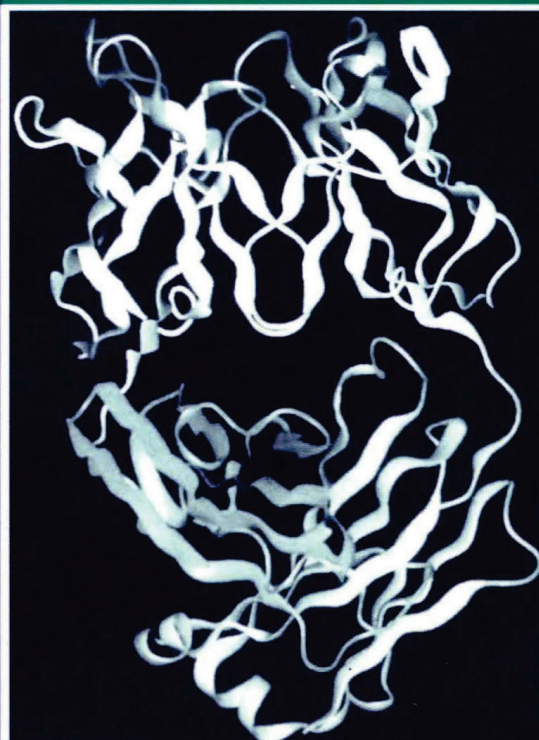


Applications and Engineering of Monoclonal Antibodies



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For example, cross-linkers containing macrocyclic ligands for the stable attachment of radioisotopes have been used to allow formation of radiolabelled antibody fragments without the need for further derivatisation of the antibody (King *et al.*, 1994).

Fv-based fragments

The two chains of the Fv fragment are less stably associated than the Fd and light chain of the Fab fragment with no covalent bond and less non-covalent interaction, but nevertheless functional Fv fragments have been expressed for a number of different antibodies. Characterisation of Fv fragments has revealed that although the antigen-binding ability of the Fab fragment is retained, dissociation of the two domains takes place at low protein concentration or under physiological conditions. Thus strategies for stabilising Fv fragments have been of great interest. Two strategies have been investigated with a number of different antibodies: firstly, mutating a selected residue on each of the V_H and V_L chains to a cysteine to allow formation of a disulphide bond between the two domains; and secondly, the introduction of a peptide linker between the C-terminus of one domain and the N-terminus of the other, such that the Fv is produced as a single polypeptide chain known as a single-chain Fv (Figure 2.5).

The production of single-chain Fv's (scFv's) was first described in 1988 (Bird *et al.*, 1988; Huston *et al.*, 1988). Since then a large number of scFv's have been produced which retain antigen-binding characteristics, although in some cases antigen-binding affinity is reduced. Expression in *E. coli* has been the most frequently used production method, with both intracellular expression and secretion enabling high yields of scFv to be made. The production of scFv molecules requires the identification of a suitable peptide linker to span the 35–40 Å distance between the C-terminus of one domain and the N-terminus of the other and allow correct folding and assembly of the Fv structure. Several different types of linker have been used and shown to result in functional scFv. Linkers have been selected from searching existing protein structures for protein fragments of the appropriate length and conformation, or have been designed *de novo* based on simple, flexible structures with perhaps the most commonly used being the 15 amino acid sequence

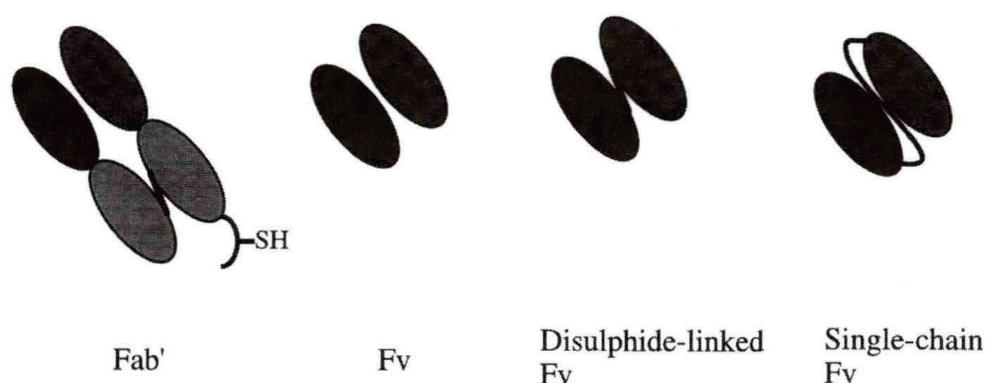


Figure 2.5 The organisation of domains in a Fab' fragment compared to Fv, disulphide-linked Fv and single-chain Fv fragments: disulphide-linked Fvs are formed when a selected amino acid residue on both V_L and V_H is mutated to cysteine such that a disulphide bond can form between the variable domains; single-chain Fvs are formed as a result of the introduction of a peptide linker into the sequence, linking the C-terminus of one variable domain to the N-terminus of the second

(Gly₄Ser)₃. Active single-chain Fv molecules have been produced in both of the two possible orientations, V_H-linker-V_L or V_L-linker-V_H; however, for some antibodies one particular orientation may be preferable as a free N-terminus of one domain, or C-terminus of the other, may be required to retain the native conformation and thus full antigen binding. For example, V_L-linker-V_H scFv's of the anti-tumour antibody B72.3 have been shown to be more active than V_H-linker-V_L with a range of different linker lengths (Desplancq *et al.*, 1994).

Many scFv's have been shown to be susceptible to aggregation with dimers and larger species formed in a concentration-dependent manner (reviewed by Raag and Whitlow, 1995). This is because the interactions between V_H and V_L are relatively weak and there appears to be an equilibrium set up between assembled and unfolded scFv in solution. This leads to domain interchange with neighbouring molecules taking place at high concentrations (Figure 2.6). This is particularly prevalent if strain is introduced into the conformation of the scFv molecule by the use of short linker peptides. Conversely, the use of longer peptide linkers relaxes any strain in the conformation and encourages monomer formation (Whitlow *et al.*, 1993). For example, the B72.3 scFv is in the form of mostly dimers and high molecular weight aggregates with linker lengths of 0, 5 and 10 amino acids but mostly monomeric with linkers of 15, 20, 25 and 30 amino acids (Desplancq *et al.*, 1994). In addition, the tendency towards dimer formation at high concentration decreases as the linker length increases (Figure 2.6). The potential of forming dimers with very short linkers, or no linker at all, can be exploited to produce stable dimeric structures which have been termed 'diabodies' (Holliger *et al.*, 1993). Such an approach can also be used to create molecules with two different binding specificities, bispecific diabodies, by fusing the V_H of an antibody of one specificity to the V_L of another and vice versa (see Section 2.5). The crystal structure of a diabody recognising a phospholipase enzyme has been solved and it has been demonstrated that the V_H and V_L domains on the two polypeptide chains associate as expected, although in this case there is also non-covalent association between two diabody molecules to form a tetrameric structure (Perisic *et al.*, 1994).

Fv's stabilised by disulphide linkages appear to be more stable structures than scFv's (Glockshuber *et al.*, 1990). The introduction of a disulphide bond between the V_H and V_L domains to form a disulphide-linked Fv requires the identification of residues in close proximity on each chain which are unlikely to affect directly the conformation of the binding site when mutated to cysteine, and will be capable of forming a disulphide bond without introducing strain into the structure of the Fv. Sites have been identified in both CDR regions and framework regions which appear to result in the formation of such disulphide bonds and allow the production of stabilised Fv fragments which retain antigen-binding characteristics (Glockshuber *et al.*, 1990; Reiter *et al.*, 1994). The use of sites in the framework region is likely to be of more general utility, and several different antibody Fv fragments have now been stabilised in this way (reviewed by Reiter and Pastan, 1996).

A different approach would be to use a single immunoglobulin domain for antigen binding. In a small number of cases a single-antigen binding domain may have antigen-binding activity. For example, V_H domains with affinity for lysozyme in the 20 nM range have been isolated which have been termed single domain antibodies or dAbs (Ward *et al.*, 1989). Light chain domains may also have antigen-binding activity in some cases (Masat *et al.*, 1994). However, single domains have been difficult to express and are poorly soluble due to an exposed hydrophobic surface which normally associates with the other V domain, and thus such small antigen binding proteins have not been widely used.

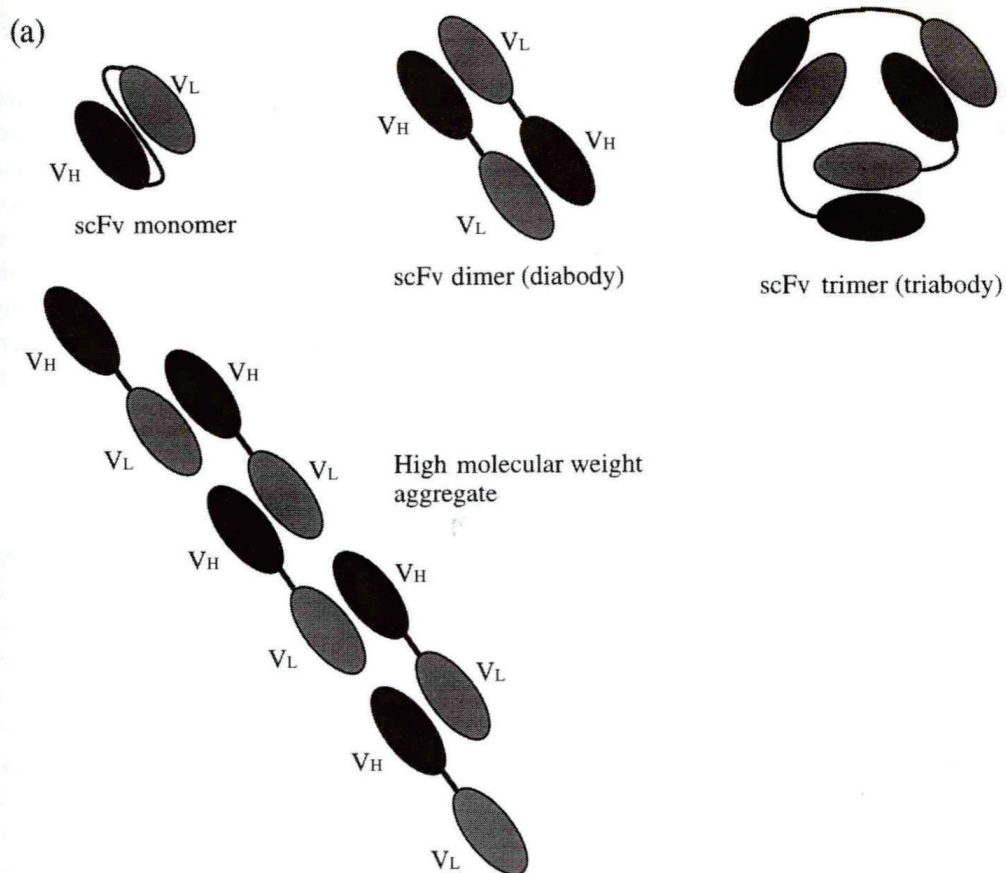
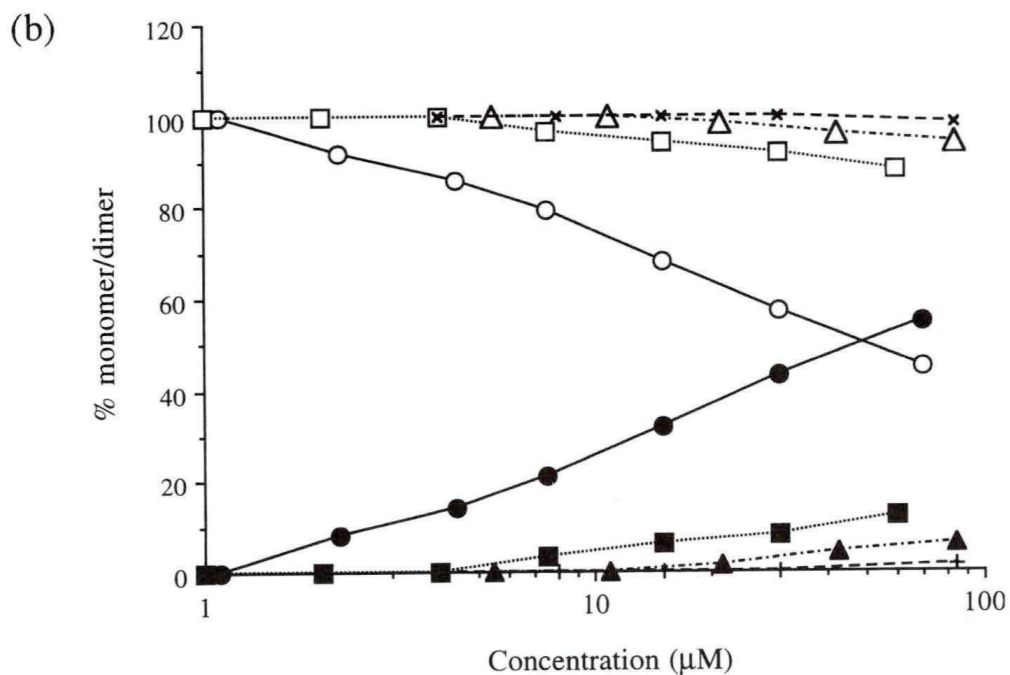


Figure 2.6 Aggregation of single-chain Fv: (a) representation of scFv monomer, dimer (diabody), trimer (triabody) and high-molecular weight aggregate; (b) extent of dimer formation with variation in linker length for scFv of the antibody B72.3, adapted from Desplancq *et al.* (1994). scFvs in the orientation V_L-V_H with linker lengths of 15, 20, 25 and 30 amino acids are shown. (○) Monomer, (●) dimer V_L-15-V_H; (□) monomer, (■) dimer V_L-20-V_H; (△) monomer, (▲) dimer V_L-25-V_H and (x) monomer, (+) dimer V_L-30-V_H.

Camels have been found to have naturally occurring antibodies without light chains, and thus attempts have been made to engineer the interface of the V_H domain to mimic camel V_H domains which may be more soluble (Davies and Riechmann, 1995). Although these attempts have been partially successful, in that antigen-binding specificities could be obtained from a library of such engineered V_H domains, only low affinity binding has been achieved to date. This is probably a consequence of the reduction in available CDR regions for antigen binding from 6 to 3 compared to authentic Fv. In some cases it may be possible to increase the number of antigen contact residues, for example by engineering an extra antigen-binding loop which can be inserted into the framework 3 region of V_H domains to increase binding affinity (Simon and Rajewsky, 1992).

Multivalent antibody fragments

As monovalent binding entities, Fv fragments suffer from relatively low avidity binding and therefore there has been much interest in producing multivalent Fv fragments which still retain a relatively small size. The production of 'diabodies' is one approach as described above, and some diabodies can be produced to high levels in the form of stable dimers (Zhu *et al.*, 1996). However, the stability of such dimeric species varies from one antibody to another (Whitlow *et al.*, 1994). In some cases, particularly with direct fusions of V_H and V_L, stable trimeric species are produced which have been termed 'triabodies' (Iliades *et al.*, 1997). Some triabodies show improved avidity for antigen as expected from the increased number of binding sites, although this is not always the case. A crystal structure of a non-functional triabody produced from the variable domains of two anti-hapten antibodies has been solved and demonstrated a dramatic alteration in the conformation of the V_H CDR3 antigen-binding loop (Pei *et al.*, 1997).

Several alternative approaches have been examined in attempts to produce dimeric species (Figure 2.7). A peptide linker can be introduced between two scFv fragments to produce a single polypeptide chain di-scFv molecule. However, attempts to produce such dimers have often resulted in reduced antigen binding for the two binding sites, even when anti-hapten antibodies were used (Mallender and Voss, 1994). One of the most successful strategies to produce dimeric species has been to introduce a cysteine residue which can be used to introduce a disulphide bond or a chemical cross-link between two scFv fragments. Either this can be introduced directly at the C-terminus of an scFv, via a linker sequence, or a natural antibody hinge region can be attached to form an scFv' fragment (Cumber *et al.*, 1992; Adams *et al.*, 1993; King *et al.*, 1994). Dimeric scFv can then be formed in a similar manner to that described for Fab' fragments above, and offers the same potential for site-specific attachment of radiolabels or other moieties (King *et al.*, 1994).

An alternative approach has been the fusion of Fv fragments with peptides that naturally form dimeric structures. Amphipathic helices can be fused to the C-terminus of scFv fragments to allow spontaneous dimerisation (Pack and Pluckthun, 1992; Pack *et al.*, 1993). These 'dimerisation domains' can be taken from, for example, a four-helix bundle structure or a leucine zipper from a naturally occurring protein. Leucine zippers are peptides of approximately 30 amino acids which naturally form amphipathic helices with leucine residues lining up on the hydrophobic face of the helix. The hydrophobic faces of two zipper peptides associate with each other through hydrophobic interactions. Therefore the association of these molecules occurs through non-covalent interactions, which require further stabilisation to form stable dimeric species. Stabilisation is achieved through engineering a disulphide bond to be formed between the two dimerisation domains when

Fc carbohydrate with conventional conjugates with ligands randomly attached to lysine residues have demonstrated significantly improved antigen-binding properties (Rodwell *et al.*, 1986). The disadvantages of this approach are the relatively harsh conditions required, which can damage and aggregate some antibody molecules. Methionine residues present in some antibody variable regions may be particularly susceptible to oxidation by periodate, which can lead to loss of antigen-binding avidity. In some cases histidine or tryptophan residues might also be affected.

Antibody engineering can be used to introduce specific attachment sites into antibody molecules, and this can be incorporated as part of the design of an engineered molecule. Extra cysteine residues can be introduced onto the surface of antibody constant domains to provide a specific attachment site without the need to disrupt native disulphide bonds. Introduction of specific cysteine sites in the CH₁ domain of the IgG heavy chain has been shown to result in sites to which ligands can be attached without any loss of antigen binding (Lyons *et al.*, 1990). Alternatively, extra carbohydrate sites can be engineered into the molecule to allow attachment via periodate oxidation. Some antibody light chains have an unusual natural glycosylation site, and thus the light chain has been used as a site to introduce a glycosylation site into antibodies which do not normally have carbohydrate attached to the light chain (Leung *et al.*, 1995). A third engineering strategy is to introduce extra lysine residues into the surface of the constant region domains (Hemminki *et al.*, 1995). Although this approach does not introduce a unique labelling site, lysine-reactive reagents are more likely to modify the antibody at the increased concentration of lysine residues in the constant region, resulting in the retention of more antigen-binding reactivity.

A more specialised approach is the use of reverse proteolysis to attach reagents specifically at the C-terminus of Fab' heavy chains (Fisch *et al.*, 1992). After production of a F(ab')₂ fragment by the protease lysyl endopeptidase, experimental conditions can be altered such that the same protease working in reverse is capable of the specific attachment of carbohydrazide groups to the C-terminus of the F(ab')₂ heavy chains. These carbohydrazide groups could then be used as an attachment point for a radiolabelled chelator reacting via an aldehyde group to form a hydrazone linkage. A similar approach has also been used to attach an aldehyde-modified enzyme to the C-terminus of F(ab')₂ (Werlen *et al.*, 1994).

Fusion proteins

An attractive alternative to chemical conjugation for antibody-protein conjugates is the direct expression of fusion proteins. This approach can be viewed as another method of site-specific attachment, as the protein to be fused is attached away from the antigen-binding site and no further modification is necessary. The antibody or antibody fragment is fused to the protein at the gene level, usually via attachment of the sequence to either full length or a truncated heavy chain. Expression of functional fusion proteins was first shown by fusion of a Fab fragment to Staphylococcal nuclease (Neuberger *et al.*, 1984). Since then the production of active antibody and antibody fragment fusion proteins with a variety of different enzymes, cytokines, and other proteins has been achieved (Table 2.6). Fusion proteins overcome problems with loss of antigen-binding ability on chemical conjugation and are also well-defined, homogeneous reagents. Disadvantages are that the flexibility of using linkers with varying chemical stability is lost, and in some cases fusion proteins have proved difficult to express in a functional form.

Table 2.6 Some examples of antibody fusion proteins

Antibody form	Fusion	Reference
Enzyme fusions		
F(ab') ₂	Staphylococcal nuclease	Neuberger <i>et al.</i> , 1984
scFv, dsFv	β-lactamase	Goshorn <i>et al.</i> , 1993 Rodrigues <i>et al.</i> , 1995
Fab'	β-glucuronidase	Bosslet <i>et al.</i> , 1992
scFv, F(ab') ₂	Alkaline phosphatase	Wels <i>et al.</i> , 1992 Ducancel <i>et al.</i> , 1993
scFv	Urokinase	Holvoet <i>et al.</i> , 1991
Toxin fusions		
scFv, dsFv	Pseudomonas exotoxin	Brinkmann <i>et al.</i> , 1991 Reiter <i>et al.</i> , 1996
scFv, Fab, F(ab') ₂	Gelonin	Better <i>et al.</i> , 1995
Fab', scFv	Angiogenin	Rybak <i>et al.</i> , 1992 Newton <i>et al.</i> , 1996
scFv	Eosinophil-derived neurotoxin	Newton <i>et al.</i> , 1994
Cytokines and growth factors		
scFv, F(ab') ₂	IL-2	Fell <i>et al.</i> , 1991 Dorai <i>et al.</i> , 1994
IgG, Fab	IL-2, GM-CSF TNFα, TNFβ	Gillies <i>et al.</i> , 1992 Gillies <i>et al.</i> , 1993
Fab, F(ab') ₂ , IgG	IGF-1, IGF-2	Shin <i>et al.</i> , 1994
Other proteins		
F(ab') ₂	Aequorin	Casadei <i>et al.</i> , 1990
Fab, F(ab') ₂ , IgG	Avidin	Shin <i>et al.</i> , 1997
Fab	Biotin carboxyl carrier protein	Weiss <i>et al.</i> , 1994
Fab, F(ab') ₂ , IgG	Transferrin	Shin <i>et al.</i> , 1995
Fab	Staphylococcal enterotoxin A	Dohlstein <i>et al.</i> , 1994
scFv	Protein A fragment	Tai <i>et al.</i> , 1990
F(ab') ₂	Metallothionein	Das <i>et al.</i> , 1992

The choice of antibody form for fusion purposes depends on the other properties of the molecule required, e.g. overall size, pharmacokinetics and valency. As shown in Table 2.6, fusion proteins with single-chain Fv, disulphide-linked Fv, Fab, F(ab')₂ and IgG have all been constructed. However, the choice of construct can have unexpected effects on the assembly and activity of the fusion protein. A series of fusions of interleukin-2 (IL-2) to the heavy chain of the chimeric anti-ganglioside antibody ch14.18 have been made with addition directly after the Fab such that the entire Fc is replaced by IL-2, after CH₂ such that the CH₃ domain is replaced with IL-2, and fusion to the end of the entire heavy chain (Gillies *et al.*, 1992). The Fab fusion was inactive in antigen binding and the fusion

to CH₂, although capable of binding antigen, did not assemble correctly to the divalent species even though a full-length hinge region was present. The fusion to the end of the entire heavy chain resulted in enhanced antigen binding of the antibody and the molecule was shown to be fully assembled. The IL-2 fused to the antibody remained active although the antibody effector functions were slightly reduced.

In some case spacer peptides have been included between the antibody and fused protein which may assist folding or activity of the fused protein. For example, a linker between an scFv toward the transferrin receptor and the RNase angiogenin was shown to be required for optimal activity of both the scFv and the enzyme (Newton *et al.*, 1996). Also, spacers can be designed to allow proteolytic release of the fused protein when internalised into cells. Other choices in the nature of the construct also need to be made, as the fusion can be made at the C-terminus or N-terminus of the antibody chain and to heavy or light chain. In a study of antibody fragment fusions to the plant toxin gelonin, fusions to Fab, F(ab')₂ and scFv were made at either the N- or C-terminus (Better *et al.*, 1995). Of these immunotoxins, the divalent F(ab')₂ variants were the most potent, as might be expected due to divalent antigen binding and possibly more effective internalisation into target cells. scFv and Fab conjugates with gelonin fused at the N-terminus or C-terminus of the light or heavy chain were all approximately equipotent. Linkers were examined with sites for trypsin-like proteases or with sites for the lysosomal enzymes cathepsin B and D. These are expected to be cleaved intracellularly and may assist gelonin release and hence cytotoxicity. However, in this study there was little difference between the cytotoxicity of the constructs with and without the cleavable linkers, suggesting that the fusion proteins themselves may be active without the need for release of free gelonin.

The types of protein used in fusion proteins includes those used to induce dimerisation (Section 2.4.2), peptide or protein tags used to simplify antibody fragment purification (see Chapter 5), enzymes for both therapeutic and diagnostic purposes, cytokines and growth factors, and many others as shown in Table 2.6. Fusions can also be used to couple some non-proteinaceous materials to antibodies via specific binding proteins. Fusion of the metal-binding protein metallothionein to antibody fragments can be used to allow subsequent binding of the radionuclide ^{99m}Tc (Das *et al.*, 1992). A small peptide which can chelate ^{99m}Tc has also been used effectively (George *et al.*, 1995). Another approach to radiolabelling is to attach a peptide sequence which can be enzymically labelled with ³²P (Neri *et al.*, 1996). Non-proteinaceous materials which are naturally bound by other proteins can be attached by fusion of a suitable 'acceptor' sequence to the antibody fragment followed by expression in *E. coli*. In this way lipid-tagged scFv fragments have been produced by fusion of the amino terminal part of the *E. coli* major lipoprotein, which is sufficient for attachment of lipid during expression (Laukkanen *et al.*, 1993). Biotin can be attached via the biotin-carboxyl carrier protein (BCCP) subunit of *E. coli* acetyl-CoA carboxylase (Weiss *et al.*, 1994). In this case a Fab-BCCP fusion was expressed in *E. coli* and biotin was attached during secretion of the fusion protein.

2.7 Engineering pharmacokinetics and biodistribution

For therapeutic applications of MAbs, the pharmacokinetics and biodistribution of the agent to be used in humans is of great importance. The degree of binding to the target antigen *in vivo* is dependent not only on binding characteristics of the antibody such as affinity and avidity which can be measured *in vitro*, but also on a variety of other factors such as the accessibility of the antigen, the concentration, and the time of exposure of the

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