The case for protein PEGylation

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2.1 Introduction

Therapeutic proteins are fast-acting and potent medicines and have proved very successful in the treatment of a wide range of indications. With increasing knowledge of the molecular mechanisms of disease, there will continue to be opportunities to develop protein therapeutics. One estimate is that the global protein therapeutics market will be approximately $315 billion by 2025 [1]. Much of this growth can be attributed to the development of monoclonal antibody therapeutics as new targets are uncovered (e.g., checkpoint blockades). While there will likely be a rise in the development of bispecific antibodies and multifunctional antibody-based medicines (e.g., antibody drug conjugates (ADCs)), there are non-antibody-based proteins such as replacement and non-endogenous proteins still being developed. Strategies have been employed to increase the half-life and duration of action of these proteins to make them viable medicines. Endogenous (e.g., blood factors) and exogenous non-antibody proteins will continue to be developed.

Therapeutic proteins are far from ideal molecules to be used as drugs. Development is often hampered by problems owing to limitations in (1) physiochemical instability (e.g., propensity to aggregate), (2) immunogenicity (e.g., ability to induce the formation of antidrug antibodies) and, (3) suboptimal circulation half-life and/or duration of action. Proteins that are rapidly cleared from the circulation must be dosed frequently, which can create toxicity and immunogenicity concerns. Poor pharmacokinetics (PK) properties are characterized by dose dumping, resulting in a subtherapeutic concentration between each repeat administration, which can have a significant impact on therapeutic efficacy.

Optimizing protein PK cannot be considered a "bolt on" step in the preclinical development process. Establishing and optimizing PK properties from the outset should be integrated in the development of protein-based medicines. Optimal PK and duration of action must be designed for the best medical outcome possible. If too short, there are problems associated with too frequent administration. If too long, there could be potential problems with loss of protein stability, lack of ability to rescue a patient if needed, and possible toxicity issues. The expectation is that the continued development of half-life strategies will continue, hand-in-hand with the development of new innovative protein therapeutics.

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IgG antibodies can display extended half-lives of 14−21 days at relatively high doses by Fc-mediated recycling by binding to the neonatal Fc receptor (FcRn). Human serum albumin (HSA) also displays a long half-life of approximately 19 days through binding to a similar FcRn. Currently, the clinically proven methods to extend the protein half-life for non-IgG drugs are dominated by strategies utilizing fusion to an immunoglobulin Fc (to exploit FcRn recycling), glycoengineering, albumin binding, and PEGylation. Fc fusion protein products, like monoclonal antibodies, need to be given in high doses to compete with endogenous immunoglobulins for FcRn receptor binding, so these benchmarked half-lives are rarely achieved unless given at a sufficiently high dose. Of these clinically proven strategies for half-life extension, PEGylation is by far the most successful. Many excellent reviews have been published to describe protein PEGylation and only a few are cited here [2−12].

Medicines that utilize the properties of macromolecules can provide clinical benefits [13]. The covalent conjugation of the water-soluble polymer, poly(ethylene glycol) (PEG), to a therapeutic protein can increase the circulation time of the protein. The process of conjugating PEG to a protein is known as PEGylation. Protein PEGylation extends the circulation time of a protein because the conjugation of PEG to a protein results in an increased solution size and reduces the renal clearance times of the conjugate compared to the unmodified protein. PEG is a flexible, random coil macromolecule that displays an extended conformation in water, which is maintained when one terminus of PEG is conjugated to a protein. Linear PEG is derived from ethylene oxide repeat units (HO-$(CH_2CH_2O)_n$-H). PEG is typically activated at one terminus for protein conjugation with a non-reactive methyl group at the other terminus. The molecular weights of PEG used in protein conjugation are well solvated in water at physiological conditions.

A PEG−protein conjugate can be linked to an A−B block polymer, where one block is the protein, which tends to be highly organized and globular in solution, and the other block is the PEG, which is more random and extended in its solution conformation. The presence of PEG when conjugated to a protein also sterically shields the protein to decrease its proteolytic degradation, opsonization, and uptake by the mononuclear phagocyte system, all of which contributes to increasing the half-life of the conjugate [14,15]. PEG steric shielding can also help to reduce immunogenicity by shielding potential antigenic sites on the protein [6,16−19] and the propensity for protein aggregation [20]. Several studies indicate that protein stability can be improved after PEGylation [12,21]. Many PEG−protein products are liquid formulations packed in ready-to-use syringes. Unconjugated PEG is also widely used in consumer and healthcare products, as an excipient in drug formulation, and in the treatment of pediatric constipation and colonoscopy [22].

PEGylated proteins have become first-line treatments and PEGylation has been used for lifecycle management as biobetter versions of existing proteins have been developed. The first PEGylated products are now coming off patent, resulting in the development of international standards [23] and biosimilar versions. Protein modification is an important area for developing new medicines and new technologies. PEGylation is also used for a wide range of other applications (e.g., to PEGylate

the surface of particulates [24]). Recent advances in PEGylation conjugation are also being used to make complex, multifunctional therapeutic proteins (e.g., ADCs [25,26]).

Many PEG−protein conjugates are often a second-generation product that is better clinically by virtue of displaying an extended duration of action compared to the unmodified protein. PEGylation of many classes of different molecules (e.g., peptides, oligonucleotides, and low-molecular-weight chemical entities) is also clinically proven and remains an active area of research [8,27,28]. Strategies in protein engineering have been developed to accelerate the development of chemically modified proteins including PEG−protein conjugates [11,29,30]. Other methods using alternative polymers and recombinant fusion of flexible polypeptide chains as PEG substitutes are reaching clinical development and may offer alternative macromolecule conjugation strategies to extend the half-life of therapeutic proteins. Countless patients have used PEGylated therapeutic proteins since the 1990s and it is anticipated that PEGylated proteins will continue to be used clinically.

2.2 Other strategies to achieve extended PK properties

As clinically successful as PEGylation has been shown to be, there has been considerable effort to optimize the PK and duration of action profiles of therapeutic proteins using alternative strategies. Chronic indications (e.g., hemophilia and endocrine disorders) are particularly appropriate for the development of extended half-life versions of therapeutic proteins.

Fully recombinant approaches, such as glycoengineering and Fc fusion, have been shown to be clinically viable, but to a lesser extent than PEGylation. Some of these other strategies may have different properties (e.g., biodistribution, toxicity, stability, manufacturing) that might be more favorable for a given indication [30]. However, it appears that more than one extension strategy may be appropriate for a given therapeutic protein.

Glycoengineering [31] was utilized to extend the circulation time of erythropoietin (EPO) in the development of darbepoetin-α (Aranesp). First-generation EPO possesses three *N*-linked glycosylation sites. Darbepoetin-α was engineered to contain two additional glycosylation sites [32], which increased the size of EPO, resulting in an increase in the serum half-life (from 8.5 to 25.3 hours) [33]. As with PEGylation there is a reduction in binding affinity, but Darbepoetin-α has proven itself to be clinically beneficial. Also, like PEGylation, glycosylation is a post translational modification and the carbohydrate structural heterogeneity associated with mammalian expression systems often occurs [34].

Fusion of a therapeutic protein to the Fc fragment of IgG to exploit FcRn recycling has been clinically established with at least 10 registered products in use. Etanercept (Enbrel, 1998) is a major product used in the treatment of inflammatory conditions (rheumatoid arthritis and psoriasis). Recent years have witnessed the approval of Fc-fusion products for other major indications including (1) cancer,

anti-angiogenesis by fusing to the Fc the extracellular binding regions of the receptors for vascular endothelial growth factor (VEGF) (Aflibercept, 2012), (2) type 2 diabetes by fusing glucagon-like peptide 1 which is an incretin mimetic to an Fc (Dulaglutide, 2014), and (3) hemophilia A and B by fusion of either factor 8 or factor 9, respectively, to an Fc (Eloctate and Alprolix, respectively, 2014). Several proteins have also been fused to albumin and have been evaluated in clinical trials including IFNα (Albuferon) [35], hGH (Albutrophin) [36], B-type natriuretic peptide (AlbuBNP) [37], and interleukin-2 (Albuleukin) [38], but none have succeeded to registration.

Many types of protein fusions are made in protein development and they generally are known to improve protein expression, secretion, and solubility. In the case of Fc fusion, downstream purification can be made more efficient with the use of protein A affinity chromatography to bind to the Fc. Protein A chromatography is widely used in the purification of monoclonal antibodies. Like PEGylation, fusion can reduce protein activity. Furthermore fusion can prevent correct protein folding and may assist in the formation of new antigenic epitopes. While the PK can be tailored to some extent by the molar amount of PEG used in the conjugate, this is less possible by Fc fusion. However, Fc mutations are being developed to increase FcRn binding interactions to optimize and better tailor PK [30].

More recently, degradable, hydrophilic poly(amino acid) sequences that lack secondary structure have been recombinantly fused to proteins as a strategy for protein modification to extend circulation times. XTEN is a recombinant hydrophilic polymer composed of six naturally occurring amino acids (alanine, glutamic acid, glycine, proline, serine, and threonine). Although these poly(amino acid)s can be chemically conjugated to a protein, they can also be recombinantly fused to the therapeutic protein. As a poly(amino acid) sequence XTEN is considered to be biodegradable. Two XTENylated products, exenatide (VRS-859) and a human growth hormone (GH) (VRS-317) conjugate, are being clinically evaluated [39–41]. To date the GH product has not met its primary endpoint in clinical trials [42]. A higher absolute molecular weight of the XTEN polypeptide is needed compared to PEG to increase the half-life, which could reduce the potency similar to albumin conjugates [28].

PAS is another recombinant poly(amino acid) that can be recombinantly fused to the therapeutic protein. PAS is composed of proline, alanine, and serine and this technology has been applied to many proteins in preclinical studies [43–45]. PAS adopts a random coil conformation in solution and has been shown to extend the half-life of the fused protein of interest during preclinical studies.

Many other strategies [46] have been evaluated in preclinical studies to extend the half-life and duration of action of protein therapeutics. Often strategies are based on the use of depots of one kind or another with the stated advantage being that an unmodified, native form of the protein can be released over time with its full bioactivity. The challenge with proteins that clear rapidly is that flux needs to be maintained, which requires high loading and therefore high concentrations of the protein is often required. Maintaining the tertiary structure of a protein under such conditions is a challenge, especially as most depot-based strategies are required to function at ambient or physiological temperatures.

Hydrogels, including stimuli-responsive gels (e.g., temperature), have been considered, and specific challenges remain for clinical translation. For example, the effective mixing of a preformed gel and protein is simply not possible, which may partially explain why most gel systems display biomodal release profiles characterized by burst release kinetics. There are currently no protein-encapsulated hydrogels in clinical use but their development is still an active area of research.

Colloidal carriers, such as liposomes or biodegradable microparticles or nanoparticles, have been widely examined for peptide and protein therapeutics. For microparticles and nanoparticles, fabrication from clinically used polymeric materials such as poly(lactic-*co*-glycolic acid) has been successful for peptides, which tend not to have the same stability constraints for maintaining a bioactive tertiary structure that a protein therapeutic has. Several approved microparticle protein delivery systems exist, including Lupron Depot, Decapeptyl, Sandostatin LAR Depot, and Somatuline LA. The clinical translation of protein-based depots has been much less successful. Nutropin Depot for GH was approved in 1999, but withdrawn in 2004 [47].

2.3 PEGylation continues to be clinically proven

Davis et al. described *PEGylation* in the 1970s [48−50] to decrease the immunogenicity of non-human proteins, and now at least 15 registered PEGylated products are in clinical use (Table 2.1) with more in clinical trials [28]. Several clinically used therapeutic proteins, such as diagnostics and ADCs, are chemically modified to add an additional function. In this regard, PEGylation is not inherently different.

Poly(*N*-vinvylpyrrolidone) (PVP) was used prior to PEG. PVP, like PEG, is a non-ionic polymer, and was used as a plasma expander during World War 2 [46,51]. Related polymers such as poly(*N*-acryloyl morpholine) and several other polymers [52] including polyglycerol, polyoxazolines [53], polyzwitterions [54], poly(vinyl alcohol), and heparosan polymer [55] and hydroxyethyl starch [56] have also been conjugated to proteins [57]. While the molecular weight dispersities are improving, much remains to be done for many of these polymers. PEG has the advantage that it can be made at scale at very narrow disparity in a wide range of relevant molecular weights for conjugation. PEG also does not have pendent chain functionality, and so does not undergo crosslinking reactions.

The first PEGylated products to appear were enzymes in the 1990s. PEGylated adenosine deaminase (Adagen) [58] is used to treat severe combined immunodeficiency (SCID) syndrome and PEGylated asparaginase (Oncaspar) is used to treat acute lymphoblastic leukemia [59]. These non-human enzymes are immunogenic, which severely limits their clinical use unless PEGylated. Both enzymes transform low-molecular-weight substrates and are conjugated with several molecules of PEG per protein molecule (i.e., hyper-PEGylated). For example, Adagen is the bovine intestinal form of adenosine deaminase and is randomly modified at the proteins ε lysines with 11−17 molecules of 5 kDa PEG succinimidyl succinate, resulting in an

Table 2.1 FDA-approved PEGylated products

Product	PEGylated molecule	Company	Date	Indication
Adagen (Pegadamese)	Adenosine deaminase (enzyme) Multiple linear 5 kDa PEG	Enzon	1990	SCID
Oncaspar (Pegaspargase)	L-asparaginase (enzyme) Multiple linear 5 kDa PEG	Enzon	1994	ALL
Pegasys (Peginterferon-α2a)	IFN-α2a (helical barrel protein) Branched 2 × 20 kDa PEG	Roche	2001	HCV
PEG-intron (Peginterferon-α2b)	IFN-α2b (helical barrel protein) Mono-linear 12 kDa PEG	Schering-Plough	2001	HCV
Neulasta (Pegfilgrastim)	GCSF (helical barrel protein) Mono-linear 20 kDa PEG	Amgen	2002	Neutropenia
Somavert (Pegvisomant)	GH antagonist (helical barrel protein) Multiple linear 5 kDa PEG	Pharmacia & Upjohn	2003	Acromegaly
Macugen (Pegaptanib)	Anti-VEGF (aptamer) Branched 2 × 20 kDa PEG	Eyetech Pharma/Pfizer	2004	Wet AMD
Mircera (Peg-epoetin-β)	erythropoietin-(epotin-β) Mono-linear 30 kDa PEG	Hoffman-La Roche	2007	Anaemia
Cimzia (Certolizumab pegol)	TNF-α inhibitor (Fab′) Branched 2 × 20 kDa PEG	Nektar/UCB Pharma	2008	RA/Crohn's
Krystexxa (PEGuricase)	Uricase Multiple linear 10 kDa PEG	Savient Pharma	2010[a]	Gout
Sylatron (peginterferon-α2b)	IFN-β1a (helical barrel protein) Mono-linear 12 kDa PEG	Merck	2011	Melanoma
Lonquex (lipegfilgrastim)	GCSF (helical barrel protein) Mono-linear 20 kDa PEG	Teva	2013	Neutropenia
Plegridy (peginterferon-β1a)	IFN-β1a (helical barrel protein) Mono-linear 12 kDa PEG	Biogen	2014	MS
Jintrolong (peg-growth hormone)	Growth Hormone Mono-linear 40 kDa PEG	GeneScience	2014	Growth deficiency

(*Continued*)

Table 2.1 (Continued)

Product	PEGylated molecule	Company	Date	Indication
Adynovate PEG-Factor VII	Factor VIII (coagulation factor) ~2 Linear PEG	Shire	2016	Haemophilia A
Rebinyn® (nonacog beta pegol)	Factor IX (coagulation factor) Glycol-PEGylated (40 kDa PEG)	Novo Nordisk	2017	Haemophilia B
Omontys (peginesatide)	Peptide ESA Branch 2 × 20 kDa PEG	Affymax/Takeda	2012[b]	Anaemia
Doxil (liposomal doxorubicin)	PEG derivatised liposome	Ortho/Schering-Plough	1995	Cancer
Movantik (naloxegol)	Small molecule opoid (tablet) Mono-linear 340 Da PEG	Nektar/Astra Zeneca	2014	Opiod-induced constipation

α, alpha; ALL, acute lymphoblastic leukemia AMD, age-related macular degeneration; d, days; ESA, erythropoiesis stimulating agent; FDA, Food and Drug Adminstration; G-CSF, granulocyte colony-stimulating factor; GHR, growth hormone receptor; hrs, hours; IFN, interferon; kDa, kilodalton; mPEG, monomethoxypoly(ethylene glycol); PEG, poly(ethylene glycol); SCID, severe combined immunodeficiency diseases; $t_{1/2}$, half-life; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

[a]Withdrawn in the EU.

[b]Withdrawn for hypersensitivity.

improved circulation half-life (by a factor of 6.4) and decreased immunogenicity. Another hyper-PEGylated non-human enzyme that has been approved more recently (2010) is PEG-uricase (Pegloticase, Kystexxa). Uricase metabolizes uric acid to reduce its propensity to precipitate in chronic gout. It appears there are nine PEG molecules (10 kDa each) conjugated to each subunit of this enzyme [12].

Hyper-PEGylation is typically accomplished using what are considered to be low-molecular-weight PEG reagents of approximately 5 kDa, which once conjugated to the protein cumulatively to display a high molar mass of PEG. PEGylation serves both to reduce the immunogenicity of these enzymes while also extending their half-life. Interestingly, some of these enzymes have quaternary structures, which must form for activity. These non-human protein products would not be possible without PEGylation.

Factor VIII is an enzyme used to treat hemophilia A that must form a complex intermediate in the coagulation pathway with other proteins. Rather than being hyper-PEGylated, PEGylated factor VIII (Adynovate) has approximately two PEG molecules per factor VIII molecule. Adynovate is manufactured with most of the PEG conjugated in the B-domain, which is not required for activity of the protein [60,61]. PEGylated Factor IX (Rebinyn, nonacog beta pegol) [62,63] to treat hemophilia B is made by glycoPEGylation which involves the selective PEGylation of *O*-linked glycosylation sites using a sialytranferase to introduce a sialic acid derivatized PEG at *N*-acetyl galactosamine residues [64]. Since PEGylation at these sites effectively substitutes the carbohydrate chains' conjugation, it is thought to have little effect on protein activity while providing associated protection from proteases and enhanced stability.

Clinically used therapeutic proteins that bind to cell surface receptors have been non-specifically mono-PEGylated. Often these proteins must bind to heterodimeric receptor pairs to elicit their biological effects, so much of the protein solvent assessable surface is involved in the interactions needed for biological activity. These products are mixtures of PEG positional isomers with a single PEG molecule conjugated at different nucleophilic sites along the protein primary structure [65,66]. The majority of the PEGylated products available are non-specifically mono-PEGylated. Products include PEGylated interferon-α-2a and α-2b (Pegasys and PEG-Intron), interferon-β1a (Plegridy), erythropoeitin (Mircera), granulocyte colony stimulating factor (GCSF) (Neulasta [67] and Longuex) and GH (Jintrolong) [68]. Efforts to decrease the number of positional isomers in these products have used reagents targeting the amine of the terminal amino acid using aldehyde base PEG reagents in reductive amination [69] (e.g., PEG−GCSF and PEG−IFN-β1a). Also, use of glycosylated proteins (e.g., EPO and IFN β-1a) can decrease the number of positional PEG isomers in the final product.

Many of aforementioned PEGylated products with one or two conjugated PEG molecules per protein molecule are derived from the PEGylation of already clinically used protein products. This has been important to demonstrate improved efficacy of the PEGylated product compared to the unmodified protein. Biosimilar versions of these PEGylated products are now appearing, e.g., PEG−IFN (peginterferon alfa-2b, Sylatron) and PEG−GCSF (lipegfilgrastim, Lonques).

In addition to the hyper-PEGylated enzymes, mono-PEGylated products are being developed where the protein has not previously been used clinically simply because without having being PEGylated, the product would not have been clinically beneficial or commercially feasible. For example, Cimzia (certolizumab peg) is a PEGylated antibody fragment (Fab′) that targets tumor necrosis factor alpha (TNF-α) to treat inflammatory conditions (e.g., rheumatoid arthritis and Crohn's disease) [70].

Cimzia is also an example of a clinical product that has been recombinantly engineered to possess an unpaired cysteine for thiol-targeted conjugation to reduce the number of positional isomers [70]. Free cysteine can readily be conjugated in mild conditions where the protein nucleophiles (e.g., lysine amines) are not modified. Most therapeutically useful proteins to date have disulfide bonds which can undergo reactions with the free cysteine thiol. Also, proteins with a free cysteine require care during downstream processing to avoid aggregation. Often protein dimers due to intramolecular disulfide bond formation have to be reduced, but this can also cause reduction of the protein disulfide bonds.

Recombinant engineering and site-specific conjugation can be used to minimize the loss of biological activity that is associated with protein modification. Non-specific conjugation is characterized by the production of a heterogeneous mix of conjugates with a range of activities. Engineering a specific conjugation site makes it possible to fabricate more homogeneous PEGylated protein products with a more narrow activity range. Site-selective conjugation at sites distal from the intended biological binding sites on the protein can result in maximal biological activity. The combination [71] of recombinant and chemical modification approaches will increasingly be used to site-specifically and selectively modify therapeutic proteins to increase their properties for better clinical efficacy. Coupled to this are efforts to ensure PEGylation reagents are as pure as possible [12].

In the case of Cimzia the intended PEG conjugation site is distal from the binding site of the antibody fragment. There are several clinically used antibody-based TNF-α inhibitors including infliximab (Remicade), adalimumab (Humira), etanercept (Enbrel), and golimumab (Simponi). Certolizumab pegol has a similar safety profile to other TNF-α inhibitors and has a favorable low level of injection site pain. It differs from other TNF-α inhibitors as it lacks an Fc region, which reduces the Fc-mediated effects such as CDC or ADCC [72].

It is also possible to remove potential conjugation sites to ensure biological activity is at a clinically acceptable level. For example, the GH antagonist PEGvisomant (Somavert) was developed by recombinantly substituting nucleophilic amino acid residues to avoid conjugation [15,73]. Interestingly, in this product there are 4−5 PEG molecules (5 kDa) conjugated to each protein molecule. There are less PEG molecules conjugated than with the non-human enzymes, but the total molar amount of PEG (∼20 kDa) on a protein of approximately 22 kDa results in a conjugate with sufficient half-life (70 hours compared to 20 hours) to be clinically beneficial. The dramatic increase in half-life may be due to the PEGylated GH binding to circulating GH receptors.

Often it is thought that a limitation of protein PEGylation is the loss of activity due to PEG steric shielding. Most half-life-extending strategies are characterized by reductions of the biological activity of the therapeutic protein. PEGylated proteins tend to have a slower association rate with their binding partner, but once bound they have essentially the same rate of dissociation as the unmodified protein [74,75]. This means modifying the conjugation site to minimize any decrease in association rate can be achieved to tailor activity. Protein function and structure do not tend to change upon PEGylation [12]. PEG−protein conjugates essentially have a reduced association rate with their target. Hence, once the PEGylated protein is bound to its target, its properties are essentially the same as the unmodified protein [76]. Reduced association due to PEG shielding means that in vitro evaluation of PEGylated proteins, and macromolecular drugs generally [77], does not necessarily correlate with in vivo efficacy. While a log reduction in protein in vitro activity after PEGylation can be observed, most therapeutic proteins are very potent and remain sufficiently potent after PEGylation to achieve clinical benefit.

Macromolecular drugs designed to remain in the blood compartment exert their desired clinical effect primarily by being present over an extended time period with a favorable dose−response profile. In the case of cytokines (e.g., interferon) these pleiotropic immunomodulators participate in multiple functions in the body and are often found naturally at very low concentration, so a reduction of activity may be important for clinical efficacy. In the case of an antibody fragment or a non-endogenous protein [78] that binds to a single epitope to block ligand interactions, there can be a need to retain as much affinity as possible to minimize the required dose while ensuring extended duration of action is possible. Site-specific conjugation far from the site of binding is an advantage in these cases.

Although the regulatory agencies do approve heterogenous medicines that (1) may be a mixture of structures or (2) are within a complex formulation, site-specific and site-selective conjugation strategies remain sought after to accelerate the development process. Homogeneous medicines are ultimately more desirable than heterogeneous medicines.

PEGylation of nonprotein actives has also been clinically proven. Pegaptanib (Macugen) is a PEGylated RNA aptamer that binds to VEGF, which was approved in 2004 to treat wet age-related macular degeneration (AMD) [79]. The approval of pegaptanib was a milestone in drug development as it was the first aptamer to be successfully developed as a therapeutic agent for humans and was PEGylated with a branched PEG reagent (2×20 kDa) for intravitreal injection. Pegaptanib was also the first anti-angiogenic therapy indicated for the treatment of neovascular AMD [79].

Peginesatide (Omontys) is a PEGylated peptide developed as an alternative to EPO to avoid pure red cell aplasia, which is an immune reaction against EPO. Unfortunately, after registration it was found that there were cardiovascular toxicities associated with this product, resulting in its withdrawal. The withdrawal was due to the activity of the peptide and not to any toxicity associated with PEG.

Other approved non-protein PEG conjugates include a low-molecular-weight PEG (seven repeat units) conjugated to naloxol which is a low-molecular-weight

active to give Naloxegol that is administered orally to treat non-cancer opioid-induced constipation. A PEGylated liposomal formulation of encapsulated doxorubicin (Doxil) was approved in 1995 to treat various cancers including acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma, leukemia, and ovarian, breast, bone, lung, and brain cancers [80].

2.4 The simplicity of protein PEGylation

PEGylation reagent use, both established and recently developed, undergoes reaction with many different amino acid residues on proteins [3,4,6] including non-native amino acid side-chains (e.g., aldehyde, alkyne) [9–11]. Reagents designed to undergo reductive amination with the amine of the *N*-terminal amino acid (e.g., PEG–aldehyde), acylation of the lysine side-chain amine (e.g., PEG–*N*-hydroxysuccinimide (PEG–NHS)) and Michael addition of a free cysteine thiol (PEG–maleimide) have been used in most of the registered clinical products to date. PEGylation reagents with a single PEG chain (so-called linear PEG reagents) are generally in the range of 20–30 kDa because the synthesis of narrow molecular weight distribution as a PEG precursor is more feasible than at higher molecular weights. The development of "branched" PEGylation reagents [3,4,8] comprised of two PEG chains of 10 or 20 kDa often linked to a lysine which is then conjugated to the protein amine (via the active lysine ester) is used in several mono-PEGylated clinical products. Branched PEGylation reagents (often denoted PEG_2) allow greater PEG molar mass and steric shielding to be accomplished by using lower-molecular-weight PEG precursors while using a single conjugation site on the protein [5,81].

Carefully and highly defined reaction conditions with different PEG–aldehyde variants used in stoichiometric excess can sometimes be found to undergo reaction predominantly with the *N*-terminal amine on a protein to form a labile imine which must then be reduced (e.g., $NaBH_4$, $NaCNBH_3$, or $Na(AcO)_3BH$). Known as reductive amination, PEG conjugation is described as site-specific for the *N*-terminal amine, but mixtures are unavoidable. Nonetheless the PEG–GCSF and PEG–IFN-β1a products are manufactured by reductive amination conjugation processes. Other strategies designed to be more efficient and selective for the *N*-terminus (or C-terminus) of proteins have been described [29,82].

Amine reactive PEG reagents (most often PEG–NHS) tend to be used in considerable stoichiometric excess to protein. Since there are often several available nucleophilic groups on the protein (e.g., terminal amine; side groups of lysine, serine), non-selective PEG conjugation results in PEG–protein positional isomers and multiple PEGylated products, which can make purification challenging [83–85]. Reaction with the histidine imidazole side chain results in the formation of a hydrolytically labile carbonyl imidazolide, and this along with competitive hydrolysis consumes reagents such as PEG–NHS. Amine-specific reagents have been used for many products that are currently used in the clinic and are widely commercially available.

Much current research effort to address cost and regulatory issues is focused on ensuring that protein modification generally, and PEGylation specifically, is more robust, efficient and site-specific. While many PEGylation reagents undergo conjugation non-specifically at different residues resulting in PEG−protein positional isomers, each with a different biological activity, increasing number of site-specific approaches have been described [7,9,11]. In particular, the selective alkylation of an unpaired cysteine thiol is possible with a wide range of reagents, often at physiological pH.

PEG−maleimides are widely used for cysteine thiol alkylations. These reagents undergo a Michael reaction at the maleimide α,β-unsaturated carbonyl. The Michael reaction of a thiol can be rapid in mild conditions that are necessary to maintain the protein tertiary structure, while protein amine nucleophiles tend to be protonated and not reactive (e.g., pH 6−7) [86]. Thiol-selective conjugation can be accomplished with little stoichiometric excess of the PEG reagent being required, which helps to avoid tedious and expensive purification processes. During clinical development and manufacture, the cost of the PEG reagent can be more than the cost of the unmodified protein. Conjugation efficiency is important and can be achieved using thiol-selective reagents.

PEG−maleimide protein conjugates can be susceptible to deconjugation via a retro-Michael reaction and can undergo thiol exchange reactions [87−90] (e.g., with the free cysteine on albumin). PEG−maleimide protein conjugates can be stabilized by hydrolysis of the maleimide ring after conjugation [91,92]. Alternative thiol-specific reagents have also been described [93,94] including α,β-unsaturated carbonyl PEG reagents that can be locked using the same reducing agents used in reductive amination to stop the retro-Michael reaction [86].

The vast majority of therapeutic proteins exhibit their activity within the blood compartment. The extracellular milieu is an oxidizing environment, so cysteines will be paired to form disulfide bonds. The vast majority of extracellular proteins, especially those that are therapeutically relevant, have an even number of cysteines. There have been many proteins engineered to have a free cysteine for conjugation. The presence of free accessible cysteine in a protein for conjugation will tend to cause disulfide scrambling and protein misfolding, which can cause the protein to aggregate. A free cysteine will also cause intermolecular disulfide formation, which also causes protein aggregation. Protein aggregation results in a loss of biological activity and can lead to immunogenicity (e.g., generation of secondary antibodies that clear the therapeutic protein). A recombinant-conjugation strategy to add a free cysteine thiol is viable for proteins without disulfides [95].

To exploit the selectivity of cysteine thiol conjugation, without the need to have a free unpaired cysteine available, PEGylation reagents have been developed to undergo reaction with *both* cysteine thiols in an accessible disulfide bond. Since many therapeutic proteins have an accessible disulfide that can be easily reduced while maintaining protein structure, *bis*-alkylation reagents (known as PEG−*bis*-sulfones) have been found that undergo disulfide rebridging conjugation [96−99]. Disulfide bridging conjugation occurs by an addition−elimination reaction mechanism where two latent α,β-unsaturated double bonds to the same carbonyl are both

revealed during the conjugation [98]. Antibody fragments [75], enyzmes, cytokines, blood factors, and non-endogenous scaffolds have all been site-specifically PEGylated by disulfide bridging conjugation.

Disulfide bridging PEGylation with PEG−*bis*-sulfones can be accomplished during protein refolding [100] or after mild partial reduction of a disulfide in the purified protein [98]. Disulfide reduction can also be readily accomplished in situ [96] without the need to remove the reducing agent prior to PEGylation. The carbonyl can be reduced to avoid retro-Michael reactions leading to deconjugation, but many protein conjugates appear to be stable and do not require the reduction step. *Bis*-alkylation can also be accomplished using a *bis*-alkylating maleimide linker [101] but, as with other maleimides, hydrolysis is required to prevent deconjugation and exchange reactions [102].

The *bis*-sulfone PEGylation reagents are also selective for the histidine tag (His-Tag) in proteins [29], which can be placed at either terminus of the protein. His-Tag PEGylation can be accomplished with greater efficiency than is possible with reductive amination [29]. The difference in reactivity of cysteine thiols and histidine imidazoles for the PEG−*bis*-sulfone reagents *5* means it is possible to conduct a disulfide bridging conjugation in the presence of a His-tag. This can then be followed by subsequent site-specific modification at the His-tag using the same conjugation moiety.

Although yet to yield any clinical products, a number of other highly imaginative approaches have been described to achieve site-specific PEGylation. Strategies often require a combination of recombinant engineering and chemical conjugation [71]. For example, targeting of the protein C-terminus has been achieved by intein fusion and was recently applied to the PEGylation of both IFN-α2a and IFN-β1b [103,104]. Proteins are expressed as an intein fusion, which on cleavage with hydrazine generates a C-terminal hydrazide that can undergo reaction with a PEG-containing ketone or aldehyde reactive group.

The genetic insertion of unnatural amino acids with alkenyl, iodo, and keto moieties for reaction can enable the generation of selectively modified proteins [105−107]. Incorporation of non-native amino acids requires that cellular biochemical pathways probably need to be robustly modified [104,108,109] to ensure that the efficiency of protein production is not compromised by reversion to the wild-type phenotype. For this strategy to work, tRNAs with the desired codon, usually the amber stop codon, are aminoacylated with the amino acid of interest. The reengineered tRNA is then added into the expression system along with the DNA template. Yields of amino acid incorporation can also be poor because of their competition with termination codons. This approach is currently limited to the addition of a single type of non-natural amino acid because only the action of the single amber stop codon can be suppressed, but incorporation of non-natural amino acids can be inefficient [104].

In addition to glycoPEGylation (e.g., used Rebiny) enzyme-mediated conjugation strategies have also been described. Transglutaminases (TGase) have been utilized to conjugate PEG to glutamine residues. For example, TGase-mediated PEGylation of IL-2 gave a homogeneous product with only one of the six glutamine

residues present (Gln64) having underwent conjugation [110]. It is also possible that additional glycosyl groups can be added to a protein and further conjugated with a PEG chain. In one such approach a maleimide-linked galactosylglucono sugar was used to introduce a glycosyl group at the free cysteine of HSA. Peroidate oxidation of the glycosyl group generates aldehyde groups to allow the conjugation of PEG−hydrazide [111]. Showing all the benefits associated with glycoPEGylation this approach may be particularly useful for the PEGylation of those sites that cannot be targeted using glycosyltransferases. Another approach involves sortase A (SrtA), which is a thiol-containing transpeptidase. SrtA is able to catalyze the formation of a new amide bond between an inserted LPXTG motif within a protein sequence and a triglycine-derived PEG. Using this technique PEGs can be site-specifically conjugated at either terminus of the protein termini. A C-terminal PEGylated version of G-CSF and interferon-α2 have been prepared using the SrtA method [112].

2.5 Concerns about PEGylation

In spite of the widespread clinical use of PEGylated proteins, debate remains about the clinical effects of PEG accumulation and immunotoxicity [113−115]. PEG has long been extensively used in cosmetic, food and pharmaceutical preparations and is considered to be essentially non-toxic. Most PEG conjugates are cleared by the kidney and liver, and sometimes proteolysis of the protein occurs with concomitant renal clearance of the PEG [116]. Some accumulation of PEGylated drugs in Kuppfer cells of the liver has been observed when using very high concentrations of PEG leading to renal tubular vacuolization [117]. Vacuoles often disappear upon cessation of treatment, but some animal studies for five products do report that conjugates with high PEG molecular weight do form vacuoles [12]. Although no PEG-related effects due to vacuolization in humans have been observed [28,115,118], strategies to minimize vacuolization have been described [119].

Animals used for accumulation studies are often given much higher bolus and cumulative doses than are given to humans [22,117,120]. Clinical doses of PEG−proteins are much lower than required for PEG toxicity seen in animals [22]. Human PEG doses are generally less than 1 mg/kg, except for Cimzia which is a 200 mg dose, and no PEG-related toxicity has been reported. Reversible accumulation of Cimzia appeared after 26 weeks (100 mg/kg) and 52 weeks (50 and 100 mg/kg) in *Cynomolgus* monkeys [121]. Accumulation effects are more apparent with increasing molecular weight PEGs (>30 kDa) [120], so branched reagents derived from 20 kDa PEG precursors can be used to achieve the necessary molar masses of PEG needed for mono-PEGylated proteins with extended circulation times. High doses of any water-soluble polymer [122] of sufficiently high molecular weight and that is essentially nonhydrolytically degradable will be expected to accumulate in mammals (e.g., PVP).

Since PEG is widely used in consumer products, there is concern that many people have developed secondary antibodies to PEG [113]. It is thought one epitope in

PEG−protein conjugates is the terminal methyl group on PEG [123], but other epitopes have been identified (e.g., some linking moieties to the protein). PEGylation normally reduces the immunogenicity of proteins [114]. Conjugation of many PEG molecules (hyper-PEGylation) to large non-human proteins and liposomes shows a higher propensity for secondary antibody generation and accelerated blood clearance (ABC effect) [124,125], although it may be possible to attenuate these effects [126].

Often the hyper-PEGylated products (e.g., Adagen, Oncaspar, and, Krystexxa) are described as being immunogenic, but they would be of little clinical usefulness unless they had been PEGylated. Hyper-PEGylation is needed to mask the protein immunogenicity to the extent possible for clinical use. Use of a hyper-PEGylated nonhuman protein to treat chronic conditions therefore may be challenging. Anti-PEG antibodies are observed in the majority of patients treated with Krystexxa [28], which is hyper-PEGylated. This problem appears to be compounded by the presence of pre-existing PEG antibodies in up to 25% if the population [114]. Since PEG is a highly flexible molecule, the affinity of some secondary antibodies may be low, with little clinical consequence for rapid clearance, especially for mono-PEGylated proteins. Products hyperconjugated with many PEG molecules (liposomes, biomaterials, non-human enzymes) appear most likely to be questioned in terms of anti-PEG antibodies and rapid clearance, so it has become important to develop standardized assays for anti-PEG antibodies [113,127−129].

The development of anti-PEG antibodies to mono-PEGylated proteins is not as prevalent as with hyper-PEGylated proteins, and there does not appear to be any impairment to therapy caused by anti-PEG antibodies to mono-PEGylated proteins. As with any class of protein, including Fc-fusion and hyperglycosylated proteins [28], immunogenicity for PEGylated products requires monitoring on a drug-by-drug basis. As the presence of neutralizing antibodies can have a significant impact on conjugate safety and effectiveness, immunogenicity testing, which is necessary for all protein therapeutics, must be a key part of the PEG−protein development process as it is for the development of any therapeutic protein [130−133]. Although some of the commentary about anti-PEG antibodies can be quite strident [57], there is a long clinical history for the beneficial use of PEGylated proteins which cannot be ignored.

Most therapeutic proteins [134] display immunogenic effects in a proportion of patients. It appears that the vast majority of the clinically observed toxicological effects of PEGylated proteins is due to the protein [12,17,18,135]. If the unmodified protein is safe, then the PEGylated variant is expected to be safe [22], especially if mono-PEGylated. The only toxicities observed clinically with PEG−protein conjugates are those associated with the protein [22]. No albumin−protein fusion products or the alternative natural and synthetic polymers being examined have been registered for clinical use in protein conjugation. Like PEG, these polymers will need to be thoroughly evaluated from a regulatory perspective, especially in postmarketing studies.

Concerns relating to the loss of biological activity due to steric shielding on PEG conjugation have also been raised. Often the reduction in binding affinity is

offset by the improved circulation half-life to give an enhanced pharmacological effect. However, when the protein's starting potency is low PEG conjugation can lead to the generation of a therapeutic that is biologically ineffective. Site-selective strategies that allow more control over the site of PEGylation are increasingly being used to improve the retained activity.

2.6 Conclusions

Protein PEGylation continues to be a clinically proven approach that enables protein therapeutics to be better medicines. PEGylation is essentially a recombinant-chemical approach where first the protein is made and then modified by a conjugation reaction using a PEGylation reagent. Recombinant engineering will be used to help direct PEG conjugation to make more homogeneous products for the continued modification of proteins with PEG and other molecules with higher efficacy.

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