Modulating the pharmacokinetics of therapeutic antibodies

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Biotechnol Lett (2010) 32:609–622
DOI 10.1007/s10529-010-0214-z

REVIEW

Modulating the pharmacokinetics of therapeutic antibodies

A. Constantinou · C. Chen · M. P. Deonarain

Received: 23 November 2009 / Revised: 6 January 2010 / Accepted: 9 January 2010 / Published online: 4 February 2010

Abstract With the advent of antibody fragments and alternative binding scaffolds, that are devoid of Fc-regions, strategies to increase the half-life of small proteins are becoming increasingly important. Currently, the established method is chemical PEGylation, but more elaborate approaches are being described such as polysialylation, amino acid polymers and albumin-binding derivatives. This article reviews the main strategies for pharmacokinetic enhancement, primarily chemical conjugates and recombinant fusions that increase apparent molecular weight or hydrodynamic radius or interact with serum albumin which itself has a long plasma half-life. We highlight the key chemical linkage methods that preserve antibody function and retain stability and look forward to the next generation of technologies which promise to make better quality pharmaceuticals with lower side effects. Although restricted to antibodies, all of the approaches covered can be applied to other biotherapeutics.

Keywords Conjugate · Fab · Fusion protein · Immunoglobulin · PEGylation · Pharmacokinetics · Polysialylation · scFv

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Introduction

The use of monoclonal antibodies (MAbs) as targeted therapeutic agents is now firmly established with many clinical and commercial successes (Deonarain 2008). Many have become household names and in 2007, monoclonal antibodies became the largest class of commercial biotechnological agents (Aggarwal 2008).

Current MAbs used in the clinic fall largely into two categories: (1) cell-targeting antibodies which modulate receptor function and/or recruit effector functions; and (2) neutralizing agents for targets readily encountered in circulation (and to a lesser extent within tissues). However, the whole immunoglobulin format has limitations for some targeted applications. Their relatively large size makes them inefficient at penetrating deep tissue antigens (Yokota et al. 1993; Graff and Wittrup 2003; Thurber et al. 2008), while poor vascularisation within the tumor microenvironment can impede high dose antibody delivery (Jain 1999; Fukumura and Jain 2008). Further, in such cases where there is poor vascularization or if a patient is immunocompromised the Fc effector function of whole antibodies becomes largely redundant due to a limited supply of lymphocytes and macrophages (Badger et al. 1987). Moreover, the immunosuppressive microenvironment of tumors, as well as the absence of a complimentary Fc isotype, can impede appropriate Fc-directed functions (Strome et al. 2007; Dougan and Dranoff 2009). Another

concern with the Fc domain is potential cross-reactivity with normal tissues, which can also lead to unwanted side effects, particularly when cytotoxin-loaded antibodies are used. In order to address these characteristics, antibody formats, devoid of Fc-regions such as scFvs and Fabs have been engineered (Holliger and Hudson 2005). Being smaller, they are capable of more rapid penetration and clearance than whole mAbs.

When engineering antibodies for therapy, agents must generally exhibit high affinity, discriminating specificity, minimal immunogenicity and low cross-reactivity (Ober et al. 2001). Normally these factors can be addressed by making relatively small changes to the primary protein structure of the antibody (Carter 2006). In addition to this, a major consideration in antibody engineering is developing one that exhibits optimal pharmacokinetics: appropriate dosing leading to optimal bioavailability, uptake, distribution and clearance in targeted and non-targeted tissues which will lead to optimal pharmacodynamics (Beckman et al. 2007).

There are two general approaches to extending the longevity and stability of therapeutic proteins: recombinant/genetic and chemical methods (Fig. 1). Chemical methods pre-date the recombinant approaches and involve the chemical attachment of moieties which alter the physico-chemical properties of the protein. Applied to antibodies, this includes the attachment of polymers, sugars and protein domains. Recombinant methods require the genetic engineering of the target protein or host cell line to produce variants with altered structure or function. The structural changes which lead to altered pharmacokinetics include size, hydrodynamic volume, aggregation status and electric charge (pI) and the functional changes include various receptor binding abilities. This review aims to survey the key technologies for pharmacokinetic modulation and will look forward to some derivatives which promise to become the next generation of antibody therapeutics. In the context of antibodies, pharmacokinetics can be affected by many parameters, including affinity for the target and valency. Modifications to the antigen binding site or multimerisation of antibodies are beyond the scope of this review. These topics are covered in excellent articles (Pluckthun and Pack 1997; Carter 2006, Beckman et al. 2007).

The most investigated method for modulating antibody pharmacokinetics has been by conjugation

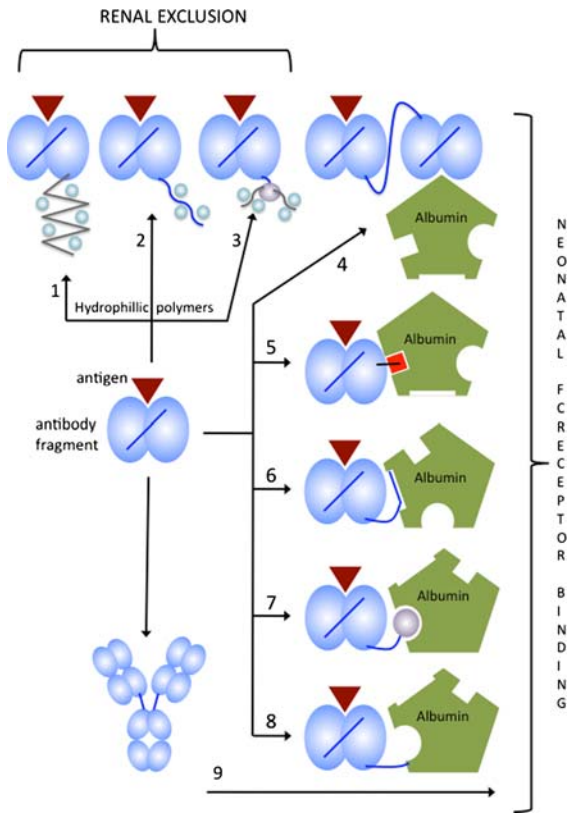


Fig. 1 Schematic diagram illustrating the different pharmacokinetic enhancing strategies. Key: *blue line* connections represent recombinant fusions and *grey line* connections represent chemical linkages. Strategies *1–3* work by increasing hydrodynamic radius (chemical PEG/polysialylation, HAP fusions and PSA carriers) whereas *4–9* utilize binding to the neonatal Fc receptor, indirectly (via albumin, albumin binding chemicals, peptides or proteins) or directly

methods where chemically inert moieties are strategically attached. This has the effect of increasing the apparent size or hydrodynamic volume of a given particle so that its pharmacokinetics are significantly altered in relation to the conjugated size. The larger the conjugate attached the greater the half-life becomes. This is most significant at around the 50–70 kDa threshold which is the molecular weight cut-off for glomerular filtration (Silbernagl 1988; Akhtar and Al Mana 2004). This equates approximately to a diameter of around 90 Å for a globular protein. However, a balance must be achieved such that lower clearance rates are not gained at the expense of significantly poorer tissue penetration and/or poor tissue to blood ratios. This is a particularly important consideration if antibodies are used as carriers of

cytotoxic agents, such as radioisotopes (Davies 2007), where high doses leading to extended systemic residency could cause damage to normal tissues. However, for unconjugated antibodies (e.g. trastuzumab and rituximab) used to treat metastatic breast cancer and non Hodgkin's lymphoma respectively (Hudis 2007; Bonavida 2007), long half lives are less significant and possibly favoured as it allows an extended window of therapy with no significant increase in cross reactivity. Certainly, for antibodies neutralising systemic factors such as vascular endothelial cell growth factor, VEGF (Panares and Garcia 2007) or tissue necrosis factor, TNFα (Tracey et al. 2008), a long half life is desirable.

Clearly there is no "one size fits all" form of conjugation when manipulating pharmacokinetics. A pharmacokinetic profile needs to be achieved that extends the longevity of the antibody, allow a more level range of bioavailability, enhances potency, and directs its particular mechanism of action specifically at the target site.

Chemical conjugation

Random (amine) versus site-specific (thiol) coupling

Chemical conjugation takes advantage of various reactive groups within the primary antibody sequence. Traditionally, lysine residues represent the favoured points of conjugation as they tend to have a high surface propensity. However, the presence of any given functional group does not automatically predispose it as a conjugation point. Reactive groups may be buried within the core tertiary structure of the antibody or sterically hindered and therefore unavailable for conjugation. Amino acids, essential for the antibody's function represent undesirable sites for conjugation. While some success has been observed through non-site specific conjugation it has now become the standard to engineer proteins that eliminate residues where conjugation is undesired and re-engineer specific sites where conjugation is desired, particularly thiol-containing cysteines (Kaushik and Moots 2005; Junutula et al. 2008). This has become increasingly favoured for conjugation purposes as, unlike the ubiquitous nature of amine groups, surface-available thiols tend to be present on proteins less frequently. In antibody Fab fragments, a free thiol is often found at the *C*-terminus of the light chain (a remnant from the whole immunoglobulin structure) or in other recombinant fragments, this can easily be introduced. Such engineering has three effects; it eliminates undesirable conjugates which would be detrimental to protein activity, secondly a more homogeneous (form of?) conjugates with specific conjugate isoforms are formed leading to more effective pharmaceutical characterisation, and thirdly, the number of reactive sites can be engineered to generate various conjugate to protein ratios with potentially different pharmacokinetics. Amine-directed chemical polysialylation (see below) was successfully used to modify an anti-tumour Fab (Constantinou et al. 2008), but the same process damaged an anti-tumour scFv which was resolved by site-specific modification (Constantinou et al. 2009). Similarly, conjugation of larger polymer chains using amine-reactive conjugation of an anti-TAG-72 scFv was also found to be more detrimental on antibody activity than conjugation to carboxylic acid moieties using PEG-hydrazine chemistry (Lee et al. 1999). Unfortunately, in the absence of structural information it is difficult to predict which residues may be important for bioactivity. While some amino acid modifications can be tolerated, others, particularly within the complementarity-determining region (CDR), may be detrimental. If known, sensitive conjugation sites can be eliminated by mutagenesis. A non-essential lysine residue in the VH CDR3 of the C6.5 scFv was mutated to a non-conjugatable alanine residue to prevent unwanted chemical modification of HER2 binding site (Adams et al. 2000).

Conjugates with modulating proteins and peptides

Chemically conjugating antibody fragments to other proteins which have a long serum half-life would, on the face of it, seem like an appropriate strategy. However, there is very little of this with most approaches focussing on recombinant fusions (see below). Due to its 19 day serum half-life (similar to immunoglobulin's 21 days, both interacting with the neonatal Fc-receptor) albumin is a common pharmacokinetic modulating protein partner. Albumin is a 67 kDa monomeric molecule and is the most abundant protein in blood serum (around 45 mg/ml in humans). Its low pI means that it has a net negative charge in the blood which hampers kidney filtration due to the

anionic kidney basal membrane. The advantages of using albumin in this way outweigh the cross-reaction and side effects from Fc-mediated interactions. The most notable example of this is the work of Smith et al. (2001) who evaluated three different albumin-binding strategies. Chemical conjugation of rat serum albumin (RSA) with an anti-TNFα Fab retained immunoreactivity but increased its bioavailability 17–200-fold. The same group also looked at a bispecific Fab which bound to endogenous RSA which produced an 8-fold increase in bioavailability.

Conjugates with modulating small molecules

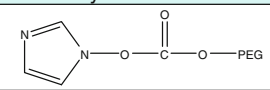
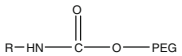
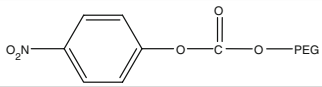
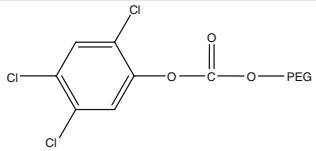
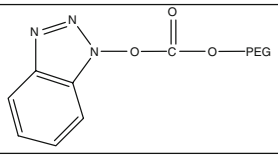
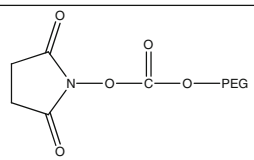
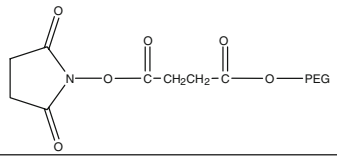
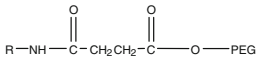
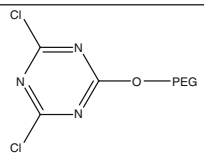
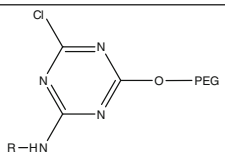
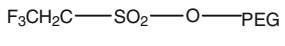
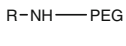
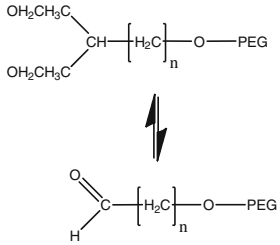
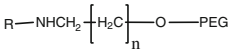
A 'portable', small albumin-binding molecule called 'Albu tag', which interacts with serum albumin and extends the half life of imaging agents, (Dumelin et al. 2008) and antibodies (Trussel et al. 2009) has been isolated from a DNA-encoded chemical library. This molecule, a 4-(*p*-iodophenyl)butyric acid derivative, was isolated from a DNA-encoded combinatorial chemical library. The portability of the 'Albu tag' overcomes the problems seen with other attempts with small molecules such as loss of albumin binding upon chemical conjugation. The nanomolar (K_d) affinity of the tag is retained upon attachment.

Conjugates with hydrophilic polymers

A wide range of polymers and attachment chemistries have been studied for their pharmacokinetic modulatory properties (Figs. 1, 2). Polymers are used because the number, length and structural complexity of the polymer can be varied so as to produce conjugates with differing properties. Polydextrans (PD), polyvinylalcohols (PVA), poly(styrene-co-maleic acid), polysialic acid (PSA), polyglycerols, polyals and polyethelene glycol (PEG) are some of the polymers that have been investigated for pharmacokinetic conjugation purposes. Their high hydrophilicity is due to their ability to interact with water molecules in solution. As such these hydrated polymers assume a much greater apparent molecular weight than that which can be applied to the polymer alone. This can be exploited pharmacologically when conjugated to therapeutic molecules to mask immunogenicity of native proteins, provide improved solubility and modulate pharmacokinetics. As the most advanced carrier system technology to date, the physicochemical properties of PEG have been the most extensively studied. Each of its ethylene oxide subunits can support up to three molecules of water (Sasahara 1995) resulting in an increased hydrodynamic volume that provides an effective molecular weight 5–10-fold greater than that of proteins or other macromolecules of a similar size (Harris and Chess 2003; Fee and Van Alstine 2004). Furthermore, the length of polymer and its conformation as linear or branched dictates how the polymer behaves pharmacokinetically (Yamaoka et al. 1994, 1995; Fee 2007; Gursahani et al. 2009). Generally, while clearance rates decrease as polymer-conjugate size increase, their permeability into potential target sites also slows (Yang et al. 2003; Cohen et al. 2001; Gursahani et al. 2009). Additionally, the charge associated with polymers and their effect on altering the charge on surface residues where conjugation takes place, can affect the overall isoelectric point of subsequent conjugates, which additionally affects its pharmacokinetic properties. As such, the pharmacokinetics of any given conjugate may be modulated according to a desired pharmacokinetic effect by altering PEG length and/or conjugation ratios, as well as charge.

A systematic study by Yang et al. (2003) used 5, 20 and 40 kDa maleimide–PEG conjugates to investigate the bioactivity and blood clearance rates of an anti-TNFα scFv site specifically conjugated at one or both of two available thiol sites. Although BIAcore binding data suggested some loss of bioactivity, cytotoxicity assays showed similar neutralization IC_{50} values for all conjugate variants. Pharmacokinetically, the half-life of conjugates increased as their PEGylation content increased, demonstrating up to 100-fold prolongation of circulating half-lives (Yang et al. 2003). When comparing various conjugation ratios using 2, 5, 10, 12 and 20 kDa polymers linked to either primary amines or carboxylic acids, Lee et al. found that an increase in polymer length was found to be more effective for extending serum half-life than using a corresponding increase in total PEG by using smaller polymer lengths at a higher conjugation ratio (Lee et al. 1999). These studies agree

Fig. 2 Conjugation chemistries used for PEGylation and other modification strategies. A variety of chemistries have been used to covalently modify antibodies. Illustrated here are the activated groups and the resulting linkages formed. Although primarily used for protein PEGylation, other agents can be attached ▶

First Generation Agent	Reactive Polymer Structure	Reactivity & Characteristics
PEG Carbonyl-Imidazole (Beauchamp et al., 1983; Veronese et al., 1985)		 ***Carbamate Bond*** ▪ N-terminal α-amines and lysine ε-amine group acylation leading to formation of carbamate linkages. ▪ Reactivity to other residues such as histidines and tyrosines may also be observed depending on the reaction conditions and the pKa effects of neighbouring residues. ▪ PEG Benzotriazole carbonate and PEG Succinimidyl carbonate are noted for forming imidazolecarbonate linkages with histidine residues. ▪ Polymer backbone ester of PEG succinimidyl carbonate can be readily hydrolyzed leading to possible immunogenicity towards the remaining tagged conjugate if used in vivo.
PEG p-nitrophenyl carbonate (Veronese et al., 1985)		
PEG Trichlorophenyl carbonate (Veronese et al., 1985)		
PEG Benzotriazole carbonate (Dolence et al., 1997)		
PEG Succinimidyl carbonate (Zalipsky et al., 1992; Miron & Wilchek, 1993)		
PEG Succnimidyl succinate (Abuchowski et al., 1984)		 ***Carbamate Bond***
PEG Dichlorotriazine (Zalipsky & Lee, 1992)		 ***Amine Bond*** ▪ Reacts with lysine, seine, tyrosine, cysteine and histidine residues. ▪ Remaining Chloride may allow cross-linking between conjugates.
PEG Tresylate (Francis et al., 1998)		 ***Amine Bond*** ▪ May form secondary conjugates with degradable sulfamate linkage.
Second Generation Agents	**Reactive Polymer Structure**	**Reactivity & Characteristics**
PEG Aldehyde: 1. Acetalaldehyde 2. Propionaldehyde (Harris & Herati, 1993) PEG Aldehyde hydrate: 1. Acetalaldehyde hydrate 2. Propionaldehyde hydrate (Bently & Harris, 1999; Kinstler et al., 1996)		 ***Amine Bond*** ▪ Reactive amination, selective for N-terminal α-amines and ε-amine groups found on lysine residues via a Shiff base. ▪ Hydrate form of the PEG aldehyde is more stable and therefore the preferred agent to use.

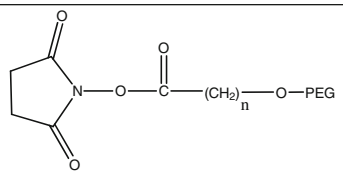
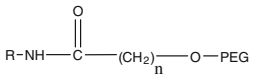
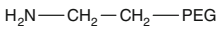
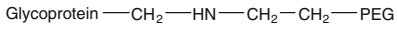
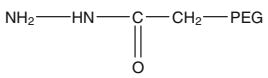
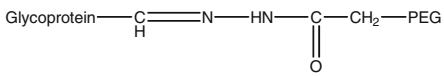
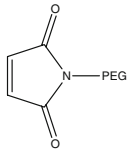
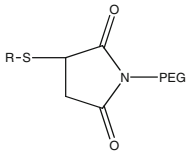
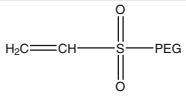
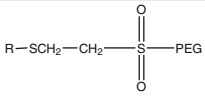
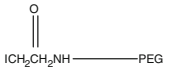
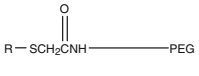
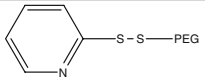
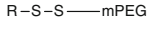
PEG NHS Esters (Zalipsky & Barany, 1990)		 ***Amide Bond*** ▪ Active esters of carboxylic acids are the most favoured acylating agents as they allow formation of very stable bonds at near physiological conditions.
Carbohydrate Specific	Reactive Polymer Structure	Reactivity & Characteristics
PEG Amine		 ▪ Conjugation to oxidised residues leads to the formation amine bonds, however reactivity with other protein amine groups can lead to the formation of cross-linked aggregates.
PEG Hydrazine (Gaertner & Offord 1996, Youn et al., 2005)		 ▪ Oxidation of carbohydrate residues, or N-terminal serine or threonine residues allows conjugation by this chemistry. The resulting hydrazone bond (shown above) can be stabilized by reduction to form a more stable amine bond.
Thiol Specific	Reactive Polymer Structure	Reactivity & Characteristics
PEG Maleimide (Goodson & Katre, 1990; Constantinou et al., 2009)		 ***Thioether*** ▪ Maleimide can react under physiological conditions but is not stable under aqueous conditions.
PEG Vinyl sulfone (Morpurgo et al., 1996)		 ***Thioether*** ▪ May also react with lysine residues
PEG Iodoacetamide (Kogan 1992)		 ***Thioether*** ▪ Reaction must be perfomed under dark conditions to limit the generation of free iodine that may react with other residues
PEG o-pyridyl didulfide (Woghiren et al., 1993)		 ***Thioether*** ▪ Forms stable disulfide bonds except under reducing conditions.

Fig. 2 conitnued

well with non-antibody studies such as those of Clark et al. (1996).

The beneficial role of using branched PEG chains over linear ones has also been investigated, with investigators agreeing that branch chain conjugates may offer greater circulatory half-lives than linear chain counterparts (Harris et al. 1999). To this end, the first FDA-approved anti-TNFα PEGylated Fab

antibody certolizumab pegol (Cimzia) is marketed as a branched PEG conjugate. Recently, it has been shown that there was no significant difference between the viscosity radii of branched and linear PEG–proteins having the same total molecular weight of PEG adducts, suggesting that any differences observed in circulatory half-lives cannot be explained by differences in hydrodynamic volume or glomerular filtration (Fee 2007). However, although both linear and branched polymers confer greater stability and protection to a protein, it has been shown than when exposed to proteolytic cleavage, branched PEG conjugates demonstrated an even greater resilience to cleavage than their linear polymer conjugate counterparts. This observation could, in part, explain why branched conjugates have a seemingly longer half-life in vivo compared to those with linear PEG chains (Monfardini et al. 1995). Since the earliest demonstration in 1977 (Abuchowski et al. 1977), therapeutic protein PEGylation has expanded into a major biotechnology industry (Fraser-Moodie 2008). Antibodies are now emerging from this pipeline, for example, AMG-885 (Amgen/UCB) an anti-sclerostin PEGylated Fab for bone loss treatment and CDP-791 (Imclone/UCB) an anti-VEGFR2 PEGylated di-Fab for solid tumours.

PEGylation chemistries

Whilst the desire to modulate antibody pharmacokinetics is clear, the type of conjugation and the ability to produce them in a homogeneous and reproducible manner is still being refined. There are many methods of activating polymers for conjugation via certain functional groups within proteins (Fig. 2). These methods have evolved over time to minimise problems encountered with their predecessors and are thoroughly covered by Roberts et al. (2002). So-called 'first generation' PEGylation chemistry, which preferentially targets *N*-terminal amines and the epsilon-amine group of lysines, react to give carbamate or amine linkages between the protein and the PEG chain. These chemistries are often limited to low molecular weight moieties and lead to unstable linkages, PEG impurities, and a lack of selectivity in modification; as such they have been used with limited success. The subsequent use of unifunctional methoxylated PEG (mPEG) has since been used to minimize the effect of cross linking between conjugates. 'Second generation' chemistries that also exploit amine conjugation have minimized considerably the difficulties described above. These include the use of PEG–aldehydes/hydrate (Fig. 2). Although not completely selective, conjugation is observed preferentially at primary amines via formation of a Schiff's base, which is subsequently reduced to give a stable secondary amine linkage (Kinstler et al. 1996). PEG–carboxylic acids have been extensively used being more stable and are favoured due to their reactivity at near physiological conditions (Zalipsky and Barany 1990).

Increasingly, conjugation through surface thiol groups have become more desirable as they offer greater conjugation selectivity than that of lysine residues (Fig. 2). Where no free thiols are available, one or more free cysteine residues can be recombinantly engineered to facilitate site-specific conjugation (Goodson and Katre 1990; Natarajan et al. 2005; Constantinou et al. 2009). PEG derivatives such as PEG–maleimide, PEG–vinylsulphone and PEG–iodoacetamide have all been used in conjugations to form stable thioether linkages (Goodson and Katre 1990; Morpurgo et al. 1996). Stable disulfide linkages have similarly been achieved using *o*-pyridyl disulfide–PEG, however such linkages are readily broken in reducing environments (Woghiren et al. 1993). Cimzia, for the treatment of Crohn's disease, recently became the first protein antibody conjugated using thiol-specific, PEG chemistry to be approved for use by the FDA (Singh et al. 2003).

The use of slow-release or hydrolysable PEGylation has also been investigated. Here, the bioactivity, previously lost upon conjugation, is restored over time (Greenwald 2001; Lee et al. 2001; Zalipsky et al. 2007; Filpula and Zhao 2008). However, this technology has not been applied to antibodies as generally, antibody immuno-reactivity is preserved upon polymer conjugation.

Of course, the same types of chemistries described above are not unique to PEG conjugates, and similar advantages and disadvantages can be cited for any other polymer activated with the same chemistry. For example polysialic acid (PSA) conjugation has been investigated using amine and thiol chemistries with similar observations made. PSA is a naturally-occurring biopolymer (α-2,8, 2,9 linked sialic acid/N-acetyl neuraminic acid) found as colominic acid (in bacteria) or PSA (in mammalian cells). PSA was proposed by Gregoriadis et al. (1993) to have biophysical

properties similar to that of PEG such that its hydrophilicity could be used to modulate the half-life of proteins. Initially demonstrated in 1993 with fluoroscein and in 1996 with an enzyme (Fernandes and Gregoriadis 1996; Gregoriadis et al. 2005), this approach was extended to antibody fragments. In the first instance, amine-based reductive amination using a PSA–aldehyde conjugation to H17E2 Fab, an antibody against the oncofoetal tumour antigen, placental alkaline phosphatase, was shown to produce conjugates that retain activity. Several coupling ratios were investigated, and all demonstrated increased blood residency in vivo compared to the parental Fab and approaching that of the whole immunoglobulin. Interestingly, the longest or highest PSA substitution ratio was not the most effective (Constantinou et al. 2008). However, the same reductive amination chemistry was applied to MFE-23, a single-chain Fv fragment directed against another oncofoetal antigen, carcinoembryonic antigen. This resulted in immunoconjugates of reduced blood clearance and high bioavailability, but poor immuno-reactivity. This was resolved using the site-specific method with a thiol-directed maleimide activated PSA (Constantinou et al. 2009).

Recombinant approaches

Antibody fragment engineering

The proliferation of antibody fragments (Holliger and Hudson 2005), and alternative binding frameworks (Ewert et al. 2004) have led to many recombinant approaches to pharmacokinetic engineering. The selection and characterisation of scFvs, diabodies, nanobodies, DARPins and anticalins is normally followed by re-engineering into bigger molecules such as immunoglobulins, SIPs (Borsi et al. 2002) or artificially multimerised proteins (Pluckthun and Pack 1997). These larger proteins have a longer blood half-life as of course, immunoglobulins have a natural retention and clearance mechanism through Fc-domain:neonatal Fc-receptor (FcRn, salvage receptor) binding via the reticulo-endothelial system (Ghetie et al. 1997; Anderson et al. 2006). There are many strategies for increasing or decreasing the affinity between immunoglobulin Fc and FcRn to increase or decrease blood serum half-life. These are beyond the scope of this review but well described in numerous recent publications.

Albumin fusion strategies

Antibody–albumin fusions have been studied as a way to both slow down and increase blood clearance (Fig. 1). ScFv–HSA fusions using a variety of linker peptides increased the blood residence times approximately 12-fold (Smith et al. 2001). These fusions expressed well in *Pichia pastoris* pointing to a strategy for low-cost expression. A radiolabeled anti-CEA scFv–HSA fusion protein ('immunobulin', expressed in mammalian NS0 cells) was designed by Yazaki et al. for tumor biodistribution and imaging studies. This scFv–HSA fusion showed a dramatic increase in tumor uptake, persistent high tumour:-blood ratios, and limited normal tissue uptake in comparison with the scFv alone (Yazaki et al. 2008). After 72 h, 27% injected dose/gram was reached with a tumour:blood ratio of almost 19:1. This is significantly better than that seen with whole immunoglobulins. A series of anti CD3 (T-cell)/anti-CEA (tumour cell) retargeting bispeciic antibodies were also fused to HSA in order to prolong their half-life for immunotherapy applications (Muller et al. 2007). These rather complex molecules were successfully expressed in HEK293 cells. Mammalian cells have been shown by the Kontermann lab and others including ourselves (unpublished) to express bispecific scFvs while yields from *E. coli* were very poor (Wright and Deonarain 2007). These constructs, bispecific scFv-, single-chain diabody- and tandem scFv–HSA fusion proteins ($scFv_2$–HSA, scDb–HSA, taFv–HSA) were all stable and had increased in vivo bioavailability, as shown by a 6–8-fold increase in the blood exposure time. In vivo therapeutic benefit of such increased residence time is yet to be shown. Conversely, 'HSAbodies' are highly glycosylated scFv–albumin fusion proteins which have accelerated blood clearance making them appropriate for 2-step drug delivery systems (Huhalov and Chester 2004).

Albumin-binding strategies

Rather than utilising the long residence time of albumin directly, there have been many recombinant

approaches that "piggy-back" on albumin, in a similar way to the albu-tag described above.

Using peptide phage display, Dennis et al. (2002) identified a specific core sequence (DICLPRWGCLW) which bound to albumin with high affinity (K_d 40 nM). They generated a peptide called SA21 that non-covalently bound to albumin with 1:1 stoichiometry at a site distinct from known small molecule binding sites (Fig. 1). Recombinantly fusing the peptide to an anti-tissue factor Fab (D3H44) significantly reduced the Fab in vivo clearance, and achieved 25–43% of the albumin half-life in mice and rabbits (Dennis et al. 2002). For tumour targeting, they incorporated the albumin-binding peptide to the Herceptin-derived Fab (Fab4D5), and showed a significant improvement in tumour deposition and retention, high tumor to blood ratios compared with Fab alone. This Genentech technology is known as AB.Fab, which has the advantage of cheap material expressed in *E. coli*. The authors also suggested that association with albumin leads to an altered route of clearance and metabolism (Dennis et al. 2007). A possible refinement of this technology is the ability to modulate the pharmacokinetics of AB.Fabs by altering the affinity of the peptide, which was demonstrated using a range of peptides with K_d values from of 4 nM to 2.5 mM (Nguyen et al. 2006).

Recently, another group applied the same albumin binding strategy to a bivalent anti-epidermal growth factor receptor (EGFR) nanobody. They also illustrated improved tumor uptake (as high as cetuximab), and reduced blood clearance rate (Tijink et al. 2008). An alternative albumin-binding approach is to fuse a homologous albumin-binding domain (ABD3-46 amino acids/6 kDa) from streptococcal protein G with a recombinant antibody. ABD3, has a broad albumin species specificity, and interacts with HSA with K_d of approx. 4 nM (Johansson et al. 2002; Linhult et al. 2002). Stork et al. (2007) applied this strategy to a bispecific single-chain diabody (scDb anti-CEA/anti-CD3) developed for retargeting of cytotoxic T cells to carcinoembryonic antigen (CEA) expressing tumor cells. They successfully showed all three parts of the chimeric protein were functional with 5- to 6-fold increase of prolonged circulation time. The drawback seemed to be a decreased immuno-stimulatory activity compared to the diabody alone (scDb). In a similar approach, a radiaolabeled anti-HER2 dimeric Affibody molecule (14 kDa) was also tested with ABD fusion for improved therapeutic efficacy. Good cellular retention, and reduced renal uptake in comparision with the non-fused dimer molecule were observed (Tolmachev et al. 2007).

Other fusion strategies

As described above, the use of inert, hydrophilic polymers represents the major strategy for pharmacokinetic engineering. Certain amino acid polymers bear this property and advantageously can also be attached to protein by genetic fusion. Schlapschy et al. (2007) investigated a glycine-rich homo-amino-acid polymer (HAP) that had an increased hydrodynamic radius. They used anti-HER2 Fab 4D5 as a model system and fused 100 and 200 residues of a repetitive sequence (Gly_4Ser) to its light chain. They showed that the 200 residue 'HAPylated' Fab acquired a hydrodynamic volume more than double that of the Fab alone, moderate rise in half-life, but lower than the enhancement made by the ABD fusion (see above). Compared with more hydrophilic polymers such a PEG or PSA, the coiled structure of HAPs may hinder its development. However, this moderate effect could be beneficial for specialized applications, such as in vivo imaging. Other sequences and more extended and hydrophilic polymer chains are currently under investigation. Poly-(Pro-Ala-Ser) fusions have been developed as alternatives (PASylation) which acquire a more hydrophilic characteristic and much enhanced hydrodynamic radius, leading to better pharmacokinetic enhancement (A. Skerra, unpublished work).

Recombinant glycosylation domains

The HAP approach opens up the idea of using other recombinant approaches to obtain hydrophilic biopolymers which could have pharmacokinetic benefits. Presently, long chain carbohydrates seem the only option and have the advantage of being attached to proteins in a site-specific manner. Neural cell adhesion molecule (NCAM/CD56), can be heavily polysialylated by up to 200 sialic acid residues with a unique α2-8 linkage in mammals (Georgopoulou and Breen 1999). We have been exploiting this to develop recombinantly polysialylatable antibodies (Deonarain et al. 2008; Chen et al., unpublished work) which could

have major improvements over chemical modification techniques.

Discussion and conclusions

The benefits of improving the longevity of therapeutic proteins are wide-ranging, from clinical benefits in terms of better dosing regimens and improved side effect profiles to commercial by extending product lifecycles and increasing their patent protection period. The emergence of fragments and alternative binding frameworks will see an increased requirement for half-life improving technologies. There are many approaches available and their features are compared in Table 1.

Table 1 Advantages and disadvantages of various pharmacokinetic enhancing strategies

Approach name	Modulation type	Advantage	Concerns
PEGylation	Chemical	Hydrophilic water attraction	Non-biodegradable
		Improved protein solubility and stability	Toxic accumulation
		Industry-established method for extending half-life	Immunogenic
			Protein inactivation
			Downstream processing
			Costs and yields
			Homogeneity control
Poly-sialylation	Chemical	Hydrophilic water attraction Improved protein solubility and stability	Protein inactivation
			Downstream processing
		Biodegradable	Costs and yields
		Potentially non-immunogenic	Homogeneity control
		Promising clinical data	
	Recombinant	Hydrophilic water attraction	Homogeneity control
		Improved protein solubility and stability	Less advanced technology
		Biodegradable	
		Potentially non-immunogenic	
		Easier production	
		Less downstream processing	
Albumin conjugation or binding	Chemical	Potentially non-immunogenic	Protein inactivation
		Biodegradable	Downstream processing
		Impressive pre-clinical data	Costs and yields
			Homogeneity control
			Less advanced technology
	Recombinant	Hydrophilic water attraction	Less advanced technology
		Improved protein solubility and stability	
		Biodegradable	
		Potentially non-immunogenic	
		Easier production	
		Less downstream processing	
HAPylation	Recombinant	Hydrophilic water attraction	Low hydrophilicity
		Improved protein solubility and stability	Long protein polymers
		Biodegradable	
		Potentially non-immunogenic	
		Easier production	
		Less downstream processing	

PEGylation is clearly the current leader but it also lends itself as a stabilising agent and provides a stealth immune evasion technique. In a study of Crohn's disease patients who had become intolerant, or no longer responsive to the market-standard infliximab (anti-TNF alpha humanised immunoglobulin), clinical data presented by UCB (Union Chimique Belge) showed certolizumab pegol was able to provide an effective and rapid clinical response in more than 60% of the group.

PEGylation technologies dominate the pharmaceutical product pipeline, with many agents due to come to market. However, the technology has matured, and in the mid to long term, a decline in PEG use is expected with the emergence of improved biodegradable, stealth polymer technologies. The primary driver for this is the concern that PEG is not biodegradable, and with use for chronic conditions PEGylated peptides or by-products could accumulate in tissues and cause unforeseen toxic effects and/or generate an immune response (Armstrong et al. 2007). Kidney vacuolization has been observed as a toxic side effect due to the inability of renal cells to degrade the PEG polymer (Bendele et al. 1998). This has already lead to the establishment of pre-emptive next generation polymers aiming to supercede PEG and is indicative of the fast pace at which the technology concept is evolving.

Lipoxen's PolyXen uses polysialic acid (PSA), a biodegradable and biocompatible human polymer, and with four non-antibody drug candidates presently in early clinical development it is currently tipped to overtake PEG in leading the stealth platform (Fraser-Moodie 2008). This is supported by Baxter's integration of Lipoxen's PolyXen technology for possible use with its propriety proteins by entering into an exclusive worldwide development and license agreement to develop improved, longer-acting forms of blood-clotting factors (Fraser-Moodie 2008).

Whilst the choice of polymer used for conjugation is paramount in producing conjugates with minimal side effects for subsequent clinical use other parameters must be taken into account and will continue to vary according to the drug being used and its target. As well as the size of the polymer, branch complexity, its charge effect, and the site(s) of conjugation will all influence how bioactive the drug remains, how well it reaches its target and how stable it remains. However, from a pharmaceutical approach, the ease in which conjugates can be produced in a large, efficient and consistent manner is also important. To this end further development in recombinant conjugation methods may provide an appealing long term objective for producing viable antibody fragment conjugates for therapy.

Acknowledgements The authors acknowledge funding from Imperial College, Cancer Research UK (Ref: C18960) and European Union FP6 (Immuno-PDT Ref: LSHC-CT-2006-037489) which fund MPD, AC and CC respectively. We thank David Leak for critically reading the manuscript.

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