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REVIEW ARTICLE

Product development issues for PEGylated proteins

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Abstract
Covalent attachment of poly(ethylene) glycol (PEG) groups to proteins, a process commonly called PEGylation, is often used to improve the performance of a protein in vivo. To date, at least eight such PEGylated peptide and protein conjugates have been approved as therapeutic agents and many more have undergone clinical trials. This review examines PEGylation from the perspective of developing a commercially viable drug product. The first section focuses on obtaining a pure and well-characterized drug substance. The latter section discusses formulation and manufacturing issues, with an emphasis on analytical methodology that provides the most detailed description of the purity and stability of PEGylated proteins.

Keywords: Protein stability, pegylation, development, chemical modification

Introduction

It is well known that post-translational modifications (PTMs) can alter the structure, stability and potency of bioactive proteins.[1,2] This is most clearly demonstrated with glycosylated proteins, many of which are now found as marketed therapeutics.[2] In addition to the PTMs that occur in vivo, proteins can be intentionally modified in vitro. The most widely employed technology is the addition of poly(ethylene glycol) (PEG) groups in a process that has come to be known as PEGylation. The general idea for PEGylating a protein is to attach a PEG covalently to the surface of a protein to improve the chemical/physical properties of the protein and to enhance the bioactivity of the conjugated protein.[3] Many reviews have appeared on PEGylated proteins, covering various aspects of their biological[4–8] and chemical behavior.[3,6,7,9–22] In this review, we seek to focus on the physicochemical properties that must be considered when developing these compounds as commercial drug therapies. For approved drug products, this effort begins with the need to control the quality of the active pharmaceutical ingredient (API) or drug substance (DS). Not only must the protein be well characterized and pure, but the PEG material must be of high quality as well. Then they must be coupled using well-controlled chemistry. Over the years, the quality of the starting materials has improved and PEGylation chemistry has become quite elaborate. Still, issues remain with specificity of PEGylation and scale-up, to name some specific concerns.

With a well-defined API in hand, development of the drug product (DP) can begin. Addition of pendant PEG groups will alter the structure and solubility of the protein, possibly causing challenges for the formulation scientist. In addition, the new entity may require a different purification approach. In parallel with these efforts, analytical methods will need to be developed to characterize the PEGylated protein conjugate as well as the initial PEG and protein starting materials. Each of these important issues is discussed below. Despite these significant challenges, a number of PEGylated proteins have been approved by regulatory authorities (see Table 1). Many more proteins of therapeutic interest have been PEGylated and evaluated, including molecules, as complex as hemoglobin and Factor VIII (Table 2).

PEGylated proteins as marketed products

There are a number of approved protein products that have used PEGylation technology to improve product

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performance, especially in terms of extending the protein half-life in vivo. These can be found in Table 1. While PEGylated proteins have been on the market for two decades now, the technology used to make these materials continues to evolve. At the same time, analytical methods have improved dramatically as well, meaning that these compounds can be characterized to a much greater degree than was previously possible. All of these technical advances are related to issues surrounding development of chemically modified proteins in general, and specifically, to PEGylated proteins as is the subject of this review article.

It should be noted that many other peptides and proteins of pharmaceutical interest have been PEGylated and evaluated as possible therapeutic agents. A list of those conjugates that have been reported in the literature can be found in Table 2. Also note that other pharmaceutically important species have been PEGylated (small molecules, DNA/RNA, liposomes, etc.), but these are outside the scope of this review.

Table 1. Approved PEGylated peptide and protein therapeutics.

Brand name	Company	Year approved
Adagen	Enzon	1990
Oncaspar	Enzon	1994
PEG-Intron	Schering-Plough	2000
Somavert	Pfizer	2002
Pegasys	Roche	2002
Neulasta	Amgen	2004
Mircera	Roche	2007
Cimzia	UCB	2008

Table 2. PEGylated peptides and proteins of pharmaceutical interest reported in the literature

Protein	Reference
Albumin	115, 216
Antibody fragments	6, 110, 190, 217, 218
Brain-derived neurotrophic factor	199
Calcitonin	219-221
EGF	117
Endostatin	172, 222
Factor VIII	223, 224
Glucagon	225
G-CSF	70, 99, 108, 116, 118, 128, 166, 171, 206
GM-CSF	186
GRF analogs	120, 226-229
Hemoglobin	74, 75, 142, 189, 203, 216, 230–234
Hirudin	177, 235
Human growth hormone (hGH)	132, 173
IL-1 receptor antagonist	210
Insulin	83, 147
Interferon-α_{2b}	31, 103, 163, 198, 236
Interferon-α_{2a}	62, 129, 191, 237, 238
Interferon-β_{1a}	239
Interleukin-2	14, 96
Interleukin-6	240
KGF-2	241
Octeotride	169, 170, 205
PTH	185
Staphylokinase	161, 242
tPA	145
Tumor Necrosis Factor (TNF)	243
TNF receptor type I	151, 152
Uricase	69, 244, 245

Production of a PEGylated protein as an API

The qualities of the PEG used for conjugation will determine the quality of the conjugate. The presence of impurities, the molecular weight (MW) distribution of the polymer, and choice of reactive group chemistry will affect the structure and quality of the final PEGylated protein. In this section, the stability and purity of PEGs themselves are described. An overview of the reagents used in the PEGylation process is provided, focusing on reaction chemistry, specificity, and the types of PEGs that can be attached, either randomly or specifically.

Purity of PEGs

Before considering PEG reagents, those activated PEGs that are capable of coupling to a protein, one should examine the stability and purity of PEG itself. One can obtain PEG in a wide range of average molecular weights, ranging from 300 to many thousands. The lower molecular weight (MW) PEGs (MW < 1000) are liquids, while the higher molecular weights (MW > 2000) exist as waxy solids with relatively low melting points (< 70°C).[16]

PEGs can often carry impurities, such as peroxides or aldehydes.[23–26] When PEG is used as an excipient or as a salting-out reagent, these impurities can have adverse effects on the API. The presence of formaldehyde in PEG 400 and PEG 300 was found to contribute to API degradation in a formulations for both O^6-benzylguanine and BMS-204352.[27,28] Chemical degradation of PEG 6000 was found to produce aldehydes, which affected the precipitation of macroprolactin for the diagnosis of hyperprolactinemia.[29] Hydrogen peroxide has been detected in various excipients, including PEG 400, by Huang et al.[30] Interestingly, there was wide variation in peroxide impurities in PEG 400 between manufacturers, and even batches from the same manufacturer, by up to an order of magnitude.[26] The use of PEG as an excipient for protein formulation has been found to cause instability, presumably due to impurities within the PEG employed. For example, addition of PEG to formulations of interferon-α_{2b} led to reduced stability.[31] Although no detailed mechanistic explanation was provided, it is likely that this was due to oxidation caused by impurities in the PEG, as this cytokine is well known to have reduced potency upon oxidation.[32] Similarly, the extent of damage for a small molecule was found to correlate with the level

of oxidative impurity in PEG as well.[33] For all of these reasons, some manufacturers recommend storage of PEG in the dark, under inert atmospheres, or at low temperature (below –10°C).[34] Some PEGs will contain antioxidants, which could be carried along through the conjugation process. Note that these oxidative instabilities in the PEG groups will still exist even after conjugation is complete.

There are reported instances of enzymatic degradation of pendant PEG groups,[34] primarily by alcohol and aldehydes dehydrogenases[35,36] and cytochrome P450 oxidases.[37,38] There are also several reports on degradation of PEG (as well as polysorbates) in the presence of light, oxygen, heat, and transition metals, often resulting in the production of oxidizing radicals.[39–42] Despite the well established issues with oxidative impurities in PEGs, it is not known if this affects PEG reagents used in PEGylation reactions nor has there yet been an investigation into the effects of PEG contaminants on PEGylated proteins. However, given these issues, PEG purity must be controlled for PEGylated proteins, especially for those for which oxidative damage is of concern.

PEG Reagents: Introduction

Most reagents used in PEGylation allow for attachment of PEG to one type of reactive site on the protein of interest. The specific types of linker groups, available chemistries, etc. are described in detail below. Typically, PEG reagents are produced by ring opening polymerization of ethylene oxide. The difficulty lies in removing trace amounts of protic solvent that could terminate the polymerization and that a number of transformations may be necessary once the polymer of the desired MW is obtained. Nevertheless, high quality mono-functionalized PEGs are commercially available. A number of commercial suppliers of PEG reagent exist, including NOF Corporation (Japan), SunBio (South Korea), JenKem (China), Chirotech (UK) and Creative PEGWorks (USA).[9]

Over time, PEGylation chemistry has become increasingly more elaborate. For example, whereas the earliest PEGylation processes has primarily employed linear PEG reagents, branched PEGs of various types are now being used, as described below.[43] The process of covalently attaching a PEG group to a protein is dependent on the physicochemical properties of the protein, the coupling chemistry and the properties of the PEG.[3,44] Typically, the PEG groups attached to proteins are less than 50 kD in size to ensure elimination by glomerular filtration.[45]

A number of good reviews have covered the coupling chemistry used to attach a PEG covalently to a protein.[13,46–48] It is important to understand that the synthesis of these protein-PEG conjugates can have a significant impact on the developability of a PEGylated protein product. In particular, it is important to understand issues related to specificity of the coupling, the stability of the linkage, and the regiospecificity of the conjugation reaction. All of these will affect the purity and quality of the final API material.

PEG reagents: Reactive groups

Conjugation chemistry for PEGylation of proteins has been well documented in several review articles.[7,10,11,47–49] While many different groups on the surfaces of proteins have been targeted for conjugation, most conjugation schemes utilize either a protein amine (lysine side chain, N-terminus) or the free sulfhydryl group of a cysteine (Cys) residue (Table 3).

Lysines (and other amines, such as the N-terminus) have been the preferred target for PEGylation over the years. The types of chemistries that have been described are certainly diverse. The succinimidyl esters and carbonates have been used for decades and are still widely used to modify amine groups (Figure 1). However, over the years, a number of PEG reagents have been derivatized for amine reactivity employing a variety of functional groups,[4,10,11,16,47,48] including:

- succinimidyl succinate;[21,50]
- succinimidyl esters;[47,51]
- trichlorotriazine;[52,53]
- epoxide;[54]
- 2-mercapto-thiazoline;[55]
- aldehyde;[56,57]
- tresylate;[58]
- succinimidyl carbonates/carbamates;[59]
- p-nitrophenyl carbonates/carbamates;[60]
- N-hydroxysuccinimide (NHS);[52,58,61–64]
- hydrazide;[65,66]
- isocyanate;[67]
- benzotriazole.[68]

Several of these schemes have been abandoned due to toxicity, lack of specificity, or low chemical stability.[11,13] Activated esters, such as NHS, are popular for amine

Table 3. Summary of reaction chemistries available for PEGylation of certain functional groups on proteins.[10,11,16,47,48]

Protein functional group	PEG reactive end
Amines (lysine, N-terminus)	N-hydroxysuccinimide (NHS) ester
	NHS carbonate
	aldehyde
	epoxide
	tresilate
	isocyanate
	hydrazide
	2-mercapto-thiazoline
	benzotriazole
Thiols (Cys)	maleimide
	pyridyl disulfide
	vinyl sulfone
	orthopyridyl
	iodoacetamide
Disulfides	$RO_2SCH_2C(CH_2)C(O)(C_6H_4)$

PEGylation (Figure 2), as is PEG-aldehyde, which is used in the production of pegfilgrastim (Neulasta®).[34,47] The type of PEG linkage used can impact the activity of the protein, even if the site of attachment and the size of the PEG group does not vary.[69]

A variety of thiol-reactive PEGs have also been examined, including PEG derivatives of maleimide, iodoacetamide, vinylsulfone, and pyridyldisulfide.[13,70–75] As free thiols are relatively uncommon, there is less use of these approaches compared to amine coupling, although this does allow for increased specificity, especially for generating mono-PEGylated proteins. Alternatively, a Cys could be introduced via site-directed mutagenesis.

PEGs have also been activated with crosslinking reagents that will release the PEG from the protein surface in vivo by hydrolysis or exposure to reducing agent.[76,77] These activated PEGs include PEG-maleic-anhydride, PEG-succinimidyl succinate, and PEG-DTSP.[72,78–83] This topic has been recently reviewed by Fipula and Zhao.[84] Labile linkers can be incorporated onto PEGs for use in cases when the PEG needs to detach from a protein once it has been delivered to a specific location in vivo.[44] An example is use of labile linkage between the protein and PEG that can be cleaved in slightly acidic environments, such as those found in tumors.[44] One can consider the use of a labile bond between the protein and PEG as a prodrug approach, with subsequent release of the unconjugated protein.[70] In the case of insulin, PEGylation with a reversible linker improves the pharmacokinetics, while using traditional PEGylation inactivates the polypeptide.[83]

More recently, PEG reagents have also been developed that allow one to couple to existing disulfide linkages. PolyTherics offers disulfide-specific using a platform they called TheraPEG, which uses a monosulfone reagent.[9] These reagents, developed by Brocchini and co-workers, use a bifunctional reagent after the disulfide has been reduced.[85–87] Bis-alkylation results in a three-carbon bridge to which the PEG is attached (Table 3).

One general approach to increasing the specificity of PEGylation is to introduce new groups into the protein (via mutagenesis)[88,89] or make use of modifications that have already been made, either in vivo or in vitro. For example, His tags are often added to a protein sequence. These short poly(His) sequences are well known for their ability to allow purification by affinity chromatography, have been viewed as possible sites for PEGylation.[9] This technique has been reported in a patent application from PolyTherics (WO 2009/047500 A1) and in an issued patent assigned to Enzon.[90] However, PEG linkages to His residues can be fairly unstable.[90]

Moreover, one could also envision introducing non-natural amino acids to allow site-specific attachment of a PEG group.[9,91] Derivatives of Ser have been prepared, allowing the use of ethynyl- and azido-PEG reagents.[92] Derivatives of Lys and Phe (*p*-amino-Phe) have been used adding terminal amino groups to allow PEG attachment to occur.[91] Companies, like Ambrx, have advanced some molecules, like a long-acting human growth hormone (hGH) using this approach.[9] Reports of PEGylation through Tyr side chains have appeared.[93,94] Another variation of this theme involves the use of PEG reagents linking through oxidized glycosides.[95] Cysteine residues were glycated, followed by PEG coupling through the remaining aldehydes group. These linkages will hydrolyze, especially under mildly acidic or basic conditions.

As well as using conventional coupling chemistry, PEGs can be functionalized via enzymatic conjugation. This has been demonstrated using an amine-terminated PEG which was conjugated to glutamine residues using transglutaminase.[96–99] This can be done with some specificity, even when the number of Gln residues is relatively large.[99] This reaction scheme promises a high degree of specificity, but the target Gln residue must reside in a flexible or unfolded region of the protein.[98] Finally, Neose has developed another enzymatic approach that allows PEGylation to occur at O-glycosylation sites.[100] There must be only a

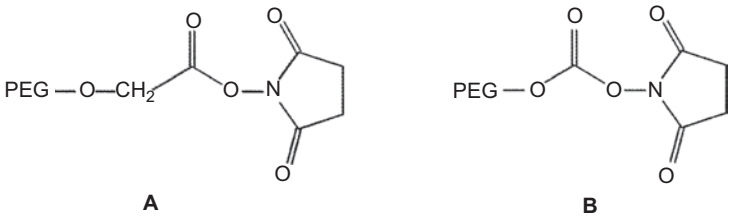

Figure 1. Succinimidyl esters (A) and carbonates of PEG.

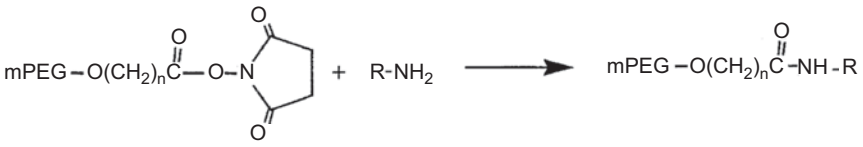

Figure 2. Coupling of NHS-PEG reagents with amine groups.

single glycosylation site that can act as an acceptor for GalNAc. The PEG is transferred using an enzyme and a sialic acid-PEG reagent. The technique has been demonstrated with G-CSF.

Linear PEG reagents

The earliest, and possibly still most common PEGylation platform, is to use a mono-functional linear PEG reagent. Three interrelated issues should be considered. First, the ability of the reaction chemistry to target a specific site is crucial. Clearly, this is more easily done with thiol-specific reagent than with reagents that couple via amine groups. Second, the issue of stoichiometry must be considered. The earliest products involved random PEGylation, where a number of PEG groups were attached, but with some control over the number and coupling efficiency from batch to batch. Third, the regiospecificity of these reagents is related to each of the first two issues. Each of these aspects of PEGylation chemistry is discussed below.

Random PEGylation

When PEGylation chemistry was in its infancy in the 1970s and 1980s, the ability to attach PEG in a site-specific manner was nearly non-existent. As a result, PEGylation was random, with more than one site being modified. In the early 90s, two PEGylated products Oncaspar® and Adagen® were both approved by the FDA as mixtures of PEGylated species.[12,101,102] Both of these products are mono-PEGylated, but the PEG groups are attached at several different sites within each protein. In other words, they exist as a mixture of positional isomers. Interestingly, Somavert, which was approved in 2002, is also prepared by random PEGylation, with the peptide containing four to six PEG groups per molecule.

Random PEGylation can still lead to a viable commercial product. However, for more recent approved products, the FDA has required characterization of each positional isomer, if possible.[11,12,103] Having a PEG-protein conjugate with different positional isomers, can cause problems with production, purification and increased work for characterization of the positional isomers (see below). Still, in 2007, the FDA approved Mircera®, which is a mixture of mono-PEGylated conjugates of erythropoietin, mostly at two different Lys residues as well as the N-terminus. On the other hand, variation in quality led to the recall of some PEG-asparaginase batches between 2000 and 2003.[34]

The key for developability of a randomly PEGylated protein is reproducibility and process control. If each batch yields a comparable mixture and the pharmacokinetic and potency properties are consistent, then the regulatory agencies will view the submission favorably. Clearly, the analytical demands and the ability to control PEGylation chemistry have improved markedly. Still, it does not obviate the development of a product containing positional isomers.

Stoichiometry

The amount of PEG linked to a protein has been shown to affect a number of properties of a conjugated protein.[14,70,104,105] The number of active PEGs that are attached to a protein and location on the protein will impact the performance of the protein-PEG conjugate.[47] It has been show in the past that the degree of modification can affect the activity of enzymes and cytokines.[21,106,107] This can be alleviated by site-specific PEGylation[73,96,108] or better control of the PEGylation process.[109,110]

A common method of improving reaction yield and control on the number of attached PEGs to a protein is the ratio of PEG vs. protein at the start of the PEGylation reaction.[110] The amount of excess PEG needed relative to the protein is dependent on coupling chemistry, the desired amount of PEG linked to protein, type of functionalized PEG and the protein.[44,110] For anti-interleukin-8 monoclonal antibody (MAb), the authors determined a ratio of 6:1 PEG to protein resulted in optimal yield and correct number of PEGs linked to the protein.[110] The need for excess PEG required for the coupling chemistry could be due to the functionalized PEG being deactivated at reaction conditions. An example is PEG-succinimidyl-propionic acid (PEG-SPA), which is easily hydrolyzed in water, and requires ratio of 50:1 PEG to protein to optimize the coupling reaction.[44]

There are a number of ways of to control the location and number of PEGs that are attached to a protein during the coupling reaction that will be discussed below. Lysine residues are a common target for PEGylation because of the high abundance of lysine on the surface of a protein (around 10%). and its absence near the active site of a protein.[47] Additionally, the reaction conditions needed to couple an activated PEG to lysine are chemically mild. When targeting lysine side chain amines for PEGylation, PEG can attach to a number of different sites resulting a large number of different positional isomers, which can jeopardize the activity of the PEG-protein conjugate.[47,111,112] Positional isomers cause a number of problems during purification process, and may result in the need to characterize each of the different positional isomers.[44,113]

The excess of the PEG over protein is an important part of optimizing the yield of a reaction, more importantly it can be used to control the number of the PEGs that are attached to a protein. The number of PEGs that attach to a protein can affect the bioactivity and alter physical properties.[44,114,115] The effects of increasing the number of PEGs attached to hGH versus the change in the bioactivity and clearance time in vitro has been studied.[114] Results of the study found that increasing the number of PEGs caused a decrease in bioactivity but increased circulating half-life, which is commonly observed.[114] The authors found that optimal performance for PEGylated hGH required the attachment of five PEGs. Others have examined the effects of increasing the weight of the

coupled PEG on the circulating half-life, bioactivity and avidity of the PEGylated protein.[116–120]

Regiospecificity

The location where the PEG attaches to the protein surface has a large effect on the bioactivity of the PEG-protein conjugate.[113] Controlling the location on the surface of the protein which the PEG attaches can be as simple as adjusting the pH for amino coupling, or as complicated by adding a non-natural functional group by mutagenesis. The second generation of coupling chemistry is the targeting of specific sites on a protein, referred to as site-directed PEGylation.[81] Site-directed PEGylation has a number of advantages over non-specific PEGylation, most importantly the elimination of positional isomers.[121] A common approach to site-directed pegylation is attachment of the PEG to N-terminal of a protein.[121–123] The most common method of controlling the location PEG attaches to the protein is adjusting the pH to target the α-amino group of the N-terminus rather than the ϵ-amino side chain of a Lys residue.[121] Decreasing the pH to 6 or so will ensure that the Lys side chains are completely protonated, while the N-terminal amino group is still deprotonated to some degree. Site-directed PEGylation on the N-terminus has been used for the approved PEGylated protein product, pegfilgrastim. The coupling is followed by treatment with sodium cyanoborohydride to reduce the Schiff base to a more stable amine linkage.[121]

The primary alternative to achieving site-specificity with amine coupling is to PEGylate using less common functional groups. Those that have been used to couple PEG to a protein with reasonable regiospecificity are thiol (cysteine) or imidazole (histidine).[13,121,124] Additionally, for glycoproteins a PEG can be attached to polysaccharides that can be oxidized under mild conditions.[125,126] Of these alternative functional groups cysteine is the mostly commonly used.

Cysteine is not commonly located on the surface of a protein in a free thiol state and when targeted for covalently attaching a PEG to protein offers a high degree of selectivity.[13,124] Cysteine can be added or subtracted to the surface of a protein by the process of mutagenesis.[13,101,124] There are number of common PEG reagents that target coupling of PEG to thiol, a common one used is PEG-maleimide, which with results in a stable covalent linkage.[12] The conditions used to covalently attach the reactive PEG to a protein is optimal around pH 5–8, but in the presence of organic solvents or pH greater than 8 could result in other nucleophilic reactions.[12,13,73] For antibodies that have been conjugated with using non-site specific coupling can result in decrease in bind up to 62%.[73] To improve the binding and extend the half-life of a Fab′ fragment, Chapman was able to couple a PEG via a cysteine at the Fab′ hinge region, resulting in a small loss of binding.[73] This strategy for attaching a PEG to a Fab′ was used for certolizumab pegol, which was approved in 2008 for treatment of the symptoms of Crohn's disease.[13]

Branched PEG reagents

Branched PEGs contain two or more PEG groups anchored to a single core molecule. These include Y-shaped PEGs, where the core may be lysine, with the PEGs connected to the two amine moieties.[82,127–129] Likewise, the original chlorotriazine structure was modified to attach two PEG groups.[130] This was then used to prepare a PEGylated version of asparaginase. These branched PEGs have the advantage of covering more of the protein surface for a single point of attachment, thereby providing increased protection against proteolysis and lesser likelihood of immunogenicity.[16,131] On the other hand, they can reduce the potency of the protein significantly, if the PEG group is large enough to hinder receptor binding. In the case of human growth hormone (hGH), attachment of a branched 40 kD PEG reduced the potency by over 20-fold.[132]

Bifunctional PEGs, with reactive groups at either end have been explored,[133] but mostly result in cross-linking of the target proteins. In fact, this approach has been used to prepared cross-linked albumin gels (ProGel). However, the use of bifunctional PEGs in dilute solution could provide a PEGylated conjugate with active proteins at either end. More commonly used are highly branched PEGs. As with the Y-shaped PEG reagents, branched PEGs allow for a single point of attachment to the protein, but provided a much larger umbrella of PEG over the surface of the protein. More recently, this idea has been extended to the use of polymeric backbones with pendant PEG chains. The term POLY PEG® has been used to describe PEGs of various chain lengths grafted onto a methacrylate backbone. The POLY PEG® can then be conjugated to the protein via the reactive end group chemistries described above.[16,134,135] The PEG architecture can become quite complex, as with the four-arm PEG developed for delivering antineoplastic agents.[136] While these have not been used for proteins yet, they illustrate the level of complexity that could arise and be used. In each case, this produces a more complicated and elaborate drug substance.

PEG reagents: Polydispersity

Any PEG reagent will exhibit some distribution of molecular weights, often described as polydispersity (the width of the molecular weight distribution). Polydispersity of a polymer can be quantified as M_w/M_n, the ratio of weight-average molecular weight to number-average molecular weight. If this ratio equals unity, then the polymer would be considered monodisperse (indicating that all of the polymer molecules are of identical molecular weight). Typical values for PEG polydispersity are between 1.01 and 1.1, where polydispersity has been found to increase with PEG molecular weight.[11,13] For a given PEG reagent, the target polydispersity value is dependent on size of the material, for material less than 20 kD, the polydispersity is

1.01 and increases to 1.1 for 50 kD material.[70] Controlling PEG polydispersity is important, as a PEGylated protein will have a polydispersity related to the starting PEG.[13]

Some monodisperse PEG regents are now commercially available, and their use in PEGylated proteins was recently demonstrated.[137] For example, monodisperse PEG-based dendrimers are commercially available, but are currently limited to less than 1000 Da.[12,138] Additionally, commercially available monodisperse PEG can be produced by a patented separation technique.[137]

The polydispersity of the PEG-protein conjugate will result in issues in downstream processes, including purification, characterization of the conjugates, and bioactivity.[12,13,34,44] A narrow molecular weight distribution will simplify the purification of material and characterization of the conjugated protein.

PEGylated API: Effect of PEG size and site of attachment

The importance of characterizing each positional isomer cannot be overemphasized, as each is a distinct chemical entity. For example, the current product PEG-IFN-α_{2a} (Pegasys) is a mixture of different positional isomers.[111] It is possible to separate and examine each positional isomer, and it has been found that two of them had improved bioavailability relative to the others.[111] Similarly, PEG-rIL-2 showed a marked decrease in the clearance rates with an increase the effective molecular size of the PEG-protein conjugate.[14,139] It has been shown that the duration of activity in vivo is directly proportional to the mass of the pendant PEG groups attached to G-CSF.[118]

It has been shown that the clearance of PEG itself decreases markedly as the molecular weight of the PEG group increases.[140] Likewise, PEGylated proteins are cleared much more slowly as the size of the PEG group increases.[4] Even a small PEG (5 kD) has been found to decrease significantly clearance rates in vivo for interferon-α_{2a},[141] while larger PEGs had even more pronounced effects. With hemoglobin, autoxidation seems to be more prevalent when the PEG is attached at Cys[93] than at any of the Lys residues.[142] It has also been shown that attachment of a larger number of small PEG groups (5 kD) is more effective at preventing autoxidation than conjugation of a comparable mass of a smaller number of large PEGs.[74]

Purification challenges

After developing a process that allows for control of the location and number of PEGs attached to the target protein, a purification process must be established. The purification process has a number of goals: Removal of unreacted protein and PEG reagent, as well as isolation of different PEGylated proteins.[143,144] For a solution-phase reaction, a number of purification steps may be required to obtain a pure drug substance. Usually, the first is to stop the coupling reaction, which is typically done by adjusting the pH of the solution.[144] Removal of unreacted PEG reagents are important, as they exhibit surfactant properties, leading to foaming and even decreased resolution during chromatography.[44]

Then, there are a number of different methods for purifying the low molecular weight and high molecular products discussed below.[15,44, 43,144] Purification of the protein-PEG conjugates often employs ion exchange (IEX) or size exclusion chromatography (SEC) resins as the first step in a purification process, with the intent of removing excess reagents and byproducts.[44,144] For an excellent overview of the different purification methods used for various PEGylated peptides and proteins, see the review by Fee and Van Alstine.[44] Currently, there is no preferred chromatographic method for purification on the preparative scale. While separation of the unreacted protein from the PEGylated species is relatively easy, separation of the various positional isomers of a PEGylated protein mixture remains a significant challenge.

Purification using SEC is widely used,[14,143,144] even though its resolving power seems to be limited for PEGylated proteins. The effectiveness of separating high molecular weight products (unreacted PEG, native protein, under- and over-PEGylated protein) from the target PEGylated protein using SEC is based on differences in size.[44,143,144] Typically, SEC works well if the species differ in molecular weight by at least a factor of two.[44] Note that PEG groups behave as if they are much larger, as they are extremely highly hydrated.

Because of its limited resolution, the use of SEC for protein purification is better suited for small batches rather than commercial scale. Still, separation of PEGylated proteins with SEC is common if the degree of PEGylation is small (i.e. only mono- or di-PEGylated conjugates are formed). Consequently, charge-based methods are preferred. Purifying the PEG-protein conjugate by IEX chromatography requires the reaction solution to be diluted and passed through the column. This allows the desired PEGylated protein to bind to the column. Elution will then resolve any excess reagents from the desired product.[44,143,145] With cation-exchange (CEX) chromatography, the PEGylated protein elutes first, presumably due to blocking charged lysine groups, while unPEGylated material elutes last.[44,113,121] Surprisingly, the same elution pattern seems to persist even when there are no charge differences.[42] Furthermore, anion-exchange (AEX) chromatography displays the same order of elution as well.[34] This suggests that PEG interferes with the interaction between the protein and the stationary phase.[42] Overall, the disadvantages of IEX chromatography are that shallow gradients must be used and low loading concentrations are required to avoid fouling.[34] Even so, IEX chromatography is widely used and is able to separate positional isomers of PEGylated proteins.[44,111]

Hydrophobic interaction chromatography (HIC) has also been described in purification of PEGylated proteins, although much less frequently than SEC and IEX.[44] Despite the numerous hydroxyl groups on PEG, it still possesses some hydrophobic characteristics and

may bind to HIC supports. It has been suggested that HIC may be a useful polishing step after IEX chromatography and SEC.[34,44]

One can also use dialysis or diafiltration (DF) to remove low molecular weight reaction products and separate PEGylated conjugates from unmodified proteins.[44,143,146] Dialysis may be preferred over DF if there are issues with membrane fouling.[44,146] Fouling of the membrane will cause a decrease in the flux across the membrane, which results from the build up of the rejected proteins on the surface of the membrane.[44,146] When removing low molecular weight reaction products, the process should be performed at room temperature and at low concentrations.[146] At high concentrations and low temperatures, DF will result in a decrease in the flux over time.[146]

Dialysis has been used to terminate the PEGylation reaction and remove unreacted starting material for insulin,[147] epidermal growth factor,[148] and β-lactoglobulin.[149] Meanwhile, DF has been used for purification of PEGylated asparaginase.[150] In addition dialysis is often combined with ultrafiltration (UF) to provide some degree of purification of the PEGylated species.[151,152]

Clearly, attachment of one or more PEG groups to a protein will result in altered physicochemical properties when compared to the protein alone. This difference can be used to separate the modified and unmodified protein by preferential salting-out.[144,146] Dialysis and purification by selective precipitation are less expensive and can be scaled up for production of commercial scale batches. An alternative purification approach is to take advantage of phase separation of the modified and unmodified proteins.[15] A two-phase system is formed by PEG and dextran and solution, resulting in partitioning of the PEG and non-PEG compounds.[15] This process will not separate out free PEG since it will partition into to the same phase as the PEG-protein conjugate.[15]

The complexity of purifying the target PEG-protein conjugate is directly related three important factors: the heterogeneity of the PEG material, the number positional isomers and the number of attached PEGs. Careful control of all three factors will decrease the complexity of the purification process, potential increasing yield and decreasing production costs.

Production of GMP material/scale-up issues

The production of GMP material is designed with the express goal of demonstrating process control. Certainly, it is known that variation in PEGylation will affect clinical performance.[4,7,21,153] So beyond concerns about the purity of the starting protein, which is important, there are the concerns about process control and engineering for steps associated with production of the PEGylated drug substance.[44]

Certainly, the first step of quality control is fully characterizing all starting material including reagents, bulk PEG material, and the protein. This section will focus on issues associated with engineering issues and process control for the PEGylation process. By starting with a pure protein, the subsequent product should be easier to isolate, purify and characterize. Effects to PEGylate protein mixtures have been reported,[154] and these lead to very complex mixtures.

A variety of reactor types have been reported to be used for PEGylation reactions with proteins.[44] Batch reactors are simple to operate, but all reactants and by-products remain within the reactor until the process is complete. This may lead to decreased yields and more extensive side reactions. Fed batch reactors have the ability to control the degree of PEGylation, especially if the protein is kept in excess. However, this can a costly, as the degree of conversion tends to be relatively low. Finally, many reports have appeared using packed bed or on-column systems to conduct PEGylation of proteins. This type of reactor has been used in the PEGylation of interferon-α_{2a}[155] and G-CSF.[121] The nature of the reaction bed may affect orientation of the bound protein as well as the strength of binding. Preferred orientation of the protein could lead to improved regiospecificity.

Another approach to PEGylation is to use size exclusion reaction chromatography (SERC).[156] In this method, the size differences of the species control the reaction rate. The protein and PEG reagent are injected, with the smallest molecular weight material being injected first. As the larger reactant catches up, the PEGylation reaction occurs. Provided the column is long enough, all species, including the purified PEGylated protein conjugate can be separated. Keep in mind that many PEGylated proteins, like interferon-α, are less than 20 kD, meaning that they behave as if they are a smaller size than most PEGs, even those with molecular weights as low as 5 kD.[44]

Independent of the reactor type, one must consider the stability of the different linkages between the protein and PEG. Some, like NHS, are quite sensitive to pH and may require special care with respect to handling.[44]

Analytical methods for PEGylated proteins

Chromatography and electrophoresis

Analytical methods can be used to determine the size, charge, degree of PEGylation, location of PEGylation, and biological activity of a PEGylated protein. The most common methods for analyzing PEGylated proteins are chromatographic techniques that separate analytes based on hydrophobicity, size, and charge.

Size exclusion chromatography (SEC) is among the most popular and robust methods for PEGylation analysis. SEC has been used to analyze the many products which are often the result of PEGylation reactions.[157] However, it has been reported that SEC does not separate PEGylated proteins simply as if they were proteins of a higher mass.[13,158] Fee and Van Alstine demonstrated that the behavior of PEGylated proteins during SEC separation is dependent on the size, not mass, of the conjugated

proteins.[159] This phenomenon limits molecular weight determination using standards. One possible alternative is the use of in-line light scattering detectors to determine molecular weight more accurately, which has been demonstrated for several PEGylated proteins.[110,160,161] Another concern when using SEC is how both the size of PEG used and the degree of conjugation affect resolution. For example, the smaller the PEG molecule, the lower the resolution between successively higher order PEG conjugates.[44] Therefore, care should be taken when using SEC for PEGylation analysis.

Ion-exchange (IEX) chromatography serves as the most widely used technique for PEGylation analysis. Cation exchange (CEX) chromatography is useful following conjugation reactions where lysine side chain and N-terminal amines are the targets for modification with PEG. Recently, Zhang et al. used CEX chromatography to analyze reaction products for the PEGylation of insulin.[162] Fractions were collected for four prominent peaks in the chromatogram, and analyzed by SDS-PAGE and MALDI-TOF. Mono-, di-, and tri-PEGylated insulin were identified in this manner. The IEX elution order followed the degree of PEGylation, with higher order conjugates eluting first. Ramon et al. also used CEX chromatography, coupled with SDS-PAGE, to identify and purify mono-PEGylated interferon-α_{2b}.[163] This process resulted in mono-PEG interferon-α_{2b} that was 96% pure. In addition, CEX chromatography was also used in this work to assess the heterogeneity between batches of PEGylated protein.

Anion exchange (AEX) chromatography is also commonly used to analyze PEGylated proteins.[44,164,165] For example, Pabst et al. used AEX chromatography to examine PEGylated products following the reaction of either 12 kD or 30 kD PEG-maleimide with bovine serum albumin (BSA), coupled via the free cysteine residue.[165] As predicted, the PEGylated products were found to elute at a lower salt concentration than native BSA. PEGylation also caused a decrease in the dynamic binding capacity on six different commercially available anion exchange columns.[165] The authors suggest that conjugation of PEG leads to attenuation of column-analyte electrostatic interactions and changes in mass transfer rates due to increased analyte size. Both CEX and AEX chromatography were used sequentially to purify PEGylated recombinant human granulocyte colony-stimulating factor (G-CSF).[166] In theory, CEX chromatography should be used first to separate PEGylated species from native protein. This was followed by AEX chromatography which separated the PEGylated protein fractions. A two-step gradient elution profile was used in the second step, and resulted in well-resolved peaks for mono-, di-, and tri-PEGylated species.[166] It has been reported that removing free PEG prior to analysis improves the resolution significantly.[164]

Hydrophobic interaction chromatography (HIC) utilizes a mildly hydrophobic stationary phase and a diminishing ionic strength gradient to separate protein mixtures on the basis of hydrophobicity.[167] Because analytes are eluted at high ionic strength when using IEX chromatography, HIC often follows IEX chromatographic separation.[44] Addition of a PEG group to a protein can impart changes in hydrophobicity to the conjugate. Snider et al. used HIC to evaluate PEGylated superoxide dismutase, and found results similar to those obtained using reversed phase HPLC.[157] Similarly, HIC was employed for the separation of a 20 kD PEGylated ribonuclease A conjugate from the native protein.[168] While ribonuclease A was easily separated from the PEGylated products, the mono- and di-PEGylated species could not be resolved. It is possible that, like SEC, HIC faces a resolution limit for increasingly PEGylated products. HIC has also been examined as a method for purification of PEG before conjugation to protein.[133]

Reversed-phase high performance liquid chromatography (RP-HPLC) has found utility in the analysis of both intact PEGylated proteins and the products of protein digests.[34] In optimizing the PEGylation of octreotide, a rapid RP-HPLC method was developed for assessment of reaction products.[169,170] The method was able to distinguish between mono-PEGylated positional isomers. In addition, RP-HPLC has been used to monitor methionine oxidation of native and PEGylated G-CSF as a stability indicating method for samples stressed at high temperature.[171]

Peptide mapping by RP-HPLC has been extended to include PEGylated proteins. Kinstler et al. identified the PEGylated N-terminal peptide of G-CSF using this method.[121] Note that PEGylation can affect the rate of proteolysis, as shown by the RP-HPLC analysis of digested native and PEGylated endostatin.[172] Peptide mapping was used to determine the extent of PEGylation of hGH.[173] At the same time, the RP-HPLC method was able to determine the amount of methionine oxidation as well.

As discussed above, SDS-PAGE has been used as a complimentary method for PEGylation analysis following chromatography with fraction collection. The presence of PEG in the sample matrix has been observed by Kudlicki et al. to cause an increase in protein electrophoretic mobility during SDS-PAGE.[174] This effect is due to the SDS micelle binding to PEG, which has been theorized to affect protein mobility through changes in the local conductivity.[175,176] Reduction of the SDS concentration and ionic strength of the buffer has been shown to eliminate this effect.[176] Micellar SDS binding to PEG and its effects should therefore also be considered when analyzing PEGylated products by SDS-PAGE. While the effects of Kudlicki et al. were observed for PEG itself,[174] one can imagine that similar interactions could be important for highly PEGylated proteins.

Kurfürst noted that the molecular weight of PEGylated hirudin could not be accurately determined using protein molecular weight standards in SDS-PAGE.[177] However, a PEG calibration curve gave a much closer result, indicating that PEGylated hirudin migrated similarly to a free

PEG of the same molecular weight. Bailon et al. observed the same behavior for a conjugate of interferon-α_{2b} and a 40 kDa branched PEG.[178] The authors suggested that the PEG could contribute to lower mobility by providing a larger hydrodynamic volume and through sieving activity in the gel matrix due to its branched nature. Zheng et al. demonstrated separation of PEGylated human serum albumin using native PAGE (no SDS) and noted higher resolution (less 'smearing') of analyte.[179] Determination of molecular weight by this method may be difficult or not possible. Staining of gels used for PEGylation experiments typically requires two dyes, Coomassie blue (protein stain) and iodine (PEG stain).[163,178] As these stains make separated species unusable for further analysis, Hardy et al. showed that zinc and imidazole salts could instead be used in an reverse staining procedure that would preserve separated PEG-protein conjugates for additional experimentation.[180]

Capillary electrophoresis (CE) is a promising technique for the analysis of PEGylation products. CE separations are performed on the basis of analyte size, charge, and shape, all parameters that are relevant to the PEGylation of pharmaceuticals. As with IEX chromatography, removal of free PEG has been found to improve the resolution of the pegylated protein species.[158] Early endeavors with CE demonstrated separation of multi-PEGylated myoglobin and superoxide dismutase.[157,181,182] Bullock et al. were able to separate seven different species following PEGylation of superoxide dismutase using CE, and the results were in good agreement with MALDI-MS data.[182,183] Li and Su used CE to monitor the PEGylation of ribonuclease A and optimize the reaction for both time and pH.[184] Na and Lee used CE for both analysis of PEGylated human parathyroid hormone (PTH) and optimization of PEGylation reaction.[185] PEGylated products in the electropherogram could be easily identified by comparison to MALDI mass spectrum. CE was also used to determine the sites of PEGylation following endoproteinase Lys-C digestion of purified mono-PEG-PTH. Positional isomers were identified by comparison of native and mono-PEG-PTH protein digests with both CE and MS. The same research group has also demonstrated capillary gel electrophoresis (CGE) analysis of interferon alpha modified with PEG of different weight and structure.[186] CE is an appealing method for analysis of PEGylation reaction products due to its low sample consumption and high speed, however its loading capacity for a single separation is too small for preparative scale work. Finally, as with all CE separation of proteins and peptides, care must be taken to prevent sample adsorption to the capillary wall.[187,188]

Sizing methods

Alternatively, as PEGylation increases the average size of the protein species, one can use methods like small-angle x-ray scattering (SAXS), to determine the effect of PEGylation on hydrodynamic radius. A recent SAXS study on PEGylated hemoglobin indicates that PEGylation compacts the size of the protein without disrupting the tertiary structure.[189] Moreover, as more PEG groups are attached, there appears to be appreciable intermolecular repulsion of the conjugates from each other. Both SAXS and analytical ultracentrifugation (AUC) were used to characterize the size of PEGylated antibody fragments.[190] Six positional isomers of interferon-α_{2a} were prepared and these were characterized for size using AUC.[191] The size of the protein is not altered, and the PEG group appears to display a similar hydrodynamic radius as the free PEG.

Spectroscopy

Although PEG itself does not possess any unique spectroscopic properties relevant to analysis, there are several spectroscopic techniques available that can determine extent of PEGylation. In a conjugation reaction where primary amines on the pharmaceutical are consumed, trinitrobenzenesulfonic acid (TNBS) assay can be used to calculate the fraction of amines involved in PEGylation.[192] Several different groups have used this assay for PEG analysis.[162,183,193,194] When conjugation is directed towards protein sulfhydryls, Ellman's Reagent assay and other disulfide exchange assays can be used in a similar manner to TNBS.[195–197]

NMR can be used to identify positional isomers on PEGylated proteins. Using a subtilisin digest of purified mono-PEGylated IFN and PEGylated synthetic peptides, Wang et al. were able to identify the major positional isomer.[198] PEGylation of histidine residues was found to cause chemical shifts for both $H^{\epsilon 1}$ and $H^{\delta 2}$. Spectral comparison between the digest and PEGylated synthetic peptides revealed the prominent positional isomer of PEGylation at His-34. The authors also concluded that this PEGylation occurs mainly at the $N^{\delta 1}$ imidazole ring nitrogen, not $N^{\epsilon 1}$. Finally, the hydrolysis of the PEG-imidazole bond in both the digest and intact protein was monitored with NMR, revealing that the intact protein conjugate was less susceptible to hydrolysis over time.

Second derivative FTIR was used by Heller et al. for structural characterization of PEGylated and native hemoglobin and brain-derived neurotrophic factor (BDNF).[199] Although this study focused on the effects of PEGylation during lyophilization, it was also noted that FTIR of the amide I region could detect small changes in secondary structure between PEGylated and native BDNF. FTIR has also been used to assess structural dynamics by monitoring of H/D exchange. Rodriguez-Martinez et al. observed changes in the rate constant and fraction of exchanging hydrogens following the PEGylation of α-chymotrypsin.[200]

Circular dichroism (CD) has also been used extensively for analysis of PEGylated products. Mabrouk used CD to show that PEGylation of cytochrome c produced structural changes to the heme site, even when the degree of PEGylation was moderate.[201] Tan et al. observed a change in percent β-turn structure associated with increased PEGlyation of endostatin.[202] In several other studies, CD

spectra showed no changes in secondary structure due to PEGylation.[115,203–206]

Mass spectrometry

PEGylation of a protein increases the protein's mass by a multiple of the mass of PEG used. Therefore, mass spectrometry can be used to determine the degree of PEGylation on a target protein. This analysis can be complicated by the polydispersity of the PEG used for conjugation.[13] Recombinant human G-CSF was mono-PEGylated with a 20 kD PEG and examined by MALDI-TOF.[207] The polydispersity of the PEG contributed to a measured mass that was approximately 1 kD less than what was expected for the conjugate. The peak width at half height for the conjugate was almost two orders of magnitude larger than that of the native protein peak, again indicating a polydisperse product. The PEGylated fragment observed from the Lys-C digest showed the same dispersity and error. Polydispersity of PEG therefore makes identification of the site of PEGylation a difficult task using mass spectrometry. In a recent publication by Mero et al, this problem was addressed through the use of an enzymatically-conjugated monodisperse PEG.[137] Using MS/MS, the authors were able to determine which amino acid residue(s) were modified by PEG. In another study, Tan et al. used MALDI to examine the reaction products for PEGylation of recombinant methioninase.[64] The resulting mass spectrum showed several broad, noisy peaks associated with different degrees of product PEGylation. As an analytical technique, the utility of mass spectrometry for PEGylation analysis may ultimately depend on the level of polydispersity in the target PEG.

Immunological assays/activity assays

PEGylation near the active site of a protein can affect binding of the conjugate to the target.[7] Immunological techniques, along with other binding assays, can yield important information about the activity of a PEGylated protein. Hershfield et al. noted that PEGylated purine nucleoside phosphorylase showed diminished binding to antisera raised against the native protein, indicating that the antigenic determinant(s) had been altered by the process of PEGylation.[88] In another study, Grace et al. noted that PEGylation of interferon-α_{2b} did not affect EIA measurements.[208] It is therefore important that the affinity reagents used for analysis are specific to the site of interest on the protein. Due to the reduced immunogenicity of PEGylated proteins, immunological analysis may be further limited by the absence of antibodies directed against PEGylated molecules.

Immunoassay methods for PEGylation analysis may be most useful when the PEGylated protein is an antibody, as the antibody-antigen interaction is likely the intended therapeutic purpose of the protein and can be easily measured. Chapman has written an extensive review of PEGylation of antibodies.[6] Provided that the PEGylation occurs far from the CDRs, binding avidity should not be affected. However, other biological molecules have shown significant decreases in potency upon PEGylation. The issue becomes whether the increases in half-life and/or solubility outweigh the loss of specific activity. For example, the bioactivity of EPO decreased to 68% of the native protein upon mono-PEGylation.[209] Likewise, the activity of mono-PEGylated IL-1ra decreased to 45% of the level before PEGylation,[210] while the activity of PEGylated uricase decreased to 75–85% of the unmodified enzyme, depending on the type of PEGylation chemistry that was used.[69] A large number of analogs of erythropoietin were prepared and the bioactivity determined, demonstrating that even mono-PEGylation can alter the bioactivity.[211] If so, then products containing mixtures of positional isomers could have species with markedly different biological activities, as well as pharmacokinetic profiles.

Formulation challenges

For proteins that have been chemically modified, as occurs with PEGylation, the physicochemical properties of the protein are altered. This has been seen with other various post-translational modifications, such as glycosylation or glycation, where the addition of a sugar moiety changes the stability, solubility, or even the potency of the protein. Likewise, PEGylation has the same effect on these important qualities. Finally, assuming that the PEGylated API is relatively pure, the formulation scientist will begin development with a molecule that has mostly the properties of a protein, but with some properties of PEG.

It is important to note that attachment of any group to a protein will generate a linkage that could suffer chemical degradation, leading to loss of that group. This has been a focus for those involved in preparing antibody-drug conjugates. The same is true for PEGylated proteins. For this reason, preformulation studies, examining the effects of pH, buffer, and temperature, will watch for loss of the PEG group(s).[171] Loss of the linkage, usually through hydrolytic damage, can lead to release of the attached PEG.[212] This will be an additional instability that must be considered as a final formulation is developed.

From a formulation point of view a number of important parameters need to be taken into account that will have a large impact on the quality of the final product. These parameters include the quality of the PEG such as the type and molecular weight, its molecular weight distribution, the number of PEGs attached to the protein and attachment location. In some cases the type of chemistry used to covalently attach the protein to the PEG can be adjusted for covalent attachment and the number of attached PEGs. The process of achieving a formulated conjugated protein that has improved physical/chemical stability, bioavailability is more complex and will be discussed below.

For liquid formulations, two concerns over physical instability are often stated.[10] First, attachment of a large PEG group or groups could lead to increased propensity for phase separation. Second, there may be increased viscosity, likely due to self-association of the PEG groups. On the other hand, PEGylation may improve solubility, due to the extensive hydration of PEG groups. At the same time, having a pendant PEG group can make the conjugate more surface active and thereby more prone to interfacial damage. As a result, surfactants may need to be added to minimize surface interactions.[171]

For lyophilized protein products, the rules for rational development of stable formulations are well established.[213] However, PEGylated proteins possess some physical properties that could be problematic for freeze-drying. For example, PEG molecules are known to phase separate, and the possibility of phase separation occurring with PEGylated proteins has been discussed.[199] In addition, each PEG unit holds many water molecules.[203, 214] This makes drying of PEGylated proteins more difficult than unmodified proteins.[215]

So, while there is little information published about formulating PEGylated proteins and peptides, we do have some general principles to consider. The physical properties, including solubility and viscosity, are altered by the addition of PEG groups. In addition, one must maintain the chemical integrity of the linker attaching the PEG to the protein. With these points in mind, liquid formulation development can proceed as for any other protein. For lyophilized products, the difficulty of removing water from PEG groups will alter the cycle and may impact the final product stability.

Summary

As with any other type of chemical modification (e.g. glycosylation, conjugation to low molecular weight compounds, etc.), the physical properties of the protein are altered during PEGylation. This affects purification and formulation strategies. Even more importantly, the chemistry of attaching PEG to a protein or peptide is the critical issue affecting product development. The quality of the starting materials must be controlled, as must the coupling process. These issues become exacerbated as the scale increases and the chemistry is performed under GMP conditions. Once the product is synthesized, there are significant issues with selecting a purification scheme. Unfortunately, there does not seem to be a consensus on what methods work the best, although the focus is usually on SEC and IEX chromatography. This is a key point, as one is dealing with a potentially complex mixture, if there are multiple isomers of the PEGylated protein. Once the purified drug substance is obtained, a number of analytical methods can be employed to characterize the material. Formulation development and preparation of the final drug product is rarely described in the literature. These have been summarized to provide some framework for developing the final dosage form. Altogether, these points illustrate the significant challenges associated with developing a commercially viable PEGylated protein product. Having said that, there are a number of approved products providing some guidance on how to bring these important compounds to the market.

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