resulted in relatively low production levels and accumulation of the L chains, probably due to precipitation of the Fd chain in the periplasmic space of *E. coli* (Adib-Conquy *et al*., 1995; Jahn *et al*., 1995). The C_H1 domain of the IgM subclass antibody is glycosylated (Davies and Shulman, 1989), and thus the lack of glycosylation in bacteria may also contribute to the low levels of IgM Fab in these cases. Interestingly, all these antibody types, IgG2a, IgG2b and IgM, share the interchain disulfide bond arrangement with the IgG3 antibody. At this point, it would be tempting to speculate that this kind of interchain disulfide bond arrangement is involved in the non-productive folding of Fab fragments in *E. coli*. However, the situation is more complex, as shown in the case of human IgE Fab fragments (Steinberg *et al*., 1996), which have a similar interchain disulfide bond arrangement to that of the IgG3 antibodies. Nevertheless, the IgE Fab fragments have been successfully expressed on the surface of bacteriophage, demonstrating the ability of the Fd chain to assemble in *E. coli* (Steinberg *et al*., 1996). It also appears that the effect of the constant domains on the production of functional Fab fragments is not limited to the Fd chain, but that C_L domains can also have a significant influence on the expression of functional antibody fragments (MacKenzie *et al*., 1994). All these results together emphasize that still more information is required to understand all the factors influencing the productive folding and disulfide bond formation of functional Fab fragments in *E. coli*.

The thermal stabilities measured for the different Fab fragments suggest that the C_H1 domain stabilizes the structure against denaturation. This is demonstrated

by the results showing that the Ox antibody fragments lacking the C_H1 domain began to loose their activity already at 40°C, whereas those antibodies having an intact C_H1 domain were active up to 60°C (paper I, Fig. 5). In another study it was observed that glycerol decreases the binding affinity of the scFv fragment, although no effect of the glycerol on the activity was observed for the Fab fragment or the whole monoclonal antibody (Müller *et al*., 1994). It was suggested that the constant domains adjacent to the V domains provide additional stabilization of the molecular structure at the antigen binding site. By using phage display technology, it is now possible to generate antibody fragments and other proteins with improved stability by performing the selection steps at elevated temperatures or in the presence of denaturants or proteases (Chowdhury *et al*., 1998; Sieber *et al*., 1998; Jung *et al*., 1999).

3.1.2 Stability of scFv and Fv fragments (III)

In an earlier work we constructed an Ox scFv in which the C-terminus of the V_H domain was joined to the N-terminus of the V_L domain by a 28 amino acid residues long interdomain peptide of the *Trichoderma reesei* cellobiohydrolase I, CBHI (Fig. 10) (Takkinen *et al*., 1991). Initially, the CBHI linker peptide was chosen because it represents a flexible linker in a naturally secreted multidomain protein. The Ox scFv was secreted into the periplasm of *E. coli* and released as an active hapten binding fragment into the culture medium. During the bacterial production we observed occasional proteolytic degradation of the linker peptide, the extent of which varied between cultivations. Even when degradation occurred it was not so extensive as to prevent the use of these scFvs fragments in our studies. The yields of the intact fragment were satisfactory and allowed functional characterization of the antibody.

The proteolytic cleavage sites (↓) of the CBHI linker were determined by N-terminal amino acid sequencing and three sites were identified: R5↓G6, R11↓R12 and H27↓Y28 (Fig. 10). In order to improve the stability of the CBHI linker, we constructed shorter linker variants of 11, 6 and 2 amino acid residues by omitting the identified proteolytic cleavage sites (Fig. 10). This truncation of the linker was prompted by the molecular model constructed earlier for the Ox Fv fragment (Holm *et al*., 1990; Hoffrén *et al*., 1992), which suggested that both the C-terminus of the V_H domain and the N-terminus of the V_L domain are

flexible enough to serve as part of the linker. In addition to these CBHI linkers, we tested the performance of two other linkers, a 15 amino acid residues long $(GGGGS)_3$ linker (Huston *et al*., 1988) and a 22 amino acid residues long IgG2b hinge sequence (Bell *et al*., 1990) (Fig. 10), in which the four Cys residues of the original sequence were replaced by Ser residues. In addition to the different Ox scFv derivatives, a corresponding Ox Fv fragment without any joining peptide between the V domains was produced.

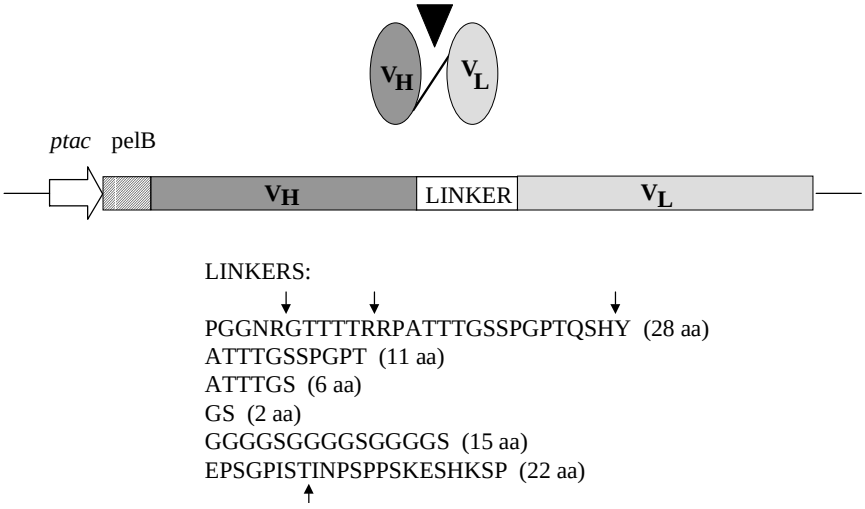


Figure 10. Linker peptide sequences used in this study. The proteolytic cleavage sites are indicated by arrows.

Attempts to improve the stability of the scFv molecule by truncation of the CBHI linker peptide revealed that the degradation could not be completely avoided simply by omitting the identified protease sensitive sites in the linker peptide. In addition it was also observed that the Ox scFv with the modified IgG 2b linker was cleaved into two smaller fragments after purification, probably due to the presence of copurified proteases (paper III, Fig. 3A). N-terminal amino acid sequencing revealed that the proteolytic cleavage site was located in the linker peptide at the site T8↓I9 (Fig. 10). One approach to minimize or avoid proteolytic degradation might be to produce the antibody fragments as insoluble proteins into the bacterial cytoplasm, as inclusion bodies are often protected from degradation (Baneyx, 1999). This strategy, however, requires effective *in vitro* refolding steps, which may be laborious and time-consuming to optimize.

In this study the observed proteolytic degradation of the Ox scFv fragment could not be eliminated by replacing the CBHI linker by the $(GGGGS)_3$ linker. Some amounts of low molecular weight proteolytic degradation products were observed in both cases (paper III, Fig. 2). However, the functionality of the $(GGGGS)_3$ linker has been demonstrated in several studies and currently the most frequently used linkers are composed of repeats of glycine and serine residues. The flexibility of the $(GGGGS)_3$ linker has been confirmed by NMR and it was also observed that the linker peptide had essentially no influence on the structure of the V domains (Freund *et al*., 1993). As well as the $(GGGGS)_3$ linker (Huston *et al*., 1988) longer derivatives even up to 30 amino acid residues, $(GGGGS)_{6,}$ have been used (Nieba *et al*., 1997). The results of several recent studies suggest that phage display combined with random mutagenesis of the linker might provide a rational means to optimize the linker sequence (Tang *et al*., 1996; Turner *et al*.,1997; Hennecke *et al*., 1998).

Since publication of our study on Ox scFv (Takkinen *et al*., 1991), the CBHI linker peptide has been applied to construct also other scFv molecules. For example, a modified CBHI´ linker containing 24 amino acid residues was employed to join two scFvs with different binding specificities in order to construct a bispecific scFv produced in *E. coli* (Mallender and Voss, 1994). In this study the antibody fragments accumulated in bacteria as insoluble inclusion bodies and although smaller antibody fragments were detected in the sample after purification, no instability of the CBHI´ linker was reported. The CHBI linker has also been used to join the V domains of a CEA specific antibody and the performance of this scFv for tumor localization *in vivo* was compared to that of an scFv with the 14 amino acid residues long "212" linker (Bedzyk *et al*., 1990). It was noticed that the scFv containing the linker "212" had better pharmacokinetic properties than the scFv with the CBHI linker. Proteolytic cleavage of the CBHI linker *in vivo* was suggested as one of the possible explanations for this difference between the two scFvs (Wu *et al*., 1996).

During this work we did not observe any proteolytic degradation of the Ox Fv fragment. However, the V domains of the Fv fragments are known to dissociate at low protein concentrations (Glockshuber *et al*., 1990). Although the Fv fragments are less stable than the scFv fragments, the apparent stability of the Fv molecules is increased with high protein concentration and in the presence of the

antigen (Jäger and Plückthun, 1999). In our study we observed instability of the Ox Fv fragment during the hapten binding studies (see 3.1.3).

3.1.3 Binding properties of the antibody fragments (I and III)

The Ox scFv constructed earlier showed hapten binding affinity comparable to that of the corresponding parental monoclonal antibody indicating that the CBHI linker peptide used to join the V domains did not disturb Ox binding of the scFv molecule (Takkinen *et al*., 1991). In this work we determined the hapten binding properties of several other recombinant antibody fragments produced in *E. coli* using ELISA, BIAcore and fluorescence quenching.

In order to study the effect of the linker peptides on the hapten binding activity of the Ox scFv molecule, the different scFv derivatives (see Fig. 10) were first measured by ELISA. The results suggested similar hapten binding activities of the original Ox scFv and the Ox scFv with the $(GGGGS)_3$ linker, whereas increased activity was observed for the shorter Ox scFv derivatives (paper III, Fig.4). Further, the binding activity appeared to be inversely proportional to the length of the linker; the shortest derivative showed the highest response, approaching that of the intact antibody. Earlier observations had suggested that the use of short linker peptides drives dimerization of the scFvs (Holliger *et al*., 1993). Gel filtration analysis of the Ox scFv derivatives revealed that the original Ox scFv and the one with a $(GGGGS)_3$ linker were monomeric, whereas the shorter derivatives existed in several different forms. Results obtained later by others supported our observation that the use of the CBHI linker allows folding of the V domains, resulting in a monomeric scFv (Wu *et al*., 1996), while with the $(GGGGS)_3$ linker both monomeric (Iliades *et al*., 1997) and multimeric (Desplancq *et al*., 1994) forms were observed. The multimerization appears to be concentration-dependent, increasing antibody concentrations driving the formation of multimers (Desplancq *et al*., 1994). In addition to dimers, trimers, tetramers or even higher molecular mass multimers have been obtained either by using short linkers or by omitting the linker peptide (Iliades *et al*., 1997; Kortt *et al*., 1997; Atwell *et al*., 1999; Le Gall *et al*., 1999; Dolezal *et al*., 2000). Competitive ELISA was used to confirm the binding specificity of all the antibody fragments (data not shown). However, in the case of the Ox Fv fragment problems were encountered in attempts to obtain a linear correlation

between sample dilution and absorbance, probably due to dissociation of the V domains during the experiment (Glockshuber *et al*., 1990; Jäger and Plückthun, 1999).

The BIAcore biosensor was used to analyze the binding kinetics of the different Ox scFv derivatives and the corresponding Ox Fv fragment. Attempts to separate the different molecular forms of the truncated Ox scFv fragments by gel filtration did not give satisfactory resolution and samples containing mixtures of the different molecular forms were taken for BIAcore analysis. Two different epitope densities, high (Ox_{16}BSA) and low (Ox_4BSA), were chosen in order to see the possible effects caused by the differences in the binding valencies of the samples. The binding data was evaluated using the 1:1 interaction model available in the kinetic software supplied by the manufacturer at that time. Although the interaction of multivalent antibodies with the antigen is much more complex this simple set-up allowed us to compare the relative binding properties between the different samples.

The analysis revealed that the Ox scFvs with the truncated CHBI linkers showed higher apparent K_A values than the original Ox scFv and the Ox scFv with the $(GGGGS)_3$ linker, which is consistent with the results obtained by ELISA. The K_A value of the Ox Fv determined on the high epitope surface was close to that of the parental monoclonal antibody ($1.5 \times 10^{-6} M^{-1}$, Takkinen *et al*., 1991) determined by the fluorescence quenching method, whereas higher K_A values were obtained with all the other samples. The K_A values on the low epitope surface were lower than those obtained on the high epitope surface (paper III, Table I) and in the case of the original Ox scFv and the Ox scFv with the $(GGGGS)_3$ they were close to those values obtained by the fluorescence quench titration (Table I). The responses of the Ox Fv on the low epitope surface were too low to rely on. The Ox scFv derivatives with the truncated CBHI linkers dissociated more slowly from the high epitope surface than from the low epitope surface due to the presence of bi- or multivalent fragments, whereas the monovalent Ox scFv fragments dissociated with similar rates from both surfaces. Slow dissociation of bivalent or multivalent antibody fragments has also been reported by other workers (reviewed in paper V). The kinetic constants determined with both low and high epitope densities for the Ox IgG1 Fab (K. Alfthan, unpublished data) were close to those obtained for the monomeric Ox scFv fragments. The K_A values determined on the low epitope surface for all the

monovalent fragments were close to those obtained with the fluorescence quenching method and were also comparable to that of the parental monoclonal anti-Ox antibody (Table 1).

Table 1. Kinetic constants, $k_{ass,}$, k_{diss} and K_A (k_{ass}/k_{diss}) determined for the monomeric Ox antibody fragments by BIAcore on the Ox_4BSA surface (paper III, unpublished results) and the K_A values determined by fluorescence quenching (FQ) (papers I, III).

	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})	K_A (M^{-1})	K_A(FQ) (M^{-1})
Ox $scFv_{CBHI}$	3.6×10^3	2.5×10^{-3}	1.4×10^6	0.9×10^6
Ox $scFv_{(GGGGS)3}$	7.4×10^3	1.5×10^{-3}	4.9×10^6	1.1×10^6
Ox IgG1Fab	7.4×10^3	2.7×10^{-3}	2.7×10^6	1.8×10^6

K_A value of parental Ox IgG1: 1.5×10^6 M^{-1} (Takkinen *et al*., 1991)

The affinities of the Ox IgG3ΔC_H and of the Ox IgG3 Fab fragments were determined by the fluorescence quench titration and found to be close to those of the parental monoclonal antibody. The observed binding activity of the Ox IgG3 Fab fragment is probably a result of noncovalent pairing of the L chain with the C-terminal degradation products of Fd chains, which are still able to form an intact and relatively stable antigen binding cavity. By contrast, attempts to determine the K_A values of the Ox Fv by fluorescence quenching gave different K_A values for different Fv concentrations, indicating instability of Fv fragments, which has been found to be concentration dependent (Glockshuber *et al*., 1990; Jäger and Plückthun, 1999).

3.2 Characterization of scFvs displayed on liposomes (II)

It was earlier shown that fusion of the signal peptide and the nine N-terminal amino acid residues of the major lipoprotein of *E. coli* (lpp) to the Ox scFv with the 28 amino acid CBHI linker led to expression of an active lipid-modified single-chain antibody, which behaved like a membrane protein (Laukkanen *et al*., 1993). The work in paper II describes the functional reconstitution of this biosynthetically lipid- and histidine-tagged Ox lpp-scFv-H6 in liposomes. The Ox lpp-scFv-H6 was expressed in *E. coli* and purified in two steps by applying IMAC followed by a hapten affinity chromatography. The purified, detergent solubilized Ox lpp-scFv-H6 was reconstituted in active form into liposomes by dialysis. The average number of antibody molecules per liposome was calculated to be about 2000. In addition the detergent dialysis method was thought to lead to a 50/50 distribution in terms of inward/outward orientation of the reconstituted protein.

In therapeutic and diagnostic approaches both the strength and specificity of the antibody-antigen interaction mediating the binding of liposomes to the antigen is of crucial importance. The multivalent binding obtained by display of many antibody molecules on the surface of liposomes can be thought to lead to higher binding avidity. In this study the Ox binding of the immunoliposomes was analyzed in BIAcore by running the liposomes on a sensor chip surface with immobilized Ox_{16}BSA. The results showed increased binding of the immunoliposomes to Ox_{16}BSA compared to BSA used as a control surface (paper II, Fig. 5B). Also liposomes lacking the antibody did not bind to either surface. The binding of the Ox scFv displayed on liposomes was inhibited in the presence of the soluble hapten in a concentration dependent manner which confirmed the specificity of the binding (paper II, Fig.6). We also tested the hapten binding activity of the soluble lipid-tagged Ox scFv in BIAcore in the presence of different detergents (Triton X-100, n-octyl β-D-glucopyranoside). In contrast to the soluble Ox scFv, no reproducible binding of the lipid-tagged Ox scFv was observed. As demonstrated by both the successful affinity purification of the fragment and ELISA assay, the presence of the detergent as such allows the lipid-tagged Ox scFv to bind the hapten (paper II, Fig. 3). The reason for this discrepancy is not clear.

Dissociation of the immunoliposomes from the hapten surface analyzed in BIAcore was extremely slow compared to that of the soluble Ox scFv (paper II, Fig.5C), which is in good agreement with the expected multivalent binding of immunoliposomes. The determination of the affinity constant for the interaction between the hapten and the scFv fragments displayed on the liposomes was not possible in the assay format used in this study. This was because the concentration of the active antibody molecule has to be known, but this was not possible in our experimental design. At this point further studies on binding kinetics were not performed. However, it is possible to analyze interaction kinetics of molecules displayed on liposomes by capturing the intact liposomes on a sensor surface (Masson *et al*., 1994; MacKenzie *et al*., 1997; Harrison *et al*., 1998). For the kinetic analysis, the other binding partner can then be supplied in different concentrations over this liposome surface. New modified sensor chips designed either to immobilize intact liposomes or to form a lipid layer generated from the liposomes on the sensor surface may provide another useful approach in future studies (Cooper *et al*., 1998; Erb *et al*., 2000). This development in surface chemistry allows analysis of biomolecular interactions in a membrane environment, and thus may open new possibilities in membrane protein research.

The potentiality of the immunoliposomes as specific, stable and signal-amplifying immunodiagnostic reagents was demonstrated in later studies, in which either europium-chelate (Laukkanen *et al*., 1995) or carboxyfluorescein (Kobatake *et al*., 1997) was encapsulated as a fluorescent marker in immunoliposomes harboring the lipid-tagged Ox.

3.3 Characterization of scFvs displayed on baculovirus (IV)

Recombinant baculoviruses can be used for efficient production of proteins with post-translational modifications such as glycosylation, phosphorylation, acylation and palmitylation and therefore serve as potential candidates for a eukaryotic display system. Baculoviruses are not inherently pathogenic for mammalian cells and thus possess potential for use as gene delivery vehicles (Hofmann *et al*., 1995). To target the virus it is possible to introduce cell specific molecules on the viral surface. One approach for the specific targeting is presented in paper IV, in which the ability of baculovirus to display functional scFv fragments and

synthetic IgG-binding domains (Z and ZZ) was studied. Three different scFvs were chosen for this work, two of which were murine Ox scFvs, one with the 28 amino acid CBHI linker and the other with the $(GGGGS)_3$ linker (Fig. 10). The third scFv containing the $(GGGGS)_3$ linker peptide was specific for CEA and was isolated from a naive human scFv library (see below). CEA is expressed on different cancer cells, e.g. colorectal carcinoma cells and it has been used as a marker in determining and monitoring the status of malignant diseases (Konishi *et al*., 1998).

3.3.1 Isolation of scFvs from a phage library

To isolate CEA-specific scFvs (CEA scFv), a mixture of the human scFv/κ and scFv/λ phage libraries (~ 10^{11} phage particles) was incubated on a CEA-coated microtitre plate well after preincubation on a blocked well without the antigen. After five rounds of selection the DNA pool was isolated and transformed to a non-suppressor *E. coli* strain (HB2151) to produce soluble scFvs. The expression of the scFv was confirmed from both the culture supernatant and the periplasmic fraction by immunoblotting, and the CEA specific clones were identified from the culture supernatants by ELISA. Three CEA-binding clones were identified from the twenty clones analyzed. These three positive clones were sequenced and found to have identical DNA sequences. The deduced amino acid sequences of the CDRs found in this clone were similar to those of the CEA scFvs isolated earlier by other groups from naive human scFv libraries (data not shown).

The antigen binding activity of the CEA scFv was tested in BIAcore by running the periplasmic fraction isolated from the induced RV308 culture on the sensor chip surface with immobilized CEA. A low but significant binding response was observed, compared to a control (untreated) sensor chip surface (K. Alfthan, unpublished data) (Fig. 11). The specificity of the isolated CEA scFv was confirmed by competition ELISA from the culture supernatant (paper IV, Fig. 2).

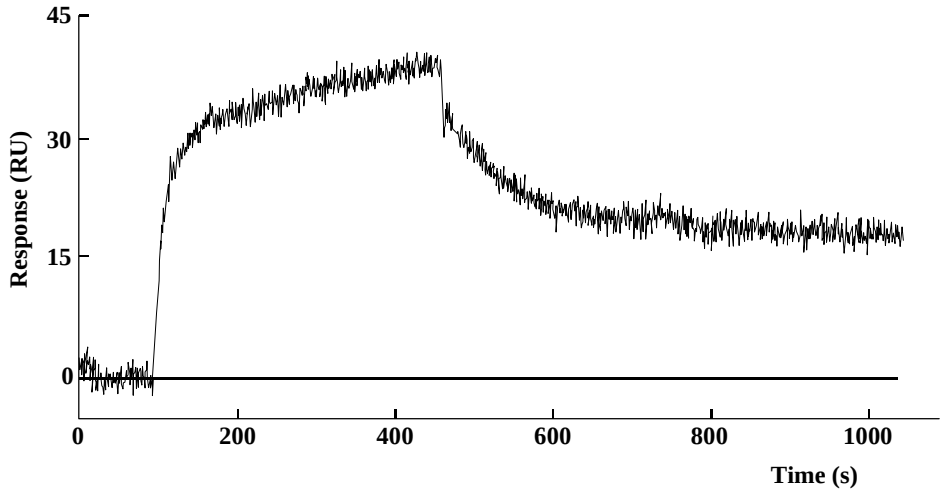


Figure 11. Binding of the isolated scFv on immobilized CEA analyzed by BIAcore. The background signal obtained on the control surface has been subtracted.

3.3.2 Binding properties of the scFvs displayed on baculovirus

For the expression on the surface of virions, the two Ox scFvs and the CEA scFv were fused to the N-terminus of the major envelope protein, gp64, of the baculovirus AcNPV. All the antibodies were incorporated into the virion, although the amount of the Ox scFv with the CHBI linker was significantly lower than that of the two other scFvs with the $(GGGGS)_3$ linker peptide (paper IV, Fig. 4). These two Ox scFv fragments differ from each other only with respect to the linker, and the Ox scFv with the 28 amino acid CBHI linker is known to be produced as a soluble and active antibody in *E. coli* (Takkinen *et al*., 1991; paper III). The CBHI linker is rich in Ser and Thr residues, which in the filamentous fungus *Trichoderma reesei* are known to be O-glycosylated (Tomme *et al*., 1988; Salovuori *et al*., 1987). It is possible that the high degree of the O-glycosylation in the linker peptide during expression in insect cells interferes with the incorporation of this scFv into the baculovirus particle.

The antigen/hapten binding ability of the scFvgp64 fusions was first demonstrated by ELISA from the cell lysates from virus-infected insect cells

(paper IV, Fig. 5). The expression of the scFv fragments on viral particles and the specificity of the binding were then confirmed from the viral supernatants using competitive ELISA (paper IV, Fig. 6). The only scFv fusion protein, which failed to give ELISA signals above the background, was the Ox scFvgp64 with the CBHI linker. This is consistent with the low incorporation of this fusion protein into the virus particle (paper IV, Fig. 4). Throughout the ELISA experiments the CEA scFvgp64 fusion showed the highest signal of all the scFv fusion proteins tested. The CEA scFv was originally isolated from a phage display library, demonstrating that this scFv is well expressed in bacteria. The results obtained in this study suggest good expression of this fragment also in the eukaryotic display system applied. It has been shown earlier that selection of the scFv phage libraries on microtitre plate wells favours enrichment of scFv fragments forming dimers (Schier *et al*., 1996a). Neither the presence of dimers nor the binding affinity of the CEA scFv was studied in this case. As another approach to confirm the specific hapten/antigen binding of the scFvgp64 fusions, the virus particles were analyzed in BIAcore by running the infected cell culture supernatants on immobilized Ox_{16}BSA or CEA. However, interpretation of the results was hampered by non-specific binding of the virus on the sensor chip surface. The gp64 fusion proteins are transported onto the surface of infected insect cells, from which they are then incorporated into the budded virus particles. Thus, one possibility to control production of functional pg64 fusions is to analyze by FACS the binding of the proteins expressed on the infected insect cells rather than the virus particles themselves (Ernst *et al*., 1998).

The baculovirus display system may prove to be a useful display technology for large, complex eukaryotic proteins that cannot be processed post-translationally correctly in bacteria. Currently, however, the construction of large baculovirus display libraries is limited by the relatively low transfection efficiency (6.6×10^5 clones/μg viral DNA, Ernst *et al*., 1994). Further, the amount of recombinant fusion protein present on the viral envelope compared to the native gp64 protein is relatively low, necessitating means to increase the display efficiency. Due to the fragile nature of the AcNPV budded viruses (Summers and Volkman, 1976; Mottershead *et al*., 1997), the selection and screening methods used to isolate binders from the phage display libraries are most probably not applicable to baculovirus display system and thus the development of new, gentle but efficient selection and screening methods is needed. Despite these apparent limitations, a proof of the principle of the display of active antibodies on the surface of

baculovirus was demonstrated in this work, and future development of the methodology will eventually show the usefulness of the baculovirus system both in the display technology and in gene therapy.

4. Conclusions

Antibodies have been targets of intensive protein engineering due to their practical importance in medical, diagnostic and research applications. As shown in this work and by others, a number of different antibody fragments can be expressed in *E. coli* as active antigen-binding proteins. By utilizing the bacterial secretory machinery the proteins are produced in their native state and no *in vitro* refolding is needed. However, the expression levels of the functional antibodies may vary widely. This work demonstrated that the stability of the antibody fragments against proteolysis during their production in bacteria is one of the factors, which influences the yield of the functional fragments. The susceptibility to proteases may arise from incomplete folding of the antibody fragments or the presence of proteolytic cleavage sites in the engineered antibodies. Two findings of specific importance were made. Firstly, it was observed that the production of stable and active mouse Fab fragments depends on the first constant domain of the heavy chain. Comparison of the IgG3 and IgG1 Fab indicated the superiority of the latter for the production of active properly assembled Fab fragments in *E. coli*. Secondly, the results indicated that a linker peptide is necessary to stabilize the Fv fragment, but that the linker region may be susceptible to proteolytic degradation. In addition it was demonstrated that linker peptides shorter that 12 amino acids promote the formation of dimeric or multimeric scFvs, which showed increased binding activities compared to the monomeric scFvs.

The development of antibody phage display technology for generating antibodies with improved or novel binding properties has opened new possibilities in the entire field of antibody technology. In this work, two alternative antibody display systems, liposome display and baculovirus display, were explored. Liposomes displaying antibody fragments have potential applications in immunoassays, targeted drug delivery and gene therapy. This work demonstrated that a biosynthetically lipid-tagged scFv can be immobilized in a stable and orientated manner onto liposomal membranes, which allows reconstitution of active hapten-binding immunoliposomes. Biosynthetic lipid-tagging of bacterially expressed antibody fragment provides an alternative for chemical conjugation of antibodies to liposomes. Expression of foreign proteins on the baculovirus surface provides a means for functional display of complex eukaryotic proteins. In this work the baculovirus display system was studied

using single-chain antibodies as model proteins. Baculoviruses carrying a cell-targeting function, such as antibodies, have aroused interest as a gene transfer vehicle. It was shown that fusion of the scFv fragments to the baculovirus major envelope protein led to the expression of active scFv fragments on the surface of the virus particles.

This work and many others show that antibodies are molecules which are compatible with a number of expression systems and that they tolerate significant structural modifications without loss of the binding function. The enormous application potential, limited only by the imagination, will thus continue to make antibody engineering increasingly popular in many areas of research as well as in medical and industrial applications.

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Author(s)
Alfthan, Kaija

Title

Structural stability and binding properties of soluble and membrane-anchored recombinant antibodies

Abstract

Antibody engineering and advances in microbial expression systems have enabled production of small, active antibody fragments. However, the functional expression yields of these recombinant antibodies vary widely. The results described in this thesis elucidate factors influencing the expression of stable and active antibody fragments in bacteria. The model antibody used throughout this study was a mouse monoclonal antibody binding to 2-phenyloxazolone (Ox). It was shown that the first constant domain of the heavy chain (C_H1) has a remarkable effect on secretion of functional and stable Fab fragments. Comparison of the production of the Ox IgG1 and Ox IgG3 subclass Fab fragments in bacteria demonstrated the superiority of the Ox IgG1 Fab compared to the Ox IgG3 Fab. In addition to its effect on secretion, the C_H1 domain contributes to the thermal stability of the antibody fragments. To study the effect of a linker peptide on both proteolytic stability and binding activity of single-chain antibodies, six different Ox scFv derivatives and an Ox Fv fragment with no joining peptide between the variable domains were constructed. It was shown that joining of the variable domains with a linker peptide improved hapten binding properties compared to the Ox Fv fragment, but may expose the fragment to proteolytic degradation. Truncation of the linker peptide to less than 12 amino acids induced formation of dimers or multimers. The binding affinities determined for the monomeric Ox scFv and Ox IgG1 Fab fragments using the BIAcore biosensor and fluorescence quenching methods were close to each other and comparable to that of the parental monoclonal antibody.

In addition to studies with soluble antibody fragments, this work was extended to cover characterization of antibody fragments displayed on liposomes and on the surface of baculovirus. Immunoliposomes have potential applications both in therapy and in immunodiagnostics. In this work liposomes displaying antibodies were generated by incorporation of purified lipid-tagged Ox scFvs expressed in *E. coli* into phospholipid liposomes. It was demonstrated that the biosynthetically lipid-tagged Ox scFv molecules can be immobilized in a functional, stable and oriented manner onto liposomal membranes, resulting in immunoliposomes showing specific hapten binding. BIAcore analysis of the immunoliposomes revealed very slow dissociation from the Ox surface, which is in good agreement with the predicted multivalent nature of the immunoliposomes. The baculoviral display approach holds a promise as a candidate for a eukaryotic display system. In this work single-chain antibodies were used as a model to investigate functional expression of foreign proteins on the surface of baculovirus. Viral vectors displaying cell targeting moieties such as single-chain antibodies have aroused interest as gene transfer vehicles. Two Ox scFv derivatives and a human scFv specific for carcinoembryonic antigen (CEA) were fused to the major envelope protein (gp64) of the *Autographa californica* nuclear polyhedrosis virus (AcNPV). It was shown that the two single-chain antibodies which contained a $(GGGGS)_3$ linker peptide were incorporated into the budded virus particles and expressed as functional hapten/antigen binding fragments on the viral surface. In the case of the third scFv containing a natural linker peptide derived from a fungal cellulase, less efficient incorporation into the virus particles was observed.

Keywords

recombinant antibodies, antibody engineering, structural stability, binding, expression, *Escherichia coli*, display

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