Expert Opinion

Modulating antibody pharmacokinetics using hydrophilic polymers

Chen Chen, Antony Constantinou & Mahendra Deonarain†
Imperial College London, Department of Life Sciences, Faculty of Natural Sciences, Exhibition Road, London, UK

Introduction: The use of hydrophilic polymers as a substitute for the Fc-domain in immuno- or non-immuno-based binding proteins is accelerating. Chemical PEGylation has led the way and is still the most advanced and clinically-approved approach. Hydrophilic polymers act by maintaining a flexible conformation and hydrogen bonding to a network of water molecules to acquire a larger hydrodynamic volume and apparent mass than their actual molecular mass suggest. The benefits are increased blood half-life and bioavailability, stability and reduced immunogenicity. In the case of PEG, there is also evidence of enhanced targeting and reduced side effects, but drawbacks include the fact that PEG is non-biodegradable.
Areas covered: This report reviews the state of the art for antibody PEGylation in terms of approaches and effects. Additionally, non-biological (such as *N*-(2-hydroxypropyl)methacrylamide) and potentially superior biological alternatives (such as polysialylation) are described, ending with recombinant approaches (such as hydrophilic peptides and glyco-engineering), which promise to circumvent the need for chemical modification altogether.
Expert opinion: The emergence of many small, antibody fragment-like mimics will drive the need for such technologies, and PEGylation is still the choice polymer due to its established use and track record. However, there will be a place for many alternative technologies if they can match the pharmacokinetics of PEG-conjugates and bring addition beneficial features such as easier production.

Keywords: antibody, fragment, hydrodynamic, pharmacokinetics, polyethylene glycol, polysialic acid

Expert Opin. Drug Deliv. (2011) 8(9):1221-1236

1. Introduction

For many years, the 'next generation' of antibody technology has been proposed to be recombinant antibody fragments (including their fusions and conjugates) [1]. We are now seeing the reality of this with some of these newer antibody formats reaching advanced clinical trials or marketing approval. Not far behind are alternative binding scaffolds: non-antibody-like binding proteins which have a number of advantages over immunoglobulins [2]. Whole mAbs of which there are now around 30 approved (http://csdd.tufts.edu; http://fda.org) have brought clinical and commercial success [3,4]. However, for reasons such as lack of potency, functional resistance, side effects, poor tissue penetration and sub-optimal pharmacokinetics, they have been less effective, especially for solid tumours [4]. The last three reasons are related to the presence of an Fc region/domain. Fc interaction with the neonatal Fc receptor (FcRn) on cells of the reticulo-endothelial system accounts for the immunoglobulin long half-life (3 – 4 weeks in humans) but also its crossreaction to non-target tissues and slow tissue penetration [4]. This has led to the development

informa
healthcare

10.1517/17425247.2011.602399 © 2011 Informa UK, Ltd. ISSN 1742-5247

Article highlights.

- The driver for half-life/pharmacokinetic modulation is the requirement for non-Fc-bearing immuno-proteins with retained bioavailability and the proliferation of alternative scaffold-binding proteins.
- The approaches can be random/site-specific chemical modification or protein engineered recombinant technologies, all of which have their merits and limitations. However, site-specific conjugations or recombinant fusions are preferred due to reduced polydispersity and better product definition.
- Hydrophilic polymers act primarily by increasing the hydrodynamic volume of the target protein and excluding renal clearance, but other features, such as charge, also contribute to the mechanism of action.
- PEGylation is the accepted and leading method due to its track record and accessibility but the other chemical approaches need more clinical validation to support their expansion.
- From the next-generation approaches, polysialylation and hydrophilic peptide extensions could emerge as commercially viable technology.

This box summarises key points contained in the article.

of non-Fc bearing antibody (or antibody-like) derivatives. Such small proteins of biotechnological interest are rapidly eliminated from plasma circulation by renal filtration. The kidney glomeruli act as a molecular sieve, preventing proteins larger than around 60 – 70 kDa (around 40 Å) to pass through to be degraded and excreted [5]. A direct modulation approach is to increase their apparent molecular size to above the glomerular filtration cutoff limit. To achieve this, multimerisation of recombinant antibody fragments increases the molecular mass (MM) leading to overall size enhancement, which also offers extra options such as increased valency (functional affinity or avidity) and specificity [6]. However, multimerisation opens up issues such as complex formation or receptor crosslinking leading to agonist functions. A more preferable and common way is to attach biocompatible hydrophilic polymers to increase the hydrodynamic radius under physiological conditions. Generally, these materials are flexible chains with low MM (10 – 40 kDa) and high water attraction capacity. Such 'hydrated' polymers then appear to have a much greater apparent MM and hydrodynamic volume (due to a greater Stokes radius), and their attachment to the small molecules leads to an overall hydrodynamic size enlargement. Water soluble polymers such as polydextrans, polystyrene-co-maleic acid, polylactides, poly (lactide-co-glycolides), polysialic acid (PSA), poly(L-glutamic acid), *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer and PEG have been studied previously as effective drug carriers/modulators [7-9]. Many of these have been utilised in biotherapeutics with additional benefits including masking immunogenicity (humoural and cellular), protease protection, solubility improvement, controlled release, reduced absorption, reduced injection volume and conjugational linking (**Figure 1**) [7,8]. From a commercial viewpoint, such modifications can also extend patent life and help compete against biogenerics/biosimilars, thus increasing revenue. They can even lead to improved generics (known as 'biobetters'). Covalent attachment of PEG, PEGylation is one of the best validated and practically acceptable chemical conjugation methods to date. Although many more non-antibody examples exist (e.g., PEGylated IFN-2β, PEGylated erythropoietin and PEGylated insulin), the first approved protein PEGylated product was in the 1970s [10]. Antibody fragments are a significant group (the first example approved in 2008 [10]) with a great deal of research that can be compared. Polymer modification of antibody fragments tend to have a greater pharmacokinetic effect than pharmacodynamic effect as antibodies tend to be relatively more stable in serum than other proteins. Although the above-mentioned chemical approaches make up the majority of the technologies, recombinant approaches are also making progress. Chemical manipulation and conjugation of proteins can suffer from a number of limitations including protein inactivation, loss of stability, heterologous products, loss of affinity or specificity, low yields and costly downstream processing to remove non-reacted materials. This has led to the development of competing 'recombinant PEG/PEG-like' technologies where hydrophilic polymers are genetically encoded in some way, leading to engineered cell lines expressing the target protein already bearing the pharmaco-modulatory biopolymer. Such 'biopolymers' are naturally-occurring and thus are biodegradable overcoming potential toxicity, immunogenicity and side effect issues [11-13]. This includes recombinantly-encoded PSA, homo- and hetero-amino-acid polymers.

This review summarises the state of the art in hydrophilic polymer technology for antibody fragment pharmacokinetic modulation, evaluating both chemical and recombinant approaches and looks ahead to how these will be used with the many antibody/antibody-like proteins on the horizon.

2. Chemical conjugation

2.1 Conjugation residues

Chemical conjugation occurs through reaction with various chemical groups within the primary antibody sequence. Typical reactive groups are amines, thiols, imidazole rings, carboxylic acids and hydroxyls. Amine groups on lysine or N-terminal residues are often used as 'random' conjugation points as they have a high surface propensity. Reactive groups may be sterically hindered, as they are often embedded within the core tertiary structure of the antibody and, therefore, unavailable for conjugation. Moreover, amino acids essential for the antibody's function represent undesirable sites for conjugations. Although non-site-specific, random conjugation has shown some success [14,15]; the more prevalent and preferred approach is to engineer proteins that eliminate residues where conjugation is undesired

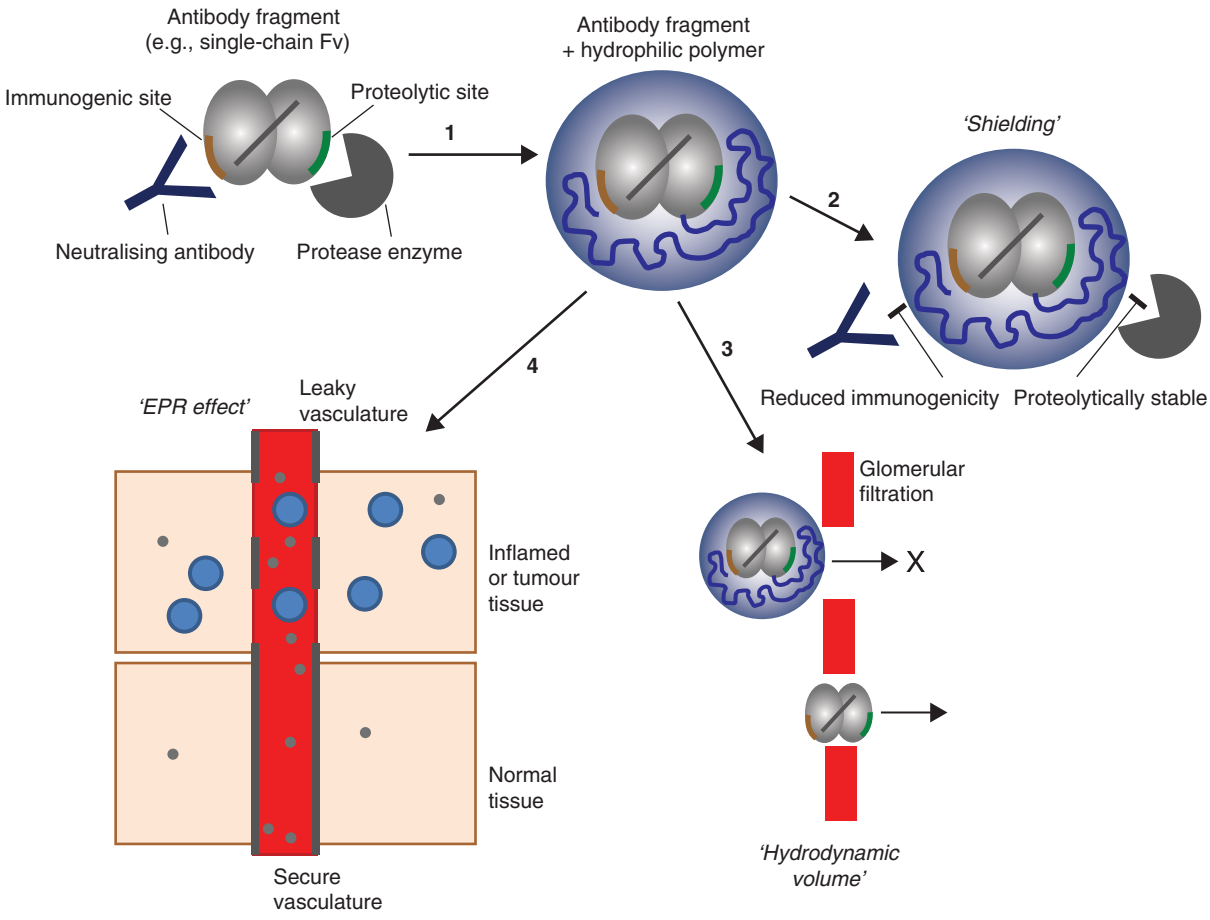

Figure 1. Schematic illustration of how hydrophilic polymers impart the many benefits described. Using a single-chain Fv as an example, protein sequences may be exposed which are immunogenic or proteolytically-sensitive. On chemical modification with a polymer (1), the increased hydrodynamic volume acts to shield the protein from neutralising antibodies or proteases (2), reduce renal clearance by glomerular filtration (3) and preferentially exude into inflamed tissues due to the presence of more leaky vasculature and poor lymphatic drainage (EPR effect, 4).
EPR: Enhanced permeability and retention.

and re-engineer specific sites where conjugation is desired, particularly thiol-containing cysteines [16-18]. Unlike the ubiquitous nature of lysine residues, surface-available cysteines are not frequently expressed on the surface of proteins, and modification/manipulation of thiol groups are becoming increasingly favoured for conjugation purposes. As a remnant of whole immunoglobulin hinge structure, a free thiol is often found in antibody Fab′ fragments at the C terminus of the heavy chain, or indeed the light chain, or it can be easily be introduced in other recombinant fragments [19]. Three major benefits can be achieved through such engineering for chemical conjugations: i) conjugations to undesirable sites that are detrimental to protein activity are avoided, ii) more homogeneous conjugates with specific conjugate isoforms are established making more desirable pharmaceuticals and iii) the number of conjugation-reactive sites can be engineered for potential pharmacokinetic manipulation. In comparison, random amine coupling is a more feasible and direct approach for chemical conjugation. In a direct comparison of the two approaches, lysine-directed PSA conjugation reduced the antigen recognising function of an anti-carcinoembryonic antigen (CEA) scFv by a factor of 20, which was resolved by site-specific coupling modification [18,20]. However, as shown in previous studies, amine-directed PSA conjugation was successfully used to modify the pharmacokinetic of an antitumour Fab′ [18,21], whereas conversely, conjugation of PEG chains was found to be more detrimental on antibody activity after amine coupling for an anti-TAG-72 scFv [15]. This practically demonstrates the fact that antibody bioactivity is dependent on different residues within or near the complementarity-determining region (CDR) which is difficult to predict in the absence of structural information. If known, sensitive conjugation sites can be eliminated by mutagenesis. Such an approach has been successfully utilised to mutate a non-essential lysine residue in the V_H CDR3 of the C6.5 scFv into a non-conjugatable alanine residue (K100A mutation) to prevent unwanted chemical conjugation

in the anti-HER2 scFv antigen-binding site leading to a fully-function radio-immunoconjugate [22]. Lysine-60 in the heavy-chain CDR2 was mutated to arginine in the anti-CD20 s/44 mAb to allow bioconjugation without loss of activity [23]. Despite a wide range of 'chemistries' being available within a protein's primary sequence, including post-translational modifications such as glycosylation, amine and thiol groups are by far the most preferred, particularly for antibodies.

2.2 PEGylation

To date, the physicochemical properties of PEG have been the most extensively studied, making it the most advanced carrier system technology to date (**Figure 2**).

Water-attracting features, basic chemistry as well as other factors including conjugation number, length and structural complexity have been studied in terms of pharmacokinetic impact. PEG has a long history as a non-toxic, inert, hydrophilic, uncharged and non-biodegradable polymer. PEG has been approved by the FDA and Europe's EMEA for therapeutic uses in many conjugates [24]. Each ethylene glycol unit (**Figure 2**) of PEG can support up to three water molecules [25] indicating PEG can carry water up to its own molecule mass, resulting in an increased hydrodynamic volume (5 – 10×) greater than the actual mass predicts [26,27]. Furthermore, the pharmacokinetic behaviour of the PEG chains can be dictated by their lengths and conformations (i.e., linear or branched structure) [27-30]. Generally, the clearance rate of the protein therapeutics is decreased when the PEG polymer-conjugate size increases; however, their permeability into potential target sites also slows [30-32]. Therefore, several variable factors have to be considered for modulation in order to find the most appropriate window for a desired pharmacokinetic effect.

There are a number of non-antibody therapeutic agents benefiting from PEGylation including Adagen™ (PEGademase bovine), the treatment of immunocompromised individuals awaiting bone-marrow transplantation; Oncaspar™ (PEGasparaginase) for the treatment of lymphoblastic leukaemia; PEGasys™ (PEGinterferon α-2a; ribavirin) for the treatment of chronic Hepatitis C infections and Macugen™ (PEGaptanib sodium) for the treatment of macular degeneration [10]. Cimzia™ (certolizumab pegol) is the only approved PEGylated antibody but a number of others are in clinical trials (**Table 1**).

2.2.1 *Random conjugation*

Random PEG conjugation has been applied to several $F(ab')_2$ and Fab′ fragments. Generally speaking, the MM of $F(ab')_2$ is above the glomerular filtration threshold (~ 100 kDa), while the rapid clearance of Fab fragments (~ 50 kDa) is much more significant. A number of such fragments against human CEA, a cell-surface tumour marker, were used for PEGylation investigations. Kitamura *et al.* attached 4.9 (average conjugation ratio) short PEG chains (5 kDa) to a $F(ab')_2$ from murine mAb A7. This retained antigen-binding activity to that of the parent $F(ab')_2$ and demonstrated reduced blood clearance of the PEG-$F(ab')_2$ in mice (remaining for up to 24 h) with ~ 1.6-fold increase in $t_{1/2}\alpha$ (α-phase half-life: tissue distribution) and 1.3-fold increase in $t_{1/2}\beta$ (β-phase half-life: systemic elimination) [33,34]. Another murine anti-CEA $F(ab')_2$ and its Fab′ fragment from A5B7 mAb were PEGylated with two 5 kDa PEG chains. Dramatically improved circulation levels were observed by the two mAb fragments over 6 days after antibody administration. At 3 h, the PEGylated $F(ab')_2$ had a 24.7% injected dose/g tumour compared to 11.4 for the unmodified antibody fragment. The increase was even more dramatic for the Fab; 20.6 versus 4.5%. However, as expected, the tumour:blood ratios were lower than for the Fab. However, only 12 and 20% reduction in immunoreactivity was found respectively in comparison with the un-PEGylated $F(ab')_2$ and Fab′ [35]. Delgado *et al.* [36] PEGylated a chimeric recombinant Fab′ (F9) against CEA using 5 kDa PEGs. They demonstrated increasing apparent molecular size by size exclusion chromatography (SEC) by increasing the active PEG molarity ratio during conjugation. The corresponding hydrodynamic radii of the PEGylated Fab′ species were estimated in the range 29 – 40 Å rather than 22 Å of the Fab′ alone [36]. The subsequent biodistribution study further verified the improved retention of the PEG-Fab′ in tumour with ~ 5.7-fold increase in the tumour exposure/bioavailability (determined as the AUC in an uptake vs time plot) over a 6-day post-injection period. However, an almost 50% loss of antigen binding was also concluded with the PEGylated Fab′ [36]. Chapman *et al.* randomly conjugated a humanised Fab′ with 1, 3 and 6 PEG chains (5 kDa). The conjugates *in vivo* circulation half-lifes were found to increase correspondingly with the attached PEG molecule numbers, while the loss of antigen-binding affinity was also found to increase with up to 83% loss in the Fab′ conjugated with 6 PEG chains [37].

High MM branched PEG moieties have been investigated for bioconjugation. Compared to linear PEG, branched PEG chains have been generated with promising improvements: It is claimed that a branched PEG can act as if it were much larger than a corresponding linear PEG of the same MM [26,38]. Koumenis *et al.* conjugated 20 kDa linear PEGs and 40 kDa branched PEGs (20 kDa of each branch) to a neutralising $F(ab')_2$ against IL-8. Dramatic differences of the conjugate MMs were determined via different measurements. Using SEC, the apparent MM increase of $F(ab')_2$ was about 7-fold by adding one 20 kDa linear PEG and ~ 11-fold by adding one 40 kDa branched PEG in comparison with the parent $F(ab')_2$, whilst light-scattering detection gave the same values for the theoretical MMs of the conjugates [39]. Following this, they also reported that the PEGylated $F(ab')_2$ had ~ 16-fold blood exposure (AUC) increase by adding one 40 kDa branched PEG and an ~ 18-fold increase by having two branched PEG, as well as ~ 5.3- and 5.6-fold longer circulation half-lifes than the parent $F(ab')_2$ in rabbits. Additionally, a slower clearance rate was also observed by coupling with one 40 kDa branched PEG compared with same MM

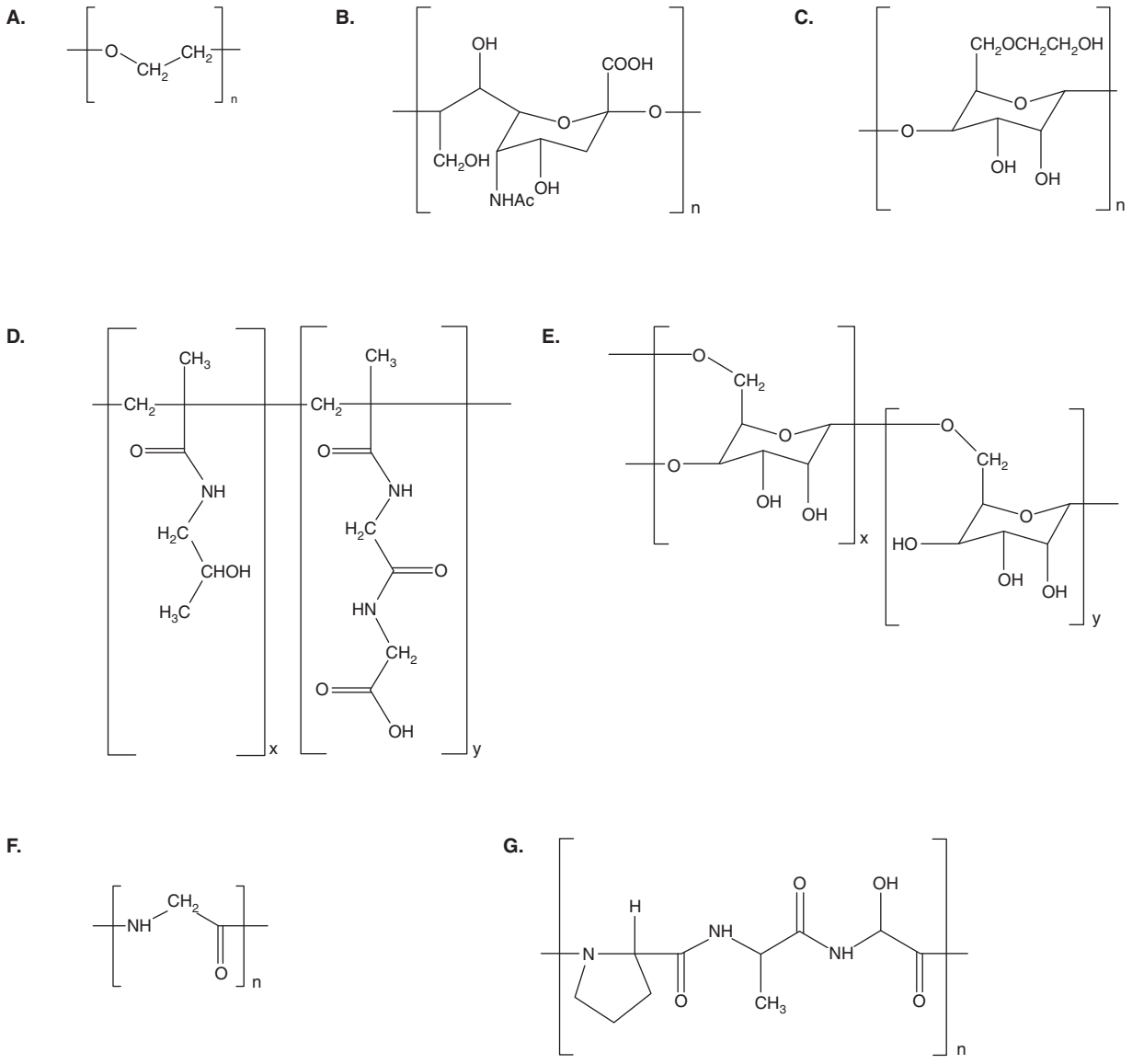

Figure 2. Pharmacokinetic-modifying polymers. A selection of the polymers is shown illustrating the structure of repeating units only (n, or x, y for co-polymers). These all attract water to varying degrees leading to an increased hydrodynamic volume. **A**: PEG, **B**: poly(sialic acid), **C**: HES, **D**: HPMA, **E**: dextran, **F**: poly(glycine), and **G**: poly(proline-alanine-serine).
HES: Hydroxy-ethyl starch; HPMA: (2-Hydroxypropyl)-methacrylamide.

linear one. In terms of antibody targeting, the affinity was retained for up to two PEG molecule conjugates (both linear and branched), but considerable affinity loss was seen beyond this coupling ratio [39].

The effect of PEGylation for prolonging the circulation lives was also investigated on smaller antibody fragments such as scFv. Lee *et al.* PEGylated a mucin-binding scFv through random amine and carboxyl site coupling [15]. A series of coupling reactions with PEG derivatives with a range of MWs (2-20 kDa) were created. The circulation half-life progressively increased as the PEG size and conjugation ratio increased, such that a half-life around 13 – 14 h was reached with both 12 and 20 kDa amine-coupled PEGs under similar conjugation ratios, whilst the unmodified scFv only showed a 0.7 h half-life. However, ~ 23 and 40% binding affinity reduction was reported to the two long PEG-conjugates whereas the short PEG-conjugates retained binding activity [15].

2.2.2 Site-specific modification

As seen above, random conjugation of PEG moieties to small antibody fragments elevate the protein *in vivo* blood residence time particularly through increasing the hydrodynamic radius of PEG–protein complex; however, the general theme is that

Table 1. PEGylated antibodies and alternative frameworks in the clinic.

Name	Molecular species	Indication/Phase	Company	Ref.
Certolizumab pegol (Cimzia®)	Humanised Fab against TNF-α	Rheumatoid arthritis/Approved 2008/9 Psoriasis/Ph III Ankylosing spondylitis/Ph III	UCB/Nektar	[59-61]
CDP-791 abciximab pegol	Chimeric Fab against VEGFR2	NSCLC/Ph II/discontinued?	UCB/Nektar	[53]
CDP-860	Fab against PDGF	Cancer/discontinued?	UCB/Nektar	[42]
PRS-050	Anticalin against VEGF	Solid tumours/Ph I	Pieris AG	Unpub.
CT322	Adnectin against VEGFR2	Solid tumours/Ph I	Adnexus/BMS	[56]
DX1000	Kunitz domain against plasmin	Breast cancer	Dyax	[58]
Nanobody	Single domain antibody from Llama against foot and mouth disease virus	Foot and mouth disease	Ablynx	[57]
CDP-7657	Humanised Fab against CD40L	Systemic lupus erythematosus	UCB/Nektar	[52]

PDGF: Platelet-derived growth factor.

antibody affinity is lost particularly with longer PEG molecule or increased conjugation ratios [14]. More precise technologies were needed for delivering better quality PEGylated antibody fragments with retained binding affinity and longer half-lifes. The engineering of site-specific thiol groups on antibody fragments for PEG coupling was predominately used in order to avoid the interference to antigen-binding site from random chemical conjugation. By positioning such thiols distal to the CDRs, normally straight-forward for antibody fragments, binding function can be retained. There is clearly a kinetic effect due to the presence of a large hydrodynamic polymer. Plückthun's group [40], Mabry *et al.* [41] and Deonarain's group (unpublished) demonstrated a slower antigen-binding association rate for PEGylated and polysialylated scFvs, respectively, when analysed by surface plasmon resonance using a BIACore biosensor. This can be as much as 10-fold. However, the charged, dense matrix may exaggerate this phenomenon, which has not been seen routinely under physiological conditions of binding. However, there are clearly some viscosity or diffusion effects associated with the large hydrodynamic volume. With more site-directed coupling, PEGylation has now been effectively developed to a much more mature technology for affinity-binding proteins.

To prevent the masking effect of large PEG moieties to antigen-binding site from antibody fragment, Chapman *et al.* site-specifically linked PEG chains to remaining free cysteine residue in the hinge region (which is furthest away from the antigen-binding site) of a humanised Fab′ fragment against the β-subunit of PDGF receptor [37]. Blocking this receptor was shown to have anti-proliferative and chemotherapy-enhancing effects (by reducing tumour interstitial pressure), thus having application in oncology [42]. They found the conjugates half-lives were significantly increased in rats with blood exposure values raised almost 7-fold for a single 25 kDa PEG and 13.5-fold for a single branched 40 kDa PEG compared to the unconjugated Fab′. Moreover, > 21-fold bioavailability increase was achieved when two 25 kDa PEG chains were conjugated site-specifically [14,37]. Non-human primate studies further demonstrated the 40 kDa PEG–Fab′ conjugate had blood exposure value approaching that (78%) of the parent IgG (which is almost double the size and benefits from FcRn binding/retention) and a slower elimination phase ($t_{1/2}\beta$). It was also suggested that the PEGylated Fab′ benefited from protection of the anti-idiotypic response [37]. This conjugate (formerly CDP860, UCB) was eventually dropped from clinical development due to lack of efficacy, most probably due to the target selection rather than poor pharmacokinetics. In contrast to the random conjugation, all site-specifically modified Fabs showed consistent binding retention by BIAcore analysis [37]. This contrasts with more recent and detailed kinetic studies of PEGylated protein binding which suggest on-rate limitations (see below). From the clinical trials using the 40 kDa PEG-conjugated Fab′, an average of 5 days plasma half-life was reported [42]. Similarly, Trakas and Tzartos conjugated a 20 kDa linear PEG to the free hinge thiol group of a rat Fab′ targeting the main immunogenic region in autoimmune disease treatment. Compared to the 3 – 6 h circulation time of the normal Fab′, the PEGylated Fab showed up to 4 days longevity in rats, and significant immunosuppressive activity compared with the original Fab′ [43]. Moreover, anti-IL8 Fab′ fragments were thiol site-specific modified with PEGs having MMs of 5 – 100 kDa (linear) or 40 kDa (branched), and dramatic increases of apparent MMs versus theoretical MMs of each conjugate were analysed according to the results from HPLC SEC. Improved pharmacokinetic parameters were showed alongside the increase of PEG MM after injection into rabbits, and the branched PEGs (40 kDa) were found to generate doubled blood exposure and circulation half-life levels compared to the linear PEGs with the same MM. Relative to the native Fab′, the clearance rates of the PEGylated molecules were decreased by 44- to 175-fold [44]. In addition, the therapeutic efficacies of all PEG-conjugates were found to be retained *in vitro* and *in vivo*. The authors also suggested the

possibility of customising the Fab′ pharmacokinetic properties via PEGylation [44]. This latter point if often mentioned as being a strength of the PEGylation technology or other polymers with low polydispersity. However, there has yet to be a published study where an optimal therapeutic effect has been observed on tailoring the half-life of biopharmaceutical protein.

The interference and masking effect to the antigen-binding site becomes more severe as the antibody fragments become smaller. To overcome this detrimental problem, site-specific PEGylation was investigated in a number of single chain Fvs. Tsutsumi *et al.* PEGylated a scFv-immunotoxin fusion protein (LMB-2) via a cysteine residue specifically introduced on the linker connecting both components of the fusion protein [45]. Both 5 and 20 kDa linear PEGs were such conjugated and five and eightfold increments in plasma half-life (LMB-2 has a rapid β half-life of around 13 min) as well as substantially increased bioavailability (3.5- and 4.6-fold increased AUC) and mean residence time were detected correspondingly. An almost unchanged cell-binding profile was observed by the two PEGylated proteins in comparison with the unmodified fusion protein, and the overall therapeutic window was increased by > 20-fold in a preclinical antitumour study. Nonspecific toxicity was seen to decrease despite the increased residence time of the toxin [45]. A systematic study by Yang *et al.* used 5, 20 and 40 kDa maleimide–PEG-conjugates to investigate the bioactivity and blood clearance rates of an anti-TNF-α scFv site specifically conjugated at one or both of two available, engineered thiol sites. Although BIAcore-binding data suggested some loss of bioactivity (see below), cytotoxicity assays showed similar neutralisation IC_{50} values for all conjugate variants. Pharmacokinetically, the half-life of conjugates increased as their PEGylation content increased, demonstrating up to 100-fold prolonged circulating half-lifes [32]. An anthrax exotoxin neutralising scFv was also site-specifically (via an incorporated C-terminal cysteine) PEGylated by a 20 or 40 kDa PEG. Their pharmacokinetic analysis in guinea-pigs showed that the 40 kDa PEG-conjugated scFv increased the serum half-life of the construct beyond that of full-length immunoglobulin in a guinea-pig anthrax model [41] (β half-lifes of 108 vs 96 h). In addition, similar overall equilibrium dissociation constants (K_d) were measured for all constructs as well as *in vitro* toxin challenge potency, and a significant increase in survival and an improved overall mean time to death in the guinea-pig model challenged with a relatively high dose of anthrax spores was also reported [41]. This was especially impressive considering there was no active mechanism for the clearance of the toxin–antibody complex, other than general uptake in the reticulo-endothelial system and degradation. Interestingly, the authors investigated the binding of the scFv by BIACore and noticed that the affinities were 10- (20 k PEG) or 100-fold (40 k PEG) reduced, all due to slower on-rates. This was seen when the antigen was immobilised. However, when the PEGylated scFv was on the chip surface, the affinities were generally retained. This supports the idea that the increased hydrodynamic radius slows down diffusion (see below). Also, PEGylated via chemical coupling to an engineered cysteine residue on the C terminus of an anti-HER2 scFv, Kubetzko *et al.* showed the enlargement of hydrodynamic radius of the 20 kDa PEG-conjugated scFv, which was a ~ 4-6-fold increase compared to the SEC measured apparent MM with the calculated MM. Animal studies in a nude mouse model bearing human carcinoma SKOV3 xenograft showed improved tumour uptake levels with the PEGylated scFv (1.8 – 9.5% ID/g) and the scFv dimer (2.1 – 11.6% ID/g) by 24 h compared to the corresponding scFv, due to significantly increased blood circulation time, but three to eightfold reduction in specificity ratios [46]. The authors also constructed bivalent and tetravalent mini-antibodies, which showed increased *in vitro* avidity for cellular binding, while the PEGylated scFv resulted in a fivefold decreased affinity. However, the multivalent formats only had a moderate improving effect in tumour localisation in comparison with the PEGylated scFv. As seen before, PEGylation has a more dramatic effect than multimerisation. These studies were more complex to evaluate due to the effect of valency on tumour binding and uptake, but there were also observations of decreased association rates. Finally, the authors suggested the prospect of combined use of multimerisation and PEGylation for improving both pharmacokinetic properties of therapeutic antibodies [46]. A cysteine-coupling site has also been specifically engineered into the linker connecting the VH and VL domains of an scFv targeting the CEACAM6 antigen found in pancreatic ductal adenocarcinoma. The scFv such conjugated with 20 kDa PEG showed prolonged plasma half-life, and improved therapeutic efficacy from tumour-bearing mouse models [47].

The current trend of PEGylation for advanced produced has focused on using: i) branched PEG chains over linear ones, as the common observation is that branched-chain conjugates may offer a greater circulatory half-life than the linear chain counterparts [26,48] and ii) site-specific conjugation methods preserving better antigen-binding efficacy. To this end, the first marketing-approved anti-TNF-α PEGylated Fab antibody certolizumab pegol (Cimzia) is a branched-PEG-conjugate (using the technology from Nektar Therapeutics, see below). Recently, it was shown that there was no significant difference between the viscosity radii of branched and linear PEG–proteins having the same total MM of PEG adducts, suggesting that any differences observed in circulatory half-lifes cannot be explained by differences in hydrodynamic volume or glomerular filtration [27]. Since the earliest demonstration in 1977 [49], therapeutic protein PEGylation has expanded into a major biotechnology industry [50,51]. Antibodies are now emerging from this pipeline, but not as many as one would expect. For example, CDP-7657, an anti-CD40L PEGylated Fab is in Phase I clinical trials for SLE [52] but CDP-791 [53] (Imclone/UCB), an anti-VEGFR2 PEGylated di-Fab for solid tumours, recently completed a Phase II clinical trial NSCLC in combination with carboplatin and paclitaxel chemotherapy. Although the PEGylation had the right affect on the pharmacokinetics at a dose of 20 mg/kg, there was a 17.7% tumour response rate without any corresponding increase in progression-free survival [54]. No further news has come forth and the fact that CDP-791 is missing from the UCB pipeline suggests that this has been dropped.

With a number of approved PEGylated biotherapeutics already existing in the market today, PEGylation is recognised as the most mature and practised pharmacokinetic modulation technique so far, and is normally the first choice for increasing the blood half-life for novel or alternative framework-binding proteins (**Table 1**) and peptides such as nanobodies or DARPins (for reviews, see [2,55]). An anticalin-based alternative framework-binding protein against VEGF, chemically PEGylated with a 40 kDa polymer (Angiocal-PRS-050), was shown to be more effective than bevacizumab in an A673 rhabdomyosarcoma xenograft model when administered every other day. Early clinical data from the Phase I trial initiated in June 2010 showed good tolerability (Hohlbaum, Pieris AG, Next Generation Protein Therapeutics conference, 2010). A 40 kDa PEGylated adnectin (fibronectin domain-based binding protein being developed by Adnexus/Bristol Myers Squibb), CT322, showed good tolerability in a Phase I clinical trial. This protein has a high affinity (11 nM) and is specific for VEGFR-2. Some clinical pharmacodynamic evidence was seen with a small number of patients (4/24) showing decreased tumour volume and 24 patients showing stable disease [56]. A small, 15 kDa nanobody against foot and mouth disease virus was PEGylated using Nektar's 40 kDa polymer [57]. Although virus neutralisation was increased 1000-fold, no enhanced protection was seen in guinea-pig models of infection suggesting the need for additional effector functions [57]. Dyax's kunitz domains are also promising agents but require pharmacokinetic modulation to reduce their blood clearance. Four 5 kDa PEG chains were attached via lysine residue amine coupling to a potent inhibitor of plasmin (DX1000, K_i 99 pM). The potency was generally retained (K_i 232 pM), but the half-life was increased from 0.45 to 12.5 h (mice) and 1 to 59 h (rabbits). In the MDA-MB-231 metastatic breast cancer xenograft model, there was a 43% decrease in primary tumour growth and correspondingly reduced metastases at a dose of 10 mg/kg [58].

2.2.2.1 *Certolizumab pegol (Cimzia)*

Certolizumab pegol, formerly CDP-870, is still the only approved PEGylated antibody fragment and has had a tough route to market. Its half-life in preclinical trials is almost as slow as a whole immunoglobulin, thanks to its single 40 kDa PEG chain on the C-terminal thiol of the heavy chain of the anti-TNF-α Fab fragment. It is starting to take a foothold in various inflammatory disease markets, with Phase III results for psoriatic arthritis and axial spondyloarthritis expected at the end of 2011. There is a lot of published material on this product (see [10,11] and references within, [59]), but recent data suggest that certolizumab pegol can bind to membrane-bound TNF-α as well as soluble cytokine. This could lead to a more potent anti-inflammatory effect due to 'reverse signalling' [60]. Additionally, the PEG component could have advantages over whole antibody competitors by allowing preferential targeting to inflamed joints due to the EPR effect (**Figure 2**) [61] and not form complexes of TNF-α as has been observed with bivalent antibodies. Additionally, unpublished work has suggested that certolizumab pegol is a more tolerable drug, with lower injection site pain due to reduced mast cell degranulation. All these observations point to the yet to be appreciated benefits of replacing an antibody Fc with an inert polymer.

2.2.3 *Next generation chemical PEGylation technologies*

Based on the commonly used cysteine-directed site-specific coupling approach, Shaunak *et al.* initiated selective conjugation via two the sulphur atoms in the disulfide bond of an anti-CD4 Fab using a thiol-specific PEG monosulfone. By this method, a 20 kDa PEG was coupled and the PEGylated Fab showed improved size, retained function but > 10-fold reduced antigen-binding capacity [62]. No pharmacokinetic data have yet been described. This technology is being developed as 'TheraPEG', by Polytherics Ltd [63]. Other innovative ways of PEGylating proteins to emerge from Polytherics include 'HiPEG' which involves PEGylating His-tag peptides specifically. C-terminal His-tags are attractive targets for site-specific PEGylation due to their common use in recombinant protein expression. Unpublished work showed that the HiPEG approach was able to increase the half-life of an anti-TNF domain antibody 200-fold, using two 20 kDa PEG chains [64]. Polytherics also have a proprietary thiol-specific PEGylation technology called CyPEG which is claimed to be 37% more stable than standard maleimide conjugation methods [65].

Alternatively, Humphreys *et al.* demonstrated a Fab–PEG product conjugated under strongly reducing conditions without retaining the Fab's inter-chain disulfide. This method enabled efficient (90% coupling efficiency), multi-PEGylation of long PEG chains to an anti-cytokine Fab, and showed unchanged affinity *in vitro* and efficacy *in vivo*. Comparable pharmacokinetic factors were also determined for such PEGylated Fabs, and the bioavailability and overall blood residence time were improved alongside the length and number of coupled PEG chains [66].

Some ingenious approaches have been devised for site-specific conjugations. One by Gao *et al.* [67,68] is where a PEG polymer can be 'grown' onto the N terminus or C terminus of a small protein using *in situ* atom transfer radical polymerisation and could also be applied to both termini of antibody fragments such as scFvs or Dabs. Another is from Ambrx technologies where they use their proprietary ReCODE technology to incorporate unnatural amino acids into proteins. One such residue is para-acetyl-phenylalanine, a derivative of phenylalanine which has been modified to include a ketone functional group. This ketone is chemically inert but reacts with a proprietary-derivatised PEG polymer for specific PEGylation [69].

2.3 Chemical polysialylation

The same types of conjugation chemistry and considerations described above are not unique to PEG: the same advantages and disadvantages can be applied to any other hydrophilic polymers with similar activating groups and target amino acids.

Conjugation using biodegradable organic polymers such as PSA (**Figure 2**) has been investigated using amine and thiol chemistries with similar observations made. PSA is a naturally-occurring biopolymer (⟨-2,8 or ⟨-2,9 linked sialic acid/N-acetyl neuraminic acid) found as colominic acid (in bacteria) or PSA (in mammalian cells) [70]. Furthermore, PSAs have a high degree of chemical versatility, which enables a wide variety of drugs and other molecules to be covalently linked to PSA, with preservation of much of the drug's activity [71]. Gregoriadis *et al.* first proposed that PSA possessed biophysical properties similar to that of PEG such that its hydrophilicity could be used to modulate the half-life of proteins [72]. Initially demonstrated in 1993 with fluoroscein and in 1996 with catalase [72,73], colominic acids (derived from *Neisseria meningitidis* and *Escherichia coli* K1 capsular polysaccharides) were covalently conjugated to achieve extended serum half-lifes with preserved protein function. Also suggested from the immunogenicity studies of chemically polysialylated asparaginase, polysialylation reduced protein antigenicity and as a result prolongs its circulation in the blood even in the presence of anti-asparaginase antibodies [74]. Other polysialylated proteins including insulin [75], IFN-α_{2b}, β-galactosidase, aprotinin and EPO [71] have been investigated *in vitro* and *in vivo* with increased solubility and stability, reduced immunogenicity, preserved function and improved pharmacokinetics.

Lipoxen technologies, through PolyXen®, is commercialising Gregoriadis' polysialylation technology. It has two disclosed non-antibody drug candidates presently in Phase I clinical development, three other disclosed proteins and three non-disclosed proteins (possibly antibodies) and is poised to compete with PEG in leading the stealth platform. The pharmaceutical industry is starting to take an interest in this technology [76].

Extending this application 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. Colominic acids (11 and 22 kDa, corresponding to ~ 35 and 71 units of sialic acid) with several coupling ratios to the antibody were investigated, and in average 1.8- to 3-fold increase of *in vivo* blood residency ($t_{½}\beta$ increasing from around 10 to 17 – 30 h) were demonstrated in comparison with the parental Fab. This was also reflected to an ~ 3-5-fold increase in tumour uptake and blood bioavailability. Interestingly, the longest or highest PSA substitution ratio was not the most effective [20]. However, the same reductive amination procedure was applied to MFE-23, a single-chain Fv directed against another oncofoetal antigen, CEA. This resulted in immuno-conjugates of reduced blood clearance and high bioavailability, but poor immunoreactivity. The latter effect, not surprisingly, led to low tumour uptake. This was resolved using the site-specific method with an addition of C-terminal thiol-containing peptide (Gly_4Cys) group to the scFv directing maleimide-activated PSA conjugation [22]. An > 300 kDa apparent size was obtained rather than the theoretical scFv size (30 kDa) after conjugating with a single 11 kDa PSA. Approximately 3.6- and 9.8-fold increases of $t_{1/2}\beta$ (around 4 to 15 h) and bioavailability, respectively, were observed from the polysialylated than the unmodified scFv [22].

Like PEG, there are no known receptors for PSA and, additionally, anionic molecules are less likely to be cleared through glomerular filtration due to the negative charge of the glomerular basement membrane. PSA, a highly negatively charged molecule must, therefore, be desialyated by neuraminidase-like enzymes before being cleared via either kidney or through the asialoglycoprotein receptors during hepatic elimination of large molecules [5,77]. These additional desialylation routes consequently contribute to a delayed clearance process of PSA-conjugated molecules.

2.4 HESylation

In a similar concept to chemical polysialylation, another organic polymer called hydroxyethyl starch (HES, **Figure 2**) is a modified, branched amylopectin (e.g., derived from waxy maize starch). It is composed of D-glucose units linked by ⟨-1,4-glycosidic bonds and ⟨-1,6-bonds for the branched points, and was chemically conjugated (or HESylated) to several proteins (i.e., erythropoietin and G-CSF) with improved bioactivity and pharmacokinetic properties [78,79]. Various HES derivatives can be prepared with preferable properties serving a flexible approach to modulate pharmacokinetics. Although HESylation has not been applied to or described for antibody fragments (publicly disclosed), its polysaccharide composition and biocompatibility make it an attractive alternative material to existing technologies [79]. Fresenius Kabi [80] is the world's largest producer of HES and own patents surrounding the biotechnology of HESylation. They are collaborating with large pharmaceutical company Bayer-Shering to develop HESylated products and smaller blood-products company Octapharma. It is likely that one of the alternative scaffolds has been HESylated (European Antibody Congress 2010, Geneva).

2.5 HPMA copolymerisation

HPMA copolymers (**Figure 2**) have been developed as drug carriers based on their hydrophilicity and biocompatibility, and it has been used extensively for drug delivery with antitumour activities [7,8,81-82]. A number of cytotoxic drugs, targeting peptides and proteins have already HPMA polymerised with improved therapeutic effects *in vitro* and *in vivo* (see review of [82]), based on the 'enhanced permeability and retention (EPR)' effect of HPMA. Focusing on antibody and antibody fragments, Seymour *et al.* reported conjugation of HPMA to murine IgG B72.3 and its Fab′ and $F(ab')_2$ fragments against CEA [83]. On average, five HPMA copolymer units (~ 20 kDa) were linked per antibody molecule, and in comparison with the native IgG, the antibody fragments showed substantial pharmacokinetic modifications after conjugation. Notably, the half-life of the Fab′ fragment circulation in the bloodstream has been extended from 35 min to 6 h (10-fold improvement).

Despite the pharmacokinetic benefits after conjugation as well as low immune response to the conjugates, the random amine-coupled HPMA conjugates showed no tumour-targeting activities, which was probably due to CDR-masking or disruption by polymer chains. Lu *et al.* subsequently conjugated the copolymer via a more site-specific approach by linking the maleimide group from HPMA to the thiol group created after IgG pepsin digestion. The resulted polymerised Fab′ fragment retained antigen targeting affinity *in vitro* [84] as observed in the polysialylation case story [21]. Although the authors have not specifically addressed the pharmacokinetic modification after HPMA conjugation, they managed to use HPMA as a scaffold to link mesochlorin, a cytotoxic drug on one end of the polymer, showing improved cell-killing activity of the polymerised Fab′ antibody fragment over the drug alone [84].

2.6 Dextran modification

Dextran–protein conjugates (**Figure 2**) were initially used as a chemical method to reduce the immunogenicity of proteins, and the oxidised dextran polymer form was used to permit the covalent attachment at multiple sites through linking with free amines on proteins [85,86]. Reduction of murine and rabbit IgGs' immunogenicity by covalent conjugation of oxidised short dextran polymers was reported by Fagnani *et al.* [85]; however, they also indirectly demonstrated an apparent MM increase of dextran–IgG conjugates alongside the addition of dextran chain length (500 – 2000 Da) by SDS-PAGE and HPLC. Based on this finding, they applied the same dextran modifications on an anti-CEA Fab′ fragment, which effectively demonstrated *in vivo* pharmacokinetic alternations to the Fab′ molecule, including reduced renal uptake and excretion as well as the prolonged residence time of the Fab′ in the vascular compartment. However, this conjugation method was found to reduce the immunoreactivity of the Fab′ molecule [87]. Following this successful pharmacokinetic improvement, a Fab′-enzyme (β-lactamase) conjugate was dextran-modified accordingly. The authors again showed apparent size increase (by SEC and SDS-PAGE) alongside the increase due to the dextran chain (~ 6000 Da). This time, enzyme activity was unaffected and *in vivo* biodistribution profile of the dextran modified Fab′-enzyme conjugate was also shown [88]. Interestingly, this study used variable pretreatment of the protein with methyl acetimidate, which modifies lysine amine groups to prevent conjugation. By varying the number of lysines from 39 to 1, dextran substitution ratios of 9:1 to 0.5:1 were seen which led to a range of SEC apparent masses. No pharmacokinetic data were give, but blood clearance was delayed by 2 – 3 days. At 48 h, a 19-fold higher blood and 2-fold higher tumour levels were seen. Although dextran was used to reduce protein immunogenicity, it has been shown that long dextran polymers can be immunogenic in other studies [89], while other studies suggested the immunogenic effect of dextran is markedly reduced for short dextran chains (< 50 kDa) [90], and the oxidised dextran form was non-immunogenic due the lack of the ring structure of native dextran [85]. Dextran conjugates were being developed by Hybritech, USA before being acquired by Eli Lilly in 1986.

Other polymers such as biodegradable poly-glutamic acid, polyhydroxyethyl-asparagine and polyhydroxyethyl-glutamine and non-biodegradable polymers such as poly-glycerol and poly-acrylamide are also alternatives which have not been applied to antibody fragments.

3. Recombinant approaches

Unlike chemical modulation which benefits from decades of research experience, genetically engineering therapeutic proteins with refined pharmacokinetic characteristics is still in its infancy. However, because of the versatility of recombinant technology and the vast selection of useful fusion agents (long-lived serum proteins, receptor retention and glycosylation domains), recombinant approaches for antibody fragment pharmacokinetic modulation may open many new areas that are more applicable, feasible, effective and economical. We focus on hydrophilic polymers but other approaches such as albumin-binding [18,79], N-linked glycosylation and FcRn-binding are formidable technologies [79].

3.1 Recombinant homo-amino-acid polymer

As described above, the use of inert, hydrophilic polymers represents the major strategy for pharmacokinetic engineering. Certain amino-acid polymers bear this property which can be harnessed by attaching to proteins by genetic fusion. Schlapschy *et al.* investigated a glycine-rich homo-amino-acid polymer (HAP) that was predicted to have a large hydrodynamic radius [91]. They used anti-HER2 Fab 4D5 as a model system and fused 100 and 200 residues of a repetitive sequence (Gly_4Ser, which bears some resemblance to PEG) to its light chain. This linker sequence had already been shown to be extended and disordered and has been used in recombinant antibodies for > 20 years [92]. Using SEC, they showed that the 200 residue 'HAPylated' anti-HER2 4D5 Fab acquired a hydrodynamic volume more than double that of the Fab alone, and a moderate rise in terminal half-life (5.7 h from 2.1 h), but lower than the enhancement made by other technologies. However, this moderate effect could be beneficial to specialised applications, such as *in vivo* imaging [91]. This work was extended to other sequences and a more extended and hydrophilic polymer chain has been described. Poly-(Pro-Ala-Ser) fusions have been developed as alternatives (PASylation) which acquire a more hydrophilic characteristic and a greater enhanced hydrodynamic radius. This was driven by modelling studies which showed that proline residues helped to break up helical secondary structures while alanine and serine residues imparted high flexibility and hydrophilicity. A large number of proteins and antibody fragments (e.g., Fab fragments, IFN-β, human growth hormone and exenatide) have been PASylated with significant pharmacokinetic enhancement (Skerra, unpublished).

Recently, the genetic fusion of an unstructured hydrophilic recombinant polypeptide (~ 864 amino acids), called XTEN®, to a peptide or a protein provided an extraordinary plasma half-life extension [93]. Modified exenatide (a synthetic form of

exendin-4, a glucagon-like peptide hormone that regulates insulin secretion) with a native half-life of 2.4 h showed a projected half-life of 139 h. The authors suggested a tuneable manner of XTEN for different pharmacokinetic requirements in practice [93]. Supported by data of other XTEN fusion proteins, it would be intriguing to see this method used for improving antibody fragment pharmacokinetics. Amunix are commercially developing the XTEN technology and although no antibodies have been disclosed on their pipeline website, three immunologically-related indications are described which may include antibody fragments to inflammatory cytokines [94].

3.2 Recombinantly modified glycosylation

Glycosylation has clearly been shown to influence Fc-mediated effector functions of whole IgG molecule [95] but it also has an effective contribution to the plasma half-life [79]. A few studies have investigated carbohydrate composition effects on the pharmacokinetics of recombinant antibody molecules. One study genetically engineered two or three tandem or overlapping *N*-glycosylation sites at the extended C-terminal part of a scFv and used a yeast (*Pichia pastoris*) system to produce a mannose-rich glycosylated scFv. Slightly faster plasma elimination was seen after intravenous injection into mice, and the clearance was explained by the uptake of phagocytic cells with high surface-expression of mannose receptors [96]. Twofold lower affinity compared to the parental scFv molecule was also reported by the authors, which further demonstrates that post-translational modification can affect antigen binding of small antibody fragments seen previously with the chemical conjugation approaches. The additional sugars themselves were unable to extend the half-life of the scFv through hydrophilicity, but they were used as site-specific anchoring points to chemically link PEG-5000 chains, which results in a 10-fold increase in bioavailability. Similarly, mannosylated scFv-enzyme fusion protein (anti-CEA MFE-23-linked to carboxypeptidase G2 for antibody directed enzyme produg therapy) had a more rapid clearance in animal models and humans compared to previously made bacterially-expressed versions [97]. This was a desirable feature designed to remove unbound systemic fusion protein prior to the administration of the prodrug. Conversely, the introduction of new *N*-glycans has been found to increase the hydrodynamic radius and thus the blood-residence time of the recombinant proteins. A bispecific single-chain diabody (scDb) molecule with additional three to nine *N*-glycosylation sites recombinantly introduced to the linker and C-terminal extension regions resulted in an increased hydrodynamic radius of the scDb and a moderate threefold increase in blood bioavailability over 24 h [98], in a similar approach first shown for erythropoietin [99]. This was due to a relatively modest increase in $t_{½}\beta$ (5.6 – 9 h at best) but also a bigger increase in $t_{½}\alpha$ (10 – 22 h), suggesting multiple physiological effects.

Similar pharmacokinetic-enhancing results were also observed with other *N*-linked hyperglycosylated proteins [79] such as the synthetic form of EPO (darbepoetin α) [99] and follicle stimulation hormone [100]. Terminal sialic acid residues appearing frequently in most protein glycosylation compositions can be considered to be a very important component in increasing therapeutic protein serum half-life [101]. Additional sialic acid content results in a moderate increase of blood residence period as demonstrated by research on sialylation-modified erythropoietin [99], asparaginase [74] and antibody fragments [20,21]. This effect is likely to be a combination hydrodynamic radii increase and delayed uptake by the asialoglycoprotein receptor.

3.3 Recombinant polysialylation

A novel recombinant glycosylation method is being investigated and has achieved significant increase in antibody fragment plasma half-life improvement through an extreme glycosylation feature, polysialylation (Deonarain *et al.*, unpublished; [102]). Already described above using direct chemical conjugation approaches, PSA, a long chain carbohydrate, and a good organic substitute for PEG, has been found in mammals on heavily polysialylated (to 200 sialic acid residues with a unique ⟨2-8 linkage) neural cell adhesion molecule (NCAM) [103]. Advances in cellular glyco-engineering, a greater knowledge of the sialic acid metabolic pathway and NCAM structure have led to the development of PSA-carrier domains from NCAM that can be used to attach PSA to antibodies or any other target protein (Deonarain *et al.*, submitted). Many of the features of chemical polysialylation have been demonstrated without the need to potentially damaging chemical conjugations.

4. Expert opinion

PEGylation is an established and successful method for increasing the half-life of antibody fragments. There is an abundance of published research (PubMed lists > 700 papers when searched with the phrase 'antibody therapy PEG') but to date, only one antibody fragment conjugate is approved and there are only a few in the pipeline. The reasons are unclear but we suspect they are corporate-related rather than technical. There seem to more non-antibody molecules in development as conjugates which support this view. Still, the benefits of PEGylation are clear and further work with certolizumab pegol is uncovering subtleties which make this and other similar approaches attractive. For example, if inflamed joints of tumours have more leaky vasculature, the EPR effect favours polymer conjugates over whole antibodies. For intellectual property reasons as well as scientific reasons, researchers have developed alternatives to PEG, some which can be biologically degraded and some which can be recombinantly encoded. Our 'money' is on the recombinant PEG-like approaches. Biotechnology history has always shown that chemical conjugates get superseded by recombinant fusion proteins as they have better production profiles and few side products. The costs for a recombinant protein to be produced from an optimised cell line are usually lower than for a multi-step conjugate process. This could take the form of an inert peptide or poly-amino-acid repeat or a protein with additional glycosylation. However,

Table 2. A comparison of the various hydrophilic polymer pharmacokinetic enhancing strategies.

Technology	Type	Features
PEGylation	Chemical	Industry-established method Non-biodegradable, bio-compatible and 'inert' polymer Concerns with toxic accumulation, immunogenicity, protein inactivation, downstream processing, costs and yields and homogeneity control
Polysialylation	Chemical	Emerging technology with some encouraging clinical results Biodegradable, bio-compatible and potentially non-immunogenic Concerns with protein inactivation, downstream processing, costs and yields and homogeneity control
	Recombinant	Very early technology Biodegradable, bio-compatible and potentially non-immunogenic Easier production with less downstream processing Concerns with yield and homogeneity control
HAPylation PASylation	Recombinant	Advanced preclinical technology with major commercial backing Biodegradable, bio-compatible and potentially non-immunogenic Easier production with less downstream processing Concerns with low hydrophilicity (HAPylation) and long protein polymers needed for pharmacokinetic effects
Hydroxy-ethyl starch	Chemical	Emerging technology with some with major commercial backing Biodegradable, bio-compatible and potentially non-immunogenic Concerns with protein inactivation, downstream processing, costs and yields and homogeneity control
(2-Hydroxypropyl)-methacrylamide	Chemical	Early technology but broadly used in drug delivery Bio-compatible but degradability unknown Concerns with toxic accumulation, immunogenicity, protein inactivation, downstream processing, costs and yields and homogeneity control
Dextran	Chemical	Early technology but broadly used in drug delivery Bio-compatible, likely to be biodegradable and potentially non-immunogenic Concerns with protein inactivation, downstream processing, costs and yields and homogeneity control

All the summarised approaches have the common features of hydrophilic water attraction and improved protein solubility and stability.
HAP: Homo-amino-acid polymer.

PEGylation is such a well-refined technique that the process is very efficient and economically viable. Therefore, the alternative techniques (summarised in **Table 2**) will need to exemplify and promote additional benefits such as better performance, tailored pharmacokinetics or immune-modifying functions. In addition, not all proteins may be amenable to fusion protein construction, due to N or C termini being buried and/or modification resulting in mis-folding. This would leave chemical or glyco-modification as the only option. 'Tailored pharmacokinetics' is often mentioned as being a benefit, but there is yet to be described an experiment where a range of molecules with varying half-lifes were tested in a therapeutic model *in vivo*, showing that a particular profile was optimal, and that exceeding this value was in fact detrimental. This idea has been tested for other antibody properties such as affinity, but not (as far as we can find) pharmacokinetics. Bioavailability-enhancing technologies are a fruitful area of research and development and very relevant now with various patent expiries and biosimilars on the horizon. With at least a dozen small protein antibody mimics in development, there will be place for the best of the competing technologies.

Declaration of interest

This work was supported by Imperial College, Cancer Research UK and PhotoBiotics Ltd. The authors do not have any conflict of interest.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Jain M, Kamal N, Batra SK. Engineering antibodies for clinical applications. Trends Biotechnol 2007;25:307-16
2. Gebauer M, Skerra A. Engineered protein scaffolds as next-generation antibody therapeutics. Curr Opin Chem Biol 2009;8:245-55

• **An excellent summary of this rapidly increasing area which highlights the need for pharmacokinetic-enhancing technologies.**

3. Aggarwal S. What's fueling the biotech engine-2009-10. Nat Biotechnol 2010;28:1165-71
4. Chames P, Van Regenmortel M, Weiss E, et al. Therapeutic antibodies: successes, limitations and hopes for the future. Br J Pharmacol 2009;157:220-33
5. Haraldsson B, Nystrom J, Deen WM. Properties of the glomerular barrier and mechanisms of proteinuria. Physiol Rev 2008;88:451-87
6. Pluckthun A, Pack P. New protein engineering approaches to multivalent and bispecific antibody fragments. Immunotechnology 1997;3:83-105
7. Duncan R. The dawning era of polymer therapeutics. Nat Rev Drug Discov 2003;2:347-60
8. Duncan R. Polymer conjugates for drug targeting. From inspired to inspiration! J Drug Target 2006;14:333-5
9. Kopecek JP, Kopeckova T, Minko ZR, et al. Water soluble polymers in tumor targeted delivery. J Control Release 2001;74:147-58
10. Jevsevar S, Kunstelj M, Porekar VG. PEGylation of therapeutic proteins. Biotechnol J 2010;5:113-28
11. Knop K, Hoogenboom R, Fischer D, et al. Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. Angew Chem Int Ed Engl 2010;49:6288-308

• **A detailed review of the more technical aspects of PEGylation.**

12. Gaberc-Porekar V, Zore I, Podobnik B, et al. Obstacles and pitfalls in the PEGylation of therapeutic proteins. Curr Opin Drug Discov Devel 2008;11:242-50
13. Armstrong JK, Hempel G, Koling S, et al. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. Cancer 2007;110:103-11
14. Chapman AP. PEGylated antibodies and antibody fragments for improved therapy: a review. Adv Drug Deliv Rev 2002;54:531-45
15. Lee LS, Conover C, Shi M, et al. Prolonged circulating lives of single-chain Fv proteins conjugated with polyethylene glycol: a comparison of conjugation chemistries and compounds. Bioconjug Chem 1999;10:973-81

• **A systematic comparison of different PEG lengths in the context of antibody fragments.**

16. Junutula JR, Raab H, Clark S, et al. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. Nat Biotechnol 2008;26:925-32
17. Kaushik VV, Moots RJ. CDP-870 (certolizumab) in rheumatoid arthritis. Expert Opin Biol Ther 2005;5:601-6
18. Constantinou A, Chen C, Deonarain MP. Modulating the pharmacokinetics of therapeutic antibodies. Biotechnol Lett 2010;32:609-22
19. Alzari PM, Lascombe MB, Poljak RJ. Three dimensional structure of antibodies. Annu Rev Immunol 1988;6:555-80
20. Constantinou A, Epenetos AA, Hreczuk-Hirst D, et al. Modulation of antibody pharmacokinetics by chemical polysialylation. Bioconjug Chem 2008;19:643-50

•• **The first description of antibody polysialylation.**

21. Constantinou A, Epenetos AA, Hreczuk-Hirst D, et al. Site-Specific Polysialylation of an Antitumor Single-Chain Fv Fragment. Bioconjug Chem 2009;20:924-31
22. Adams GP, Shaller CC, Chappell L, et al. Delivery of the alpha-emitting radioisotope bismuth-213 to solid tumors via single-chain Fv and diabody molecules. Nucl Med Biol 2000;27:339-46
23. Popplewell AG, Tickle SP, Ladyman HM. Biological Products. Celltech Research and Development Ltd. US20070059307; 2007
24. Veronese FM, Pasut G. PEGylation, successful approach to drug delivery. Drug Discov Today 2005;10:1451-8
25. Sasahara K. Temperature dependence of volume changes on glycine –PEG and L-alanaine-PEG in aqueous solution. Colloid Polym Sci 1995;273:782-6
26. Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov 2003;2:214-21
27. Fee CJ, Van Alstine JM. Prediction of the viscosity radius and the size exclusion chromatography behavior of PEGylated proteins. Bioconjug Chem 2004;15:1304-13
28. Yamaoka T, Tabata Y, Ikada Y. Distribution and tissue uptake of poly (ethylene glycol) with different molecular weights after intravenous administration to mice. J Pharm Sci 1994;83:601-6
29. Yamaoka T, Tabata Y, Ikada Y. Fate of water-soluble polymers administered via different routes. J Pharm Sci 1995;84:349-54
30. Gursahani HJ, Riggs-Sauthier J, Pfeiffer D, et al. Absorption of polyethylene glycol (PEG) polymers: the effect of PEG size on permeability. J Pharm Sci 2009;98:2847-56
31. Cohen O, Kronman C, Chitlaru T, et al. Effect of chemical modification of recombinant human acetylcholinesterase by polyethylene glycol on its circulatory longevity. Biochem J 2001;357:795-802
32. Yang K, Basu A, Wang M, et al. Tailoring structure-function and pharmacokinetic properties of single-chain Fv proteins by site-specific PEGylation. Protein Eng 2003;16:761-70
33. Kitamura K, Takahashi T, Takashina K, et al. Polyethylene glycol modification of the monoclonal antibody A7 enhances its tumor localization. Biochem Biophys Res Commun 1990;171:1387-94
34. Kitamura K, Takahashi T, Yamaguchi T, et al. Chemical engineering of the monoclonal antibody A7 by polyethylene

glycol for targeting cancer chemotherapy. Cancer Res 1991;51:4310-15

35. Pedley RB, Boden JA, Boden R, et al. The potential for enhanced tumour localisation by poly(ethylene glycol) modification of anti-CEA antibody. Br J Cancer 1994;70:1126-30

36. Delgado C, Pedley RB, Herraez A, et al. Enhanced tumour specificity of an anti-carcinoembrionic antigen Fab' fragment by poly(ethylene glycol) (PEG) modification. Br J Cancer 1996;73:175-82

37. Chapman AP, Antoniw P, Spitali M, et al. Therapeutic antibody fragments with prolonged in vivo half-lives. Nat Biotechnol 1999;17:780-3

•• **A landmark paper in the area of antibody PEGylation.**

38. Yamasaki N, Matsuo A, Isobe H. Novel polyethylene glycol derivatives for modification of proteins. Agric Biol Chem 1988;52:2125-7

39. Koumenis IL, Shahrokh Z, Leong S, et al. Modulating pharmacokinetics of an anti-interleukin-8 F(ab')(2) by amine-specific PEGylation with preserved bioactivity. Int J Pharm 2000;198:83-95

40. Kubetzko S, Sarkar CA, Pluckthun A. Protein PEGylation decreases observed target association rates via a dual blocking mechanism. Mol Pharmacol 2005;68:1439-54

• **An informative study of the biophysical effects of PEGylation which can be applied to other polymers.**

41. Mabry R, Rani M, Geiger R, et al. Passive protection against anthrax by using a high-affinity antitoxin antibody fragment lacking an Fc region. Infect Immun 2005;73:8362-8

42. Jayson GC, Parker GJ, Mullamitha S, et al. Blockade of platelet-derived growth factor receptor-beta by CDP860, a humanized, PEGylated di-Fab', leads to fluid accumulation and is associated with increased tumor vascularized volume. J Clin Oncol 2005;23:973-8

43. Trakas N. Tzartos, SJ. Conjugation of acetylcholine receptor-protecting Fab fragments with polyethylene glycol results in a prolonged half-life in the circulation and reduced immunogenicity. J Neuroimmunol 2001;120:42-9

44. Leong SR, DeForge L, Presta L, et al. Adapting pharmacokinetic properties of a humanized anti-interleukin-8 antibody for therapeutic applications using site-specific pegylation. Cytokine 2001;16:106-19

45. Tsutsumi Y, Onda M, Nagata S, et al. Site-specific chemical modification with polyethylene glycol of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) improves antitumor activity and reduces animal toxicity and immunogenicity. Proc Natl Acad Sci USA 2000;97:8548-53

•• **Direct evidence of the benefits of antibody PEGylation.**

46. Kubetzko S, Balic E, Waibel R, et al. PEGylation and multimerization of the anti-p185HER-2 single chain Fv fragment 4D5: effects on tumor targeting. J Biol Chem 2006;281:35186-201

47. Riley CJ, Engelhardt KP, Saldanha JW. Design and activity of a murine and humanized anti-CEACAM6 single-chain variable fragment in the treatment of pancreatic cancer. Cancer Res 2009;69:1933-40

48. Monfardini C, Schiavon O, Caliceti O, et al. A branched monomethoxypoly (ethylene glycol) for protein modification. Bioconjug Chem 1995;6:62-9

49. Abuchowski A, McCoy JR, Palczuk NC, et al. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem 1977;252:3582-6

50. Riley S. Business insights reports innovation in drug delivery. 2006

51. Fraser-Moodie I. Business insights reports. Delivery mechanisms for large molecule drugs. 2008

52. Wakefield IP, Burkly C, Garber L, et al. CDP7657, a monovalent Fab PEG anti-CD40L antibody, inhibits immune responses in both HuSCID mice and non-human primates. Arthritis Rheum 2010;62:1245

53. Ton NC, Parker GJ, Jackson A, et al. Phase I evaluation of CDP791, a PEGylated di-Fab' conjugate that binds vascular endothelial growth factor receptor 2. Clin Cancer Res 2007;13:7113-18

54. Available from: http://www.ucb.com/media-room/news-overview News archive [Accessed 5 June 11]

55. Revets H, De Baetselier P, Muyldermans S. Nanobodies as novel agents for cancer therapy. Expert Opin Biol Ther 2005;5:111-24

56. Tolcher AW, Sweeney CJ, Papadopoulos K, et al. Phase I and pharmacokinetic study of CT-322 (BMS-844203), a targeted Adnectin inhibitor of VEGFR-2 based on a domain of human fibronectin. Clin Cancer Res 2011;17:363-71

57. Harmsen MM, van Solt CB, Fijten HP, et al. Passive immunization of guinea pigs with llama single-domain antibody fragments against foot-and-mouth disease. Vet Microbiol 2007;120:193-206

58. Devy L, Rabbani SA, Stochl M, et al. PEGylated DX-1000: pharmacokinetics and antineoplastic activity of a specific plasmin inhibitor. Neoplasia 2007;9:927-37

59. Fleischmann R. The clinical efficacy and safety of certolizumab pegol in rheumatoid arthritis. Expert Opin Biol Ther 2010;10:773-86

60. Palframan R, Airey M, Moore A, et al. Use of biofluorescence imaging to compare the distribution of certolizumab pegol, adalimumab, and infliximab in the inflamed paws of mice with collagen-induced arthritis. J Immunol Methods 2009;348:36-41

61. Nesbitt A, Fossati G, Bergin M, et al. Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor alpha agents. Inflamm Bowel Dis 2007;13:1323-32

62. Shaunak S, Godwin A, Choi JW, et al. Site-specific PEGylation of native disulfide bonds in therapeutic proteins. Nat Chem Biol 2006;2:312-13

63. Available from: http://www.polytherics.co.uk/technologies/hipeg/ [Accessed 5 June 11]

64. Available from: http://www.polytherics.co.uk/technologies/cypeg/ [Accessed 5 June 11]

65. Available from: http://www.polytherics.co.uk/technologies/therapeg/ [Accessed 5 June 11]

66. Humphreys DP, Heywood SP, Henry A, et al. Alternative antibody Fab' fragment PEGylation strategies: combination of strong reducing agents, disruption of the interchain disulphide bond and

disulphide engineering. Protein Eng Des Sel 2007;20:227-34

67. Gao W, Liu W, Christensen T, et al. In situ growth of a PEG-like polymer from the C terminus of an intein fusion protein improves pharmacokinetics and tumor accumulation. Proc Natl Acad Sci USA 2010;107:16432-7

68. Gao W, Liu W, Mackay JA, et al. In situ growth of a stoichiometric PEG-like conjugate at a protein's N-terminus with significantly improved pharmacokinetics. Proc Natl Acad Sci USA 2009;106:15231-6

69. Available from:. http://www.ambrx.com/wt/page/site_matters [Accessed 5 June 11]

70. Janas T, Janas T. Polysialic acid: structure and properties. Polysaccharides: structural diversity and functional versatility. CRC Publisher; 2004. p. 1224

71. Gregoriadis G, Jain S, Papaioannou I, et al. Improving the therapeutic efficacy of peptides and proteins: a role for polysialic acids. Int J Pharm 2005;300:125-30

72. Gregoriadis G, McCormack B, Wang Z, et al. Polysialic acids: potential in drug delivery. FEBS Lett 1993;315:271-6

73. Fernandes AI, Gregoriadis G. Synthesis, characterization and properties of sialylated catalase. Biochim Biophys Acta 1996;1293:90-6

74. Fernandes AI, Gregoriadis G. The effect of polysialylation on the immunogenicity and antigenicity of asparaginase: implication in its pharmacokinetics. Int J Pharm 2001;217:215-24

• **A landmark paper introducing protein polysialylation.**

75. Jain S, Hreczuk-Hirst D, McCormack B, et al. Polysialylated insulin: synthesis, characterization and biological activity in vivo. Biochim Biophys Acta 2003;1622:42-9

76. Available from: http://www.lipoxen.com/pipeline/polyxen-product-pipeline.aspx [Accessed 5 June 11]

77. Stockert R. The asialoglycoprotein receptor: relationships between structure, function, and expression. Physiol Rev 1995;75:591-609

78. Besheer A, Hause G, Kressler J, et al. Hydrophobically modified hydroxyethyl starch: synthesis, characterization, and aqueous self-assembly into nano-sized polymeric micelles and vesicles. Biomacromolecules 2007;8:359-67

79. Kontermann RE. Strategies to extend plasma half-lives of recombinant antibodies. BioDrugs 2009;23:93-109

80. Available from: http:/www.fresenius-kabi.com [Accessed 5 June 11]

81. Duncan R, Kopeckova P, Strohalm J, et al. Anticancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. II. Evaluation of daunomycin conjugates in vivo against L1210 leukaemia. Br J Cancer 1988;57:147-56

82. Kopecek J, Kopeckova P. HPMA copolymers: origins, early developments, present, and future. Adv Drug Deliv Rev 2010;62:122-49

83. Seymour LW, Flanagan PA, al-Shamkhani A, et al. Synthetic polymers conjugated to monoclonal antibodies: vehicles for tumour-targeted drug delivery. Sel Cancer Ther 1991;7:59-73

84. Lu ZR, Kopeckova P, Kopecek J. Polymerizable Fab' antibody fragments for targeting of anticancer drugs. Nat Biotechnol 1999;17:1101-4

85. Fagnani R, Hagan MS, Bartholomew R. Reduction of immunogenicity by covalent modification of murine and rabbit immunoglobulins with oxidized dextrans of low molecular weight. Cancer Res 1990;50:3638-45

86. Wileman TE, Foster RL, Elliott PN. Soluble asparaginase-dextran conjugates show increased circulatory persistence and lowered antigen reactivity. J Pharm Pharmacol 1986;38:264-71

87. Fagnani R, Halpern S, Hagan M. Altered pharmacokinetic and tumour localization properties of Fab' fragments of a murine monoclonal anti-CEA antibody by covalent modification with low molecular weight dextran. Nucl Med Commun 1995;16:362-9

88. Mikolajczyk SD, Meyer DL, Fagnani R, et al. Starling. Dextran modification of a Fab'–beta-lactamase conjugate modulated by variable pretreatment of Fab' with amine-blocking reagents. Bioconjug Chem 1996;7:150-8

89. Richter W, Kagedal L. Preparation of dextran-protein conjugates and studies of their immunogenicity. Int Arch Allergy Appl Immunol 1972;42:885-902

90. Kabat EA, Bezer AE. The effect of variation in molecular weight on the antigenicity of dextran in man. Arch Biochem Biophys 1958;78:306-18

91. Schlapschy M, Theobald I, Mack H, et al. Fusion of a recombinant antibody fragment with a homo-amino-acid polymer: effects on biophysical properties and prolonged plasma half-life. Protein Eng Des Sel 2007;20:273-84

92. Huston JS, Levinson D, Mudgett-Hunter M, et al. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. Proc Natl Acad Sci USA 1988;85:5879-83

93. Schellenberger V, Wang CW, Geething NC, et al. A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. Nat Biotechnol 2009;27:1186-90

• **Highlights the power and potential of extended hydrophilic peptides.**

94. Available from:. http://www.amunix.com/content/products/overview.htm [Accessed 5 June 11]

95. Wright A, Morrison SL. Effect of glycosylation on antibody function: implications for genetic engineering. Trends Biotechnol 1997;15:26-32

96. Wang M, Lee LS, Nepomich A, et al. Single-chain Fv with manifold N-glycans as bifunctional scaffolds for immunomolecules. Protein Eng 1998;11:1277-83

97. Kogelberg H, Tolner B, Sharma SK, et al. Clearance mechanism of a mannosylated antibody-enzyme fusion protein used in experimental cancer therapy. Glycobiology 2007;17:36-45

98. Stork R, Zettlitz KA, Muller D, et al. N-glycosylation as novel strategy to improve pharmacokinetic properties of bispecific single-chain diabodies. J Biol Chem 2008;283:7804-12

99. Elliott S, Lorenzini T, Asher S, et al. Enhancement of therapeutic protein in vivo activities through glycoengineering. Nat Biotechnol 2003;21:414-21

100. Weenen C, Pena JE, Pollak SV, et al. Long-acting follicle-stimulating

hormone analogs containing N-linked glycosylation exhibited increased bioactivity compared with o-linked analogs in female rats. J Clin Endocrinol Metab 2004;89:5204-12

101. Byrne B, Donohoe GG, O'Kennedy R. Sialic acids: carbohydrate moieties that influence the biological and physical properties of biopharmaceutical proteins and living cells. Drug Discov Today 2007;12:319-26

102. Constantinou A. Production and study of polysialylated antibodies for improved cancer therapy. PhD thesis, Imperial College London; 2005

103. Georgopoulou N, Breen KC. Overexpression of the alpha2,6 (N) sialyltransferase enzyme in human and rat neural cell lines is associated with increased expression of the polysialic acid epitope. J Neurosci Res 1999;58:641-51

Affiliation

Chen Chen, Antony Constantinou &
Mahendra Deonarain[†]
[†]Author for correspondence
Imperial College London,
Department of Life Sciences,
Faculty of Natural Sciences,
Exhibition Road,
London SW7 2AZ, UK
Tel: +44 207 594 5318; Fax: +44 207 594 4560;
E-mail: m.deonarain@imperial.ac.uk