Journal of Pharmaceutical Sciences 105 (2016) 460–475

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Review

PEGylation of Biopharmaceuticals: A Review of Chemistry and Nonclinical Safety Information of Approved Drugs

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ARTICLE INFO

Article history:
Received 11 July 2015
Revised 1 October 2015
Accepted 30 October 2015

Keywords:
polyethylene glycol
PEGylation
PEG reagent
PEGylation chemistry
biopharmaceuticals
toxicology

ABSTRACT

Modification of biopharmaceutical molecules by covalent conjugation of polyethylene glycol (PEG) molecules is known to enhance pharmacologic and pharmaceutical properties of proteins and other large molecules and has been used successfully in 12 approved drugs. Both linear and branched-chain PEG reagents with molecular sizes of up to 40 kDa have been used with a variety of different PEG derivatives with different linker chemistries. This review describes the properties of PEG itself, the history and evolution of PEGylation chemistry, and provides examples of PEGylated drugs with an established medical history. A trend toward the use of complex PEG architectures and larger PEG polymers, but with very pure and well-characterized PEG reagents is described. Nonclinical toxicology findings related to PEG in approved PEGylated biopharmaceuticals are summarized. The effect attributed to the PEG part of the molecules as observed in 5 of the 12 marketed products was cellular vacuolation seen microscopically mainly in phagocytic cells which is likely related to their biological function to absorb and remove particles and macromolecules from blood and tissues. Experience with marketed PEGylated products indicates that adverse effects in toxicology studies are usually related to the active part of the drug but not to the PEG moiety.

Introduction

Polyethylene glycol molecules (PEG) are synthetic, highly water soluble, inert polymers that are produced in a large range of molecular weights. PEGs of various molecular weights have been used widely in consumer care products such as laxatives, toothpaste, and hair shampoos, and for the past 20 years, also in biopharmaceuticals. Chemical modification with PEG (PEGylation) is used to improve pharmaceutical properties of both small molecule and biotherapeutic drugs. PEGylation may prolong circulation time of a protein or nucleic acid molecule, enhance aqueous solubility of drug substances, protect against *in vivo* biological inactivation by proteolysis, and reduce immunogenicity of some biopharmaceuticals. Prolongation of half-life is the main reason why the PEGylation technology is used in parenteral drugs that are otherwise cleared by the kidney or other metabolic pathways.

There are several methods for PEGylation that use a variety of chemical reagents. These yield PEG biopharmaceutical conjugates with either stable covalent or degradable linkages. These degradable linkages are the basis of PEGylated prodrugs. PEGylation technology has been well-described in review articles and book chapters.[1-11] For example, Francis et al.[12] provide a summary of the early conjugation chemistry and Roberts et al.[3] provide a detailed summary of PEG reagents and a history of PEGylation technology. Other reviews of PEGylation technology are also available.[13-20]

Twelve PEGylated biopharmaceuticals have been approved based on balanced risk/benefit evaluations. Nonclinical safety

Conflicts of interest: As indicated, Peter L. Turecek, Mary J. Bossard, Freddy Schoetens, and Inge Ivens are employed in organizations that are engaged in the development of a variety of innovative therapeutic products which may include PEGylated biopharmaceuticals. The authors have no other relevant involvement (financial or otherwise) in organizations or entities that would represent a financial interest or conflict relative to the issues discussed in this manuscript. The authors are experts in the health care sciences, who are offering guidance on important issues to consider in the design of nonclinical safety assessment programs for PEGylated biopharmaceuticals. Several of the authors serve on a subcommittee of BioSafe, a committee composed of member volunteers from within the Health Section of the Biotechnology Industry Organization (BIO).

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http://dx.doi.org/10.1016/j.xphs.2015.11.015
0022-3549/

assessment of these products indicated that their toxicological effects were derived from the active part of the drug rather than the PEG moiety. The only effect attributed to PEG in nonclinical toxicology studies was cellular vacuolation observed with 5 of the 12 approved PEGylated biopharmaceuticals (for 2 drugs toxicology information is not available). This vacuolation is seen mainly in phagocytic but sometimes also in nonphagocytic cells. Phagocytic cells likely contribute to the clearance of larger PEGylated biopharmaceuticals, whereas small PEG molecules and PEG conjugates are eliminated via kidney and liver because of their hydrophilicity and size. For a detailed discussion of tissue vacuolation and the nonclinical safety of PEGylated biotherapeutics see Ivens et al., 2015.[21]

This review describes the chemistry of PEG reagents, PEGs, and linker chemistry used in 12 approved drugs. A brief summary is given describing the cellular vacuolation as seen in nonclinical toxicology studies. Further details and reviews of the safety of PEGylated biopharmaceuticals are provided in Ivens et al., 2015[21] and several other literature citations.[22-24]

Polyethylene Molecules

PEG is a linear polyether of ethylene glycol (ethane-1,2-diol) of the general structure shown in Figure 1.

Frequently one end of the polymer is capped with a methoxy group (mPEG) to prevent unwanted cross-linking during conjugation. This synthetic polymer soluble in both water and organic solvents is produced by anionic polymerization of ethylene oxide initiated by nucleophilic attack of a methoxide ion on the epoxide ring[3] (Fig. 2).

mPEG is used in all approved PEGylated therapeutic products. Historically, mPEG contained up to 15% PEG diol as an impurity, which can be removed by the use of various methods.[25,26]

Minimization of PEG diol content is critical before activation to ensure synthesis of a monofunctional PEG derivative, which avoids cross-linking of the protein. Multiple PEG derivatives with different functional end groups and a variety of architectures are commercially available.

Commercial mPEG normally has a very low polydispersity (measure of the heterogeneity of sizes of PEG molecules; Mw/Mn) in the range of 1.01 for low molecular weight PEGs (<5 kDa) to 1.1 for high molecular weight PEGs (>50 kDa).[3] PEGs are highly hydrated in aqueous solution and can bind 2 to 3 water molecules per repeating ethylene oxide unit. Based on the high water binding capacity of the polymer, PEGylation strongly influences protein hydrodynamics.[27,28]

PEGylation Chemistry

Both linear and branched-chain PEG reagents are used in currently marketed drugs. Branched PEG reagents contain 2 PEG molecules attached to a central core, from which extends a tethered reactive moiety which will bind to the drug molecule. Conjugation with branched chain PEG reagents results in products with higher PEG density per modification site. Multiarm reagents have not yet been used in Food and Drug Administration (FDA)–approved products. Branched or multiarm reagents are advantageous if the target is a mono-PEGylated product.

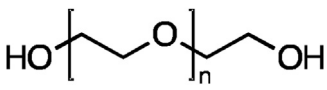

Figure 1. Structure of a single PEG chain.

Monofunctional PEG reagents are used to PEGylate proteins with a functional group attached to one end of the PEG polymer allowing reaction with N-terminal amine, lysine, cysteine, and other amino acids.[29] Which amino acids are solvent accessible depends on the specific protein. Lysines are almost always available on the protein surface but naturally occurring free thiols are very reactive and therefore usually "buried."[30] Conditions which allow reaction with a particular reagent may not be compatible with the native conformation of the protein and appropriate controls need to be made to ensure compatibility of the PEGylation conditions. Mono-PEGylation, site-directed modification and random modification strategies have been described. Random PEGylation can result in a variety of positional isomers depending on the selected chemistry. This could potentially lead to a loss of specific activity if functionally reactive sites are affected. In contrast, limited PEGylation with multiple attachment sites could improve pharmacologic properties, while reducing impact on functionality and efficacy.

When multiple PEGs have been attached, the individual species in the heterogeneous mixture are referred to as "PEGmers," and their number ranges from zero (native protein without PEG) to the number of PEGs attached. The individual PEG-protein species that differ only in location of the attached PEG are positional isomers. As the degree of substitution increased, the number of positional isomers (P) also increases according to the formula (N is the number of possible sites, and k is the number of sites modified):

$$P = \frac{N!}{(N-k)! \times k!}$$

A commercial product may consist of multiple positional isomers and each could have differential effects on pharmacologic, toxicologic, and immunogenic activity. The primary impurities in the final conjugate that need to be minimized are the native un-PEGylated protein, unreacted or free PEG, and multimers with more than the desired number of PEGs present.

It is important to ensure removal of all host cell proteins or other protein contaminants before PEGylation as they may become PEGylated as well. Ion exchange methods are usually used to separate the desired conjugate from unreacted PEG and native protein. Mock reactions of the PEGylation reaction and purification runs of unmodified protein are also performed to ensure that the conditions required to modify the drug molecule, mainly proteins, by PEG reagents and to separate the desired conjugate fractions are compatible with the stability of the native protein. As real time "in process" assays may often not be feasible, analysis of samples collected during a complete PEGylation and purification process facilitate identification of any steps that might negatively impact protein stability. Purification of monoconjugate from multi-PEGylated conjugates may be difficult as positional isomers may mask charges making it difficult to separate only the monoconjugates.

Impurities may be present in a given PEGylation reagent, which might impact the desired PEGylated product. Impurities will vary depending on the reagent's synthesis route. Failure to eliminate PEG-diol in the initial starting material can result in difunctional PEG reagents when only one monofunctional product is desired. Small amounts of PEG acid may be present from hydrolysis of an active ester because of small amounts of water present in the product. Even if present, PEG acid is nonreactive and will not generate undesired conjugation products. Other impurities will be specific to the particular PEG reagent used. The general patent literature discloses some information about synthetic methods, but the final synthetic scheme used in manufacturing according to good manufacturing practices of PEG reagents, which includes purification methods to reduce or remove impurities will be specific to a particular drug product. Size exclusion chromatographic

Figure 2. Mechanism for synthesis of mPEG by anionic polymerization.

analysis with the use of polymer standards or matrix-assisted laser desorption/ionization (technique used in mass spectrometry) can be used to verify the molecular weight of the reagent. Degree of end groups substitution of active reagent will vary depending on the type of active group and additional process-generated impurities will be specific to the synthesis process.

Effects of PEGylation on Protein Stability and Folding

Comparative studies of PEGylated proteins versus the non-PEGylated counterpart generally conclude that the addition of PEG does not significantly change the protein structure when assessed by circular dichroism (CD), ultraviolet absorption, or nuclear magnetic resonance (NMR) spectroscopy.[18,31-36] Reported changes in protein temperature stability have been both positive[37-39] and negative[40,41] depending on the coupling chemistry, the degree of PEGylation, the number of protein subunits, and formulation.[42] Only a few systematic studies have been reported.[34,39,42-44] Many examples in the literature describe the effects of PEGylation on the physical parameters of the modified protein. One should always prepare a mock reaction without active PEG to discern whether any effects are due to the reaction conditions themselves or truly due to the modification by the PEG reagent. This examination of controls is especially important for developing of pharmaceuticals as improperly folded protein material may be more subject to proteolysis or more likely to contribute to development of immunogenicity. As described in the chemistry section of this article, modification at a lysine to generate an amide bond will reduce the charge. This will change the pI. Other chemical modifications such as reductive aldehyde PEGylation at an amine (reductive amination) will be charge neutral. Additional shielding by the PEG may also mask surface charges which can alter the effective pI. General noncovalent surface interactions may be altered because of the hydrophilic nature of the PEG. These interactions may be different depending on the protein and the amount of PEG. Thus, the specific examples presented here may not extrapolate to every protein.

A few examples are given here to illustrate cases where PEGylation of peptides and proteins did not disrupt a properly folded structure. Two lysine specific conjugates (Lys12, Lys21) of growth-regulating hormone (hGRF 1-29) peptide PEGylated with a single 5 kDa PEG did not show structural distortion because of the PEG attachment[33] as measured by NMR and molecular dynamics. The presence of PEG did not interfere with the peptide's natural tendency to form an α-helix. The authors concluded that the differences in biological activity were related to steric hindrance and PEG orientation relative to the ligand-binding sites.

The secondary structure of a larger peptide, insulin (molecular weight ~ 6.6 kDa) is 53% α-helix, 8% β sheet, 18% β turn, and 21% random coil based on the crystal structure.[45] Comparison of 4 different monoinsulin conjugates by CD yielded results that indicated there were no observable structural differences because of either the PEG size or position.[34]

PEG-Intron® is a mixture of mono-PEG positional isomers as described in the following sections. CD spectra for both Intron A® and PEG Intron® were superimposable, indicating PEGylation had no effect on the secondary or tertiary structure of interferon (INF)-α2b.[46]

Pegasys®, PEG-INF-α2a has been compared to native INF-α2a. Analysis by NMR, CD, fluorescence spectroscopy, and differential scanning calorimetry all yielded results indicating that the three-dimensional structure on INF-α2a was not altered by PEGylation at any of the positional isomers.[39] The conjugate did demonstrate reduced digestion by trypsin compared with native protein. Detailed receptor-binding kinetics verified PEGylated INF had an overall reduced affinity of ~24-fold because of a change in k_{on}. Individual positional isomer differences in k_{on} could be explained by steric hindrance and the specific location of the PEG relative to the receptor-binding site.

Uricase is a tetramer with 4 identical 34 kDa subunits and 4 identical active sites. The active sites are each localized at the interface between 2 of the subunits.[47] Development of uricase as a treatment for gout has been challenging not only because humans do not have the enzyme but also because the optimal pH is ~8.8.[48] At the isoelectric point, pH 5.8, most of the catalytic activity is lost, presumably due to subunit dissociation. Previous work found PEGylation imparted partial resistance to thermal denaturation and some improved activity at acidic pH.[49] Detailed stability studies as a function of pH verified not only that unmodified uricase subunits dissociate, but that PEGylation prevented the dissociation. These authors called this a “wrapping effect” that prevents deterioration of the secondary and quaternary protein structure.

^{1}H-NMR and CD have also been used to evaluate PEGylated superoxide dismutase and ribonuclease A. Comparison of superoxide dismutase from 2 sources, modified by 4 different coupling chemistries indicated that although the reaction kinetics were different, the overall conclusion was the same.[32] Loss of enzyme activity was attributed to masking of positive charges required for channeling the superoxide ion to the active site metals. Reversibility of the metal ion activation was independent of the PEG coupling chemistry and enzyme source. At a high degree of PEGylation, a slight decrease in temperature stability was explained by the presence of forces opposite to those holding the protein chains together.

A slight reduction in thermal stability has also been seen for multiple PEGylated bovine ribonuclease A. Temperature stability was reduced from 67° to 57° at the highest degree of PEGylation; 11 methoxy 5 kDa PEGs. The effects on thermal stability were less detrimental than the steric effects of the PEG. At maximal PEGylation (11 PEGs), enzyme activity toward RNA was reduced to 3% of native but retained 33% activity toward a small nucleotide substrate. Antibody-binding affinity went from ~nM to μM consistent with the primary effect of the PEG attachment being steric rather than conformationally disruptive.[40] A detailed systematic study of PEGylated bovine serum albumin with 6 site-specific mono-PEGylated conjugates ranging in size from 5 kDa to 60 kDa demonstrated that the secondary and tertiary structure of bovine serum albumin was independent of PEGylation as measured by CD.[42] Thermal stability of a covalent PEGylated conjugate compared with native protein decreased slightly as measured by protein unfolding methodology but was independent of the PEG molecular weight. The temperature of aggregation increased slightly because of PEGylation but was also independent of PEG size. This is in agreement with results from α

chymotrypsin.[44] Biophysical characterization of recombinant human FVIIa[50] also indicated that secondary and tertiary structure were not greatly impacted by PEGylation, but the temperature of aggregation measured by light scattering increased slightly for the PEGylated conjugates independent of PEG size or architecture.

For marketed drug products the formation of aggregates is a concern not only from an immunogenicity perspective but also for determining shelf life. When liquid stability of PEGylated granulocyte-colony stimulating factor was characterized, it was found that at elevated temperature, the chemical linkage for the PEG reagent to the protein was critical. All other measurements, including activity, did not discern a meaningful difference between an acylated PEG recombinant human granulocyte-colony stimulating factor compared with an alkylated conjugate. The alkylated conjugate was more stable and less prone to aggregation than the acylated conjugate, which may be explained by loss of a charge on the N-terminal amino group.[51] In addition, the container closure and formulation may also affect conjugate stability.[41,52]

Based on the literature cited previously, if one performs the PEGylation and purification of a PEGylated protein in accordance with known sensitivities to temperature, pH, and other physical parameters, the resultant conjugate should not display any significant difference in the folded structure compared with native protein as monitored by CD, NMR, and complementary techniques to measure protein structure. Changes in thermal stability or subunit dissociation (either positively or negatively) may be observed which reflect characteristics of the protein but are not necessarily related to PEG. Steric shielding that negatively affects binding to ligands will be a function of the size and location of the PEG.

Evolution of PEGylation Technology

First-Generation PEG Chemistry

First-generation PEG reagents were usually no larger than 5 kDa.[5] Early PEG reagents were not specific for lysine and some contained degradable linkages. Cyanuric chloride (2,4,6-trichloro-s-triazine) was initially used by Davis to prepare activated PEG that was reacted with catalase and bovine serum albumin[53,54] (Fig. 3) yielding conjugates with extended circulation time and less immunogenicity than the parent proteins. PEG dichlorotriazine is not selective for lysine and will also react with serine, tyrosine, cysteine, and histidine.[9] Lack of specificity and toxicity of cyanuric chloride derivatives make this reagent unsuitable for commercial products.[9] The second chloride in PEG dichlorotriazine is less reactive than the first, but there is sufficient reactivity to generate some di-PEGylated species.

A branched PEG version of cyanuric chloride ($mPEG_2$) shown in Figure 4 is more selective toward lysine and cysteine.[3] Early synthetic methods for PEG_2 triazine yielded a mixed product contaminated with the mono-PEGylated triazine.[11] Use of branched PEG required fewer protein modification sites than a single PEG chain to reduce immunogenicity of bovine serum albumin.[53,55]

PEG tresylate was more specific for lysine than PEG dichlorotriazine, but its chemistry of conjugation was not well defined.[3] In addition to forming a stable secondary amine linkage shown in Figure 5, it can also react to form a product with a degradable sulfamate linkage[56] potentially yielding a mixed population of PEGmers.

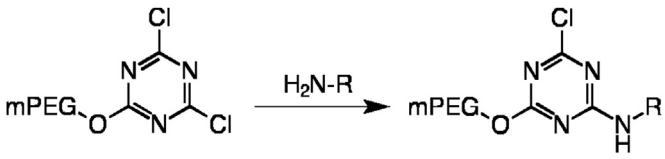

Figure 3. PEGylation of primary amines in proteins (H2N-R) by PEG dichlorotriazine.

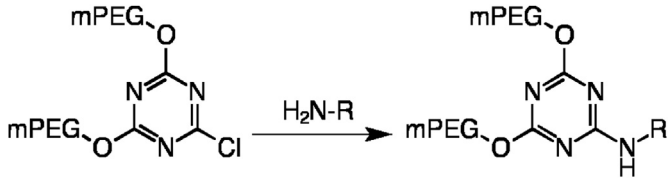

Figure 4. PEGylation of primary amines in proteins (H2N-R) by branched PEG chlorotriazine.

Lack of specificity, degradability, reduced conjugate activity, hydrolysis by water, a need for higher molecular weight PEGs, and heterogeneity of conjugates all contributed to the need for more selective next-generation PEG reagents. Second generation PEG reagents are N-terminal selective or thiol selective and demonstrate improved lysine selectivity.[3] The availability of higher molecular weight raw PEGs with low diol content permitted synthesis of high-quality conjugates $\geq$20 kDa allowing for creation of branched reagents and higher molecular weight linear PEGs suitable for protein conjugation (Fig. 6). Currently marketed products contain both early PEG reagents and newer more specific amino acid–selective reagents.

Branched PEGylation reagents with an asymmetric 1,2-substitution pattern are commercially available. Figure 7 gives an example of a reagent with an active N-hydroxysuccinimide head group (PEG-NHS), which can bind to primary amines such as accessible groups in lysines.

Commercially Available PEG Reagents

Commercially available PEGylation reagents otherwise known as “activated PEGs” generally fall into one of 3 classes: (1) acylating reagents, (2) alkylating reagents, and (3) thiol-reactive reagents.

Acylating Reagents

Coupling chemistry yields a stable bond (amide and urethane) that reduces a charge on the protein.

The acylating reagents primarily target the side-chain amine of lysine, but N-terminal amines, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, and tyrosine can also be modified.

Lysine is one of the most prevalent amino acids and can make up as much as 10% of the overall amino acid sequence in a typical protein. Reaction between electrophilically activated PEG and nucleophilic amino acids often results in substitution of several amines. The linkage to amines for commercial products forms a carbamate and/or urethane linkage or an amide linkage to the protein as illustrated in Figures 8a and 8b. In either case, the charge on the protein is reduced at the site of attachment.

PEG carbonate reagents are unstable at basic pH.[57] In a side reaction, carbonates can also react with OH groups of serine and tyrosine. Furthermore, PEG-succinimidyl carbonate can react with histidine residues to form a hydrolytically unstable carbamate linkage.

The most common acylating reagents used in commercial products are succinimidyl-activated PEG carboxylic acids. When the PEG is coupled to N-hydroxysuccinimide, the reagent is often called an NHS ester. All NHS reagents are subject to hydrolysis yielding the corresponding PEG acid and N-hydroxysuccinimide (Fig. 9).

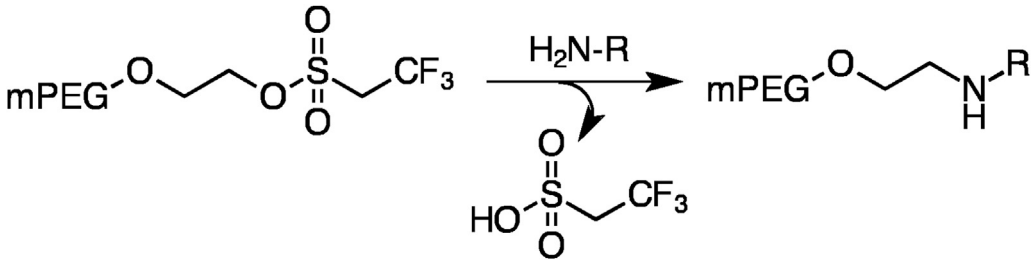

Figure 5. PEGylation of primary amines in proteins (H2N-R) by PEG tresylate.

Hydrolysis rates vary as a function of the linker (L) between the PEG and the active head group and increase as a function of pH.[58] Although aminolysis is generally faster than hydrolysis at a given pH, PEGylation of proteins requires excess reagent to compensate for the competing reagent degradation. NHS esters are generally described as lysine-specific reagents, but the imidazole group of histidine (pKa of imidazole $NH^+ = 6$) and the hydroxyl group of tyrosine can also react if conditions are favorable.

Historically PEG succinimidyl succinate (PEG-SS; Fig. 10a) was the first NHS ester, which was used for the PEGylation of asparaginase.[59] This reagent is prepared by the reaction of mPEG with succinic anhydride followed by activation of the carboxylic acid to the succinimidyl ester. The backbone of this reagent contains a second ester bond, which is susceptible to hydrolysis. This reagent together with PEG succinimidyl glutarate (Fig. 10b) is the class of first-generation PEG-NHS reagents, which contain a degradable linkage.

Later on linear stable PEGylation reagents like PEG succinimidyl propionate or PEG-succinimidyl *a*-methylbutanoate were developed. In course of further development branched reagents having a lysine core[60] were prepared, which are used in the commercial PEGylated product Pegasys® described earlier.

Alternative PEG Reagents

Besides the previously described PEGylation reagents which are commercially available, there are a variety of alternative PEGylation reagents described in the literature, which can be used for the chemical modification of amino, hydroxyl, carboxyl, or sulfhydryl groups.

An option for chemical PEGylation of proteins is the derivatization via carboxyl side chains of aspartic and glutamic acid residues. This can be performed by the use of carbodiimide chemistry. Usually treatment of proteins with carbodiimide requires harsh conditions, may lead to protein cross-linking, and therefore, is not very often used.[61-63]

Another method that can be performed under milder conditions (pH 5-6) is a coupling reaction with free COOH groups in the presence of a water-soluble carbodiimide when a PEG-hydrazide is used.

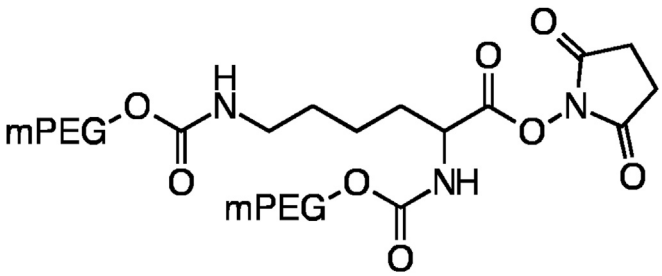

Figure 6. Branched PEG-N-hydroxysuccinimide (PEG2-NHS) lysine–based reagent for amine PEGylation.

Releasable PEGylation Reagents

Most of the reagents described in the review above are designed to form a stable covalent linkage between the PEG chain and the protein moiety. However, in many cases, the derivatization is leading to conjugates having only very low or reduced functional activity. For that reason, new concepts with degradable linkages between the protein and the PEG were designed. These releasable drug delivery systems are acting as a prodrug. In theory, the PEG polymer is released over time in the circulation and the activity of the therapeutic protein can be recovered.

The first releasable PEGylation technology described in the literature used chemistry effects based on backbone esters such as PEG-SS or PEG-succinimidyl glutarate. These backbone esters are susceptible to acid or basic hydrolysis or to enzymatic cleavage. For this type of releasable system, the ester cleavage results in formation of a "tag" staying on the protein, which could affect receptor affinity or act as a hapten, enhancing immunogenicity.

Greenwald et al. developed and published a method to avoid the formation of a tag on the protein using systems which allow a complete release of the native drug protein while maintaining full activity.[64,65] The releasable linker systems of Greenwald et al. use an ester group attached to a PEG which acts as a trigger. The release of the native drug is accomplished by a subsequent chemical reaction. The formed intermediates undergo an elimination or cyclization reaction and the native drug is released. These releasable PEG ester systems include an aliphatic Bicin linker and an aromatic benzyl elimination linker.

Another releasable PEGylation approach was described by Zalipsky et al.[66] This disulfide-based system releases the PEG polymer by a reduction process and a subsequent elimination mechanism by which the residual linker is detached from the protein drug.

A further important releasable linker system is based on 9-fluorenylmethyl carbonate (FMOC) chemistry which is used as a protecting group in the organic synthesis of peptides. This FMOC protecting group was introduced in PEGylation chemistry by Tsubery et al.,[67] who published the synthesis of 9-hydroxymethyl-2-(amino-3-maleimidopropionate)-7-sulfo fluorene N-hydroxysuccinimidyl carbonate to create a releasable linker system. This reagent was used by Peleg-Shulman et al.[68] for PEGylation of INF

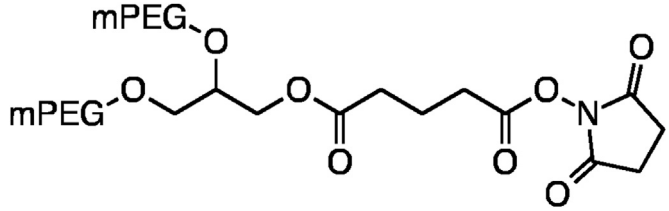

Figure 7. Structure of branched PEG-NHS reagents with an asymmetric 1,2-substitution for amine PEGylation.

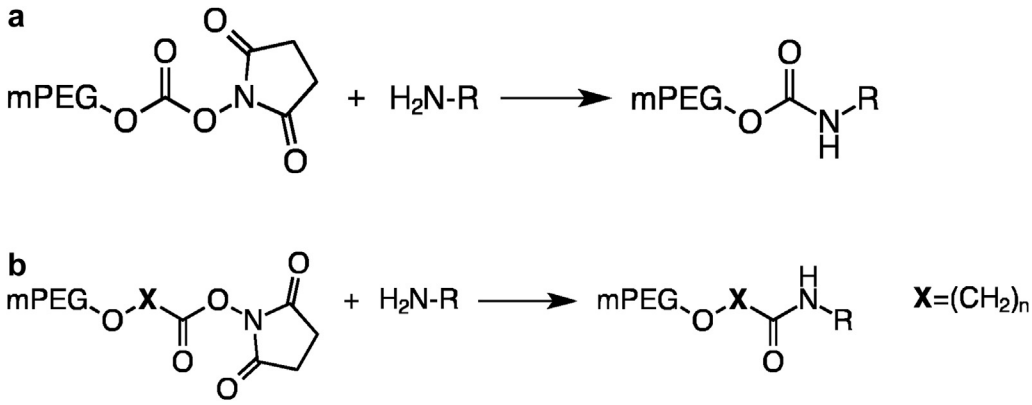

Figure 8. PEGylation of primary amines in proteins (H2N-R) leading to formation of (a) carbamate and/or urethane linkage or (b) amide linkage.

alpha-2. The FMOC ring system contains a maleimide group to which PEG-SH groups can be easily coupled. The FMOC-NHS groups can react with primary amino groups to form a releasable linkage. In circulation, the free drug can be released by base catalysis via β-elimination. The protein is released in native form, therefore, a complete recovery of the functional activity is possible, and no tag is remaining on the protein.

PEG Reagents for Carbohydrate PEGylation

Glycosylation of proteins occurs in nature either at asparagine in the case of N-glycosylation or at serine and/or threonine residues for O-glycosylation. Carbohydrate residues are attractive PEGylation sites because a PEG can substitute for some of the natural functions of glycosylation such as protease protection and improved solubility. Carbohydrate residues may have an important role in folding and trafficking of a protein from the site of synthesis to the site of function but may not be required for activity at the final location. Thus, attaching PEG to the end of a carbohydrate chain may not interfere with activity or binding as much as a direct attachment to the protein backbone. Another reason for attachment to carbohydrate residues is that these residues frequently are distant from functionally important parts of the proteins and, as a consequence, little steric hindrance may be expected when attaching a PEG polymer there. Carbohydrate chains may also provide an opportunity for site-selective PEGylation such as for B domain–deleted FVIII.[69]

A general strategy for PEGylation of carbohydrates and carbohydrate moieties in proteins is to oxidize the carbohydrate under mild conditions, e.g., with $NaIO_4$.[70] Under these conditions, the vicinal hydroxyl groups of N-glycans are oxidized in the Malaprade reaction to form an active aldehyde group, which is the target for the further modification reaction.[71] The PEGylation reaction can be performed by reductive amination to form Schiff bases by reaction with PEG amine, which can be subsequently reduced with sodium cyanoborohydride to form stable amino linkages. More common is the use of specific chemistries like the reaction with hydrazides to form hydrazones, semicarbazide, or thiosemicarbazides to form semicarbazones or the use of aminooxy groups to form a stable oxime linkage.[72]

An enzymatic method to PEGylate proteins was recently developed. This so-called GlycoPEGylation is an enzymatic strategy to directly PEGylate O-glycosylation sites of *Escherichia coli*–expressed proteins[73] or existing carbohydrate chains of proteins produced in mammalian cell culture.[74]

Two enzymes are required for GlycoPEGylation of *E coli* expressed proteins (nonglycosylated). Serine or threonine residues are conjugated to N-acetylgalactosamine (GalNAc) using recombinant polypeptide O-GalNAc-transferase. The second step requires a sialyltransferase (STgGalNAc-1) to link a sialyl PEG derivative to the conjugate. This process was demonstrated for cytokines such as granulocyte colony-stimulating factor (GCSF), INF-α2b, and GM-CSF, but none of the conjugates has been commercialized.[75]

GlycoPEGylation of a glycoprotein requires 3 steps: (1) removal of the carbohydrate terminal sialic acids using a sialidase (neuraminidase) exposing galactose residues, (2) enzymatic PEGylation at the galactose using an enzyme that attaches sialic acid-gly-PEG, (3) capping any residual galactose residues with sialic acid using the enzyme ST3Gal III and cytidine monophosphate sialic acid. This approach was used on the coagulation enzyme rFVIIa,[76] but the corresponding clinical phase III study was discontinued for reasons that are publicly unknown. It has also been applied to blood coagulation factor IX[77] and an O-glycosylation site on the truncated B domain of an engineered blood coagulation factor VIII.[69]

Marketed PEGylated Biopharmaceuticals; Chemistry and Toxicology

Since 1990, 12 PEGylated biopharmaceuticals have been introduced into the market place as drugs for human use (Table 1). Eleven are PEGylated protein biopharmaceuticals and one is a PEGylated aptamer (Macugen) administered intravitreally. The PEG components of these biopharmaceuticals vary widely in size,

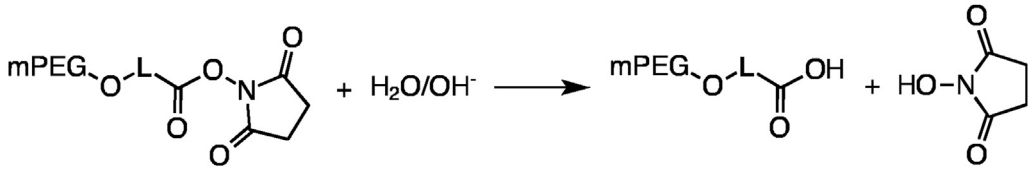

Figure 9. Hydrolysis reaction of N-hydroxysuccinimide–activated PEG reagents yielding the corresponding PEG acid and N-hydroxysuccinimide (L: linker group between PEG and active N-hydroxysuccinimide head group).

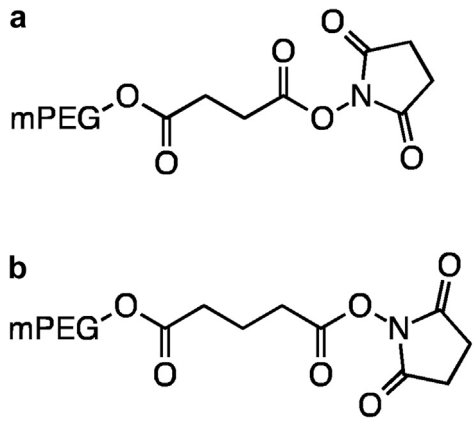

Figure 10. Structures of (a) PEG-SS and (b) PEG-SG reagent.

branching structure, and attachment type. Adagen®, Krystexxa®, Neulasta®, Oncaspar®, PEG-Intron®, Somavert®, and Plegridy® are PEGylated with PEG molecules having molecular weight of 5-20 kDa. Cimzia®, Macugen®, Mircera®, Omontys®, and Pegasys® are PEGylated with PEG molecules of 30 or 40 kDa.

Marketed PEGylated Biopharmaceuticals Modified With Acylating PEG Reagents

Adagen® (Pegademase) and Oncaspar® (Pegaspargase)

The PEGylation technology used in the first 2 marketed PEG protein products pegademase[78] (Adagen®) and pegaspargase[59] (Oncaspar®) used 5 kDa PEG,[59,79-81] which results in a stable amide linkage of multiple PEGs attached to the protein ε lysines.

Adenosine deaminase (ADA) is an enzyme involved in purine metabolism. Some mutations in ADA result in failure of expression and ADA deficiency is one cause of severe combined immunodeficiency. A 41 kDa molecular weight ADA protein of bovine intestinal origin was considered for replacement of the deficient enzyme, but it was readily cleared from human plasma. Subsequent modification with 11-17 molecules of 5 kDa SS-activated PEG (mPEG-SS) extended the circulating half-life and decreased the immunogenicity of this nonhuman protein. The resulting PEGylated molecule is marketed as pegademase (Adagen®) for treatment of severe combined immunodeficiency.

PEGylated L-asparaginase (Oncaspar®), known generically as pegaspargase (other synonyms: asparaginase, PEG-asparaginase, pegaspargasum, [monomethoxypolyethylene glycol succinimidyl] 74-L-asparaginase), is used to treat acute lymphoblastic leukemia in patients who are hypersensitive to the native enzyme.[59] The *E coli*–derived enzyme, a 141 kDa homotetramer,[82] produced high levels of hypersensitivity in children (~20%), which could be temporarily voided (~2% hypersensitive) by use of *Erwinia caratovora*–derived enzyme (134 kDa tetramer).[83] A portion of 15%-20% of children with prior reaction to *E coli*–derived enzyme eventually reacted to the *Erwinia* version as well. The allergic reactions varied from local injection site reaction to anaphylaxis. PEGylation of asparaginase (*E coli* version) was shown to reduce antibody formation in animal models compared with native drug. Early preclinical work using cyanuric chloride–activated PEG[84] demonstrated low specific activity for PEG asparaginase, but retention of activity could be improved using 5 kDa mPEG-SS (Fig. 11).[59]

Table 1
Marketed PEGylated Biotherapeutics Approved in the United States and/or Europe

Brand Name, Generic Name	Molecular Weight of Drug (kDa)	PEG Size (kDa) [Number of PEGs per Drug Molecule]	Patient Dose	Indication (Year Approved)	Human Route of Administration/ Duration of Treatment
Adagen®, pegadamase	96-126	5 [11-17]	Patient dependent (approximately 15 IU/ kg/wk)	Severe combined immunodeficiency (1990 US)	IM/chronic
Oncaspar®, pegaspargase	483-548	5 [69-82]	2500 IU/m^2	Leukemia (1994 US)	IV or IM
Somavert®, pegvisomant	42-52	5 [4-6]	Maximum daily SC dose: 30 mg. Not intended for pediatric population.	Acromegaly (2002 EU, 2003 US)	SC/chronic
Krystexxa®, pegloticase	540	10 [9 per homotetramer (4)]	8 mg/2 wk	Chronic gout (2010 US; 2013 EU)	IV infusion/once every 2 wk
Peg-Intron®, PEG-interferon alpha 2b	31	12 [1]	0.5-1.0 μg/kg SC 1×/wk (1.5 μg/kg SC 1×/wk in combo w/ribavirin)	Hepatitis C (2001 US; 2000 EU)	SC/6 mo or 1 y
Neulasta®, pegfilgrastim	39	20 [1]	6 mg/ Q 3 weeks	Neutropenia (2002 US, EU)	SC
Mircera®, CERA; PEG-EPO	60	30 [1]	0.6 μg/kg once every 2 weeks	Anemia/chronic renal failure (2007 US, EU)	IV and SC/chronic
Omontys®, voluntary recall reported 2/24/2013	45	40 [1 branched]	0.04 mg/kg body weight, once a month	ESA: anemia/chronic renal failure (voluntary recall)	IV or SC
Macugen®, pegaptanib	50	40 [1 branched]	Intravitreal 1.6 mg	Wet form of age-related macular degeneration (2004 US, 2006 EU)	Intravitreal/repeated treatment
PEGASYS®, PEG-interferon alpha 2a	60	40 [1 branched]	2.7 and 3.6 μg/kg 1× week for 48 (cycle repeated)	Chronic hepatitis C, B (2002 US, EU)	SC/chronic
Cimzia®, certolizumab pegol	91	40 [1 branched]	400 mg up to 1× per month (after loading dose)	Chronic, moderate to severe RA, Crohn's disease, axial spondyloarthritis and psoriatic arthritis (2008/ 2009 US; 2008 EU)	IV and SC/chronic
Plegridy®, peginterferon beta-1	44	20 [1]	125 μg every 14 days (after loading dose)	Relapsing forms of multiple sclerosis (2014 US, EU)	SC

All data from publicly available sources (US: SBAs, US PI; EU: EPARs and SmPC).
SC, subcutaneous.

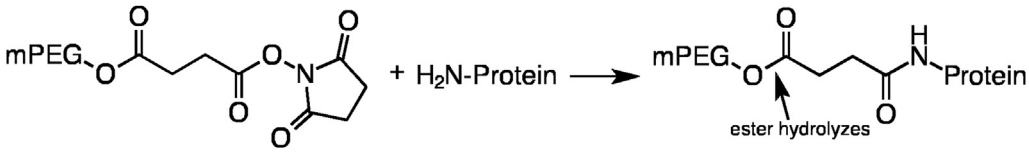

Figure 11. Conjugation of PEG to a protein by a SS linker. Ester hydrolysis may result in removal of PEG at the position indicated by the arrow.

The SS linker between PEG and the protein group contains an ester group, which has limited stability *in vitro* at neutral pH and is subject to *in vivo* hydrolysis by endogenous esterases.[85] This mPEG-SS protein conjugate hydrolysis can result in a "tag" still bound to the protein amines[10,86-88] (Fig. 11). For Adagen® and Oncaspar®, no information on toxicology studies was publicly available on the FDA or European Medicines Agency (EMA) web pages.

Krystexxa™ (Pegloticase, Formerly Puricase®)

Krystexxa® is mammalian engineered uricase (urate oxidase) used to treat gout. Humans do not naturally have the enzyme, and non-mammalian sources of the enzyme are highly immunogenic. PEGylation of the mammalian-derived enzyme may reduce some of the immunogenicity but does not eliminate it completely.[89-91] The exact number of PEGs bound to the protein varies according to the literature. The protein is a tetramer of four 34 kDa subunits (~136 kDa total protein). According to the BLA submission,[92] each subunit has approximately 9 molecules of 10 kDa PEG after lysine conjugation (approximately 360 kDa of PEG) with a total conjugate size of approximately 500 kDa. Publicly available documents do not describe the PEG reagent used, but the statement of a nonproprietary name adopted by the United States Adopted Name council shows a carbamate linkage to the protein with the conjugate structure shown in Figure 12.

Pivotal nonclinical intravenous (IV) toxicology studies were conducted in dogs, whereas shorter term studies were conducted after subcutaneous (SC) and/or intramuscular (IM) administration in rats and dogs (Krystexxa FDA SBA, EMA EPAR). Dose- and time-dependent cellular vacuolation of some cells attributed to PEG was observed. In dogs, observations of vacuolated cells in the splenic red pulp were found at all doses (0.5, 1.5, 5.0 mg/kg/week) in the 12-week study without reversal after 6 weeks of treatment-free period.

Partially reversible vacuolation was also seen in the spleen of the rat. Vacuolation was considered to be nonadverse because of the lack of cell damage or inflammatory infiltration. Another review concluded that the effect should be evaluated further.

In a 39-week study, followed by 12 weeks of recovery in dogs (0.4, 1.5, 10 mg/kg/week intravenously) cellular vacuolation was observed in hematoxylin and eosin–stained tissues, mainly in the high dose in the adrenal cortex, duodenum, jejunum, liver Kupffer cells, and heart without recovery of vacuolation after 12 weeks. Detailed immunohistochemistry evaluation determined that PEG and uricase were present in vacuoles in macrophages in tissues of duodenum and/or jejunum, liver, and spleen. Vacuoles observed in the adrenal cortex and heart may not be associated with macrophages. There was also an indication of uricase and/or PEG staining in these organs not inside vacuoles. Importantly, immunohistochemistry staining determined that the presence of uricase and PEG in vacuoles and tissues at the end of the recovery period was absent or reduced. It was assumed that vacuoles in macrophages were not of toxicologic relevance, whereas the impact of the vacuoles in adrenal cortex and heart was unclear, and additional information may be needed.

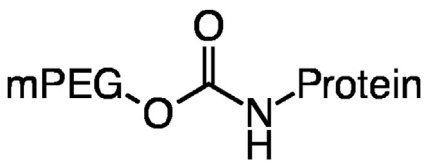

Figure 12. Conjugate structure of pegloticase (Krystexxa®).

In the same experiment, intravascular and/or interstitial proteinaceous fluid was collected at sacrifice (Krystexxa® FDA, SBA) from organs where vacuolation was seen and analyzed for uricase and PEG staining. At the end of the dosing period, a dose-dependent increase in staining was seen for both. Staining was mostly absent after 12 weeks of recovery. Vacuolation was also observed in the embryo–fetal development rat study in 1 dam (1 of 8) at 10 mg/kg and in all dams at 40 mg/kg. No other effects or any health impact due to the vacuolation was seen in any of the animals.

To further assess vacuolation, cells from rats dosed for 4 weeks with Krystexxa® doses of 4.3, 10.2, and 34 mg/kg were tested *in vitro*. Significant decreases in the tumor necrosis factor (TNF) response to a lipopolysaccharide (LPS) challenge were seen in total splenocytes and macrophages. The effects were most prominent in the high-dose group but were also observed for the lower dose groups. Because there were no vacuoles reported in the low- and mid-dose groups, it was difficult to establish a functional relationship between TNF-release on LPS challenge and the presence of vacuoles. Vacuolation of nonphagocytic cells (aortic endothelium and adrenal cortex) along with the reduced TNF response to LPS was discussed in the US package insert. For a detailed discussion of PEG-related cellular vacuolation and nonclinical safety, see the review by Ivens et al.[21]

PEG-Intron® (PEG INF-α2b)

PEG-Intron®, a mono-PEGylated INF-α2b, is synthesized using a 12-kDa succinimidyl carbonate PEG reagent (mPEG SC-12 kDa). The mPEG SC reagent forms a covalent carbamate and/or urethane linker with amine groups on the protein (Fig. 13).

The commercial product PEG-Intron® is a mono-PEGylated product but consists of 14 positional isomers. The distribution of positional isomers and activity of resultant conjugates is dependent on the PEGylation reaction pH. The final product PEG-Intron® is modified at histidine[34] (48%), cysteine[1] (13%), and the remainder at various lysines,[31,49,83,112,121,131,133,134,164] serine,[163] tyrosine,[129] and histidine[7].[93]

The optimum reaction condition for 5-kDa PEG-SC with the model compound N-acetyl lysine is pH 9.3,[58] whereas under stronger base conditions, the reagent is unstable. Even at pH 9.3, degradation via Lossen rearrangement yielding β alanine and β alanine derivatives from ring opening side reactions is possible.[57] The PEGylation reaction with INF-α2b was tested from pH 5.4 to 10. Above pH 8, the PEGylation was random and the side chains of lysines were modified. Performing the PEGylation at lower pH minimized the side reactions.

Under acidic conditions, the PEGylation of INF occurred predominately at the N-terminus. Screening of PEG sizes, reaction conditions, and resultant conjugate properties led to selection of pH 6.5 for the PEGylation.[93-96] At pH 6.5, PEGylation occurred largely at histidine 34[46,97,98] (Fig. 14). This linkage to the imidazole

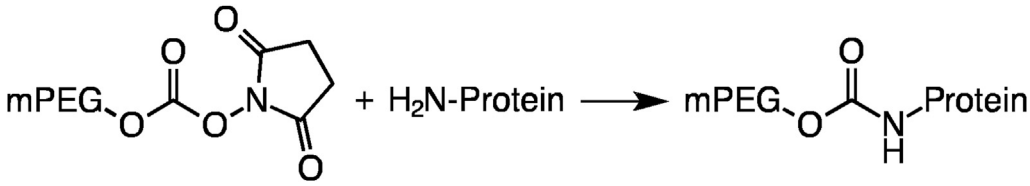

Figure 13. PEGylation chemistry used for synthesis of PEG-Intron®; use of succinimidyl carbonate PEG reagent (mPEG SC).

ring, although covalent, is labile under *in vitro* basic (hydroxylamine) conditions, but the half-life of the conjugate was sufficiently long *in vivo* to allow for a once-a-week dosing schedule.

The acute and chronic toxicity of PEG-Intron was assessed in mice, rats, and monkeys. A 4-week monkey toxicity study with SC dosing every other day and several studies with SC and IM administration were conducted. Different to most toxicology programs conducted for the marketed drugs, mPEG alone was also tested in acute IV and SC studies in mice and rats, in 13-week toxicology studies in rats (SC administration twice weekly at doses up to 2276 μg/m^2/week) and monkeys (SC administration twice weekly at doses up to 2276 μg/m^2/week) and in embryo–fetal development studies in rats and rabbits. No cellular vacuolation or other effects related to PEG were seen (PEG-Intron® EMA EPAR, FDA SBA).

Pegasys® (PEG INF-α2a)

Pegasys® is approved for the treatment of patients with chronic hepatitis C or chronic hepatitis B. Initially, the protein was PEGylated via a urea linkage with an mPEG 5-kDa reagent.[99-101] A cation exchange method was developed to separate all 11 mono-PEGylated isoforms. Peptide mapping was used to verify that all 11 lysines on the native protein were PEGylated but not the N-terminus. Unlike the positional isomers described previously for INF α-2b, the individual conjugates did not show a particular differentiation in terms of biological activity.

To avoid rapid renal filtration of small proteins and peptides with a cutoff at approximately 60 kDa (albumin),[13] mitigation of renal clearance is obtained by increasing the hydrodynamic volume of a PEGylated peptide or protein. A series of INF-α2a conjugates were prepared to determine the effect of PEG size and architecture using second-generation PEG reagents in a rat pharmacokinetic model. Using medium-sized PEGs, it was found that a single linear PEG 20 kDa had the same effectiveness as a 20-kDa branched reagent. Increasing the total PEG size to 40 kDa using either di-PEGylated 20 kDa, branched reagent or PEGylating the protein with a single 40-kDa branched reagent showed improvement over the mono-PEGylated 20-kDa conjugates. The branched PEG displayed a smaller volume of distribution, was more resistant to proteolytic digestion, had greater thermal stability, and improved pH stability relative to linear PEG conjugates. A single large PEG was preferred when PEGylation occurs at multiple sites.[102]

PEGylation of INF-α2a (Pegasys®) with a 40-kDa branched PEG N-hydroxysuccinimide yielded a stable amide linkage to the protein via the surface accessible ε-amine of lysine residues. The branching PEG structure was achieved by using lysine to link the 2 PEG chains as shown in Figure 15. One PEG chain is attached to the lysine α-amino group and the other chain to the ε-amino group.[103] Although the urethane bonds are generally stable, site-specific hydrolysis of the lysine branched PEG can occur under basic conditions.[104]

Pegasys® is mono-PEGylated with a single 40-kDa branched PEG but consists of 4 major positional isomers, Lys^{31}, Lys^{121}, Lys^{131}, and Lys^{134} accounting for 94% of the total synthesis yield. The remaining minor positional isomers are PEGylated at Lys^{70} and Lys^{83}. As with the smaller 5-kDa conjugate, the N-terminal cysteine, which is disulfide bonded to Cys^{98} was not PEGylated.[105] The final conjugate contained 4 major mono-PEGylated species of 11 possible lysines and the N terminus. The overall *in vitro* bioactivity of the 40-kDa conjugate was only 7% of native protein.[106] Structural and biophysical studies found that PEGylation did not disrupt the three-dimensional structure of any of the 6 positional isomers.[39] The biophysical studies provided evidence that PEGylation increased the effective hydrodynamic volume, yielded slightly higher temperature stability, and reduced tendency toward aggregation. Additional structural kinetic and thermodynamic analysis determined that the PEGylation of the conjugate as a whole and the individual positional isomers did abolish receptor binding, but reduced the affinity primarily due to a change in the association rate.[107] Changes in conjugate-binding affinity correlated well with the location of the PEG relative to the known receptor-binding site residues.

The toxicology program for Pegasys® was designed to bridge to non-PEGylated INF α-2a. Studies included SC studies in Cynomolgus monkeys, an SC 4-week monkey study with twice weekly doses up to 562.5 μg/kg or daily up to 600 μg/kg (PEG up to 400 μg/kg) and a 13-week monkey study dosed SC twice weekly up to 150 μg/kg of Pegasys® (resulting in PEG up to 100 μg/kg). Toxicity observed was characteristic of INF-α, and no PEG-related histologic or other changes were observed in the toxicity studies (Webster et al., 2007, 2009[82]; Pegasys® FDA SBA, EMA EPAR).

Pegasys® was cleared mainly via the liver, its target organ, and the kidney excreted the metabolic products. In a route of excretion study with ^{14}C-lysyl-labeled PEG (40 kDa) IFN-α-2a, 51% of the total radioactivity dose was found in urine, and 9.6% in feces within 14 days after dosing. SC and IV doses gave similar results.

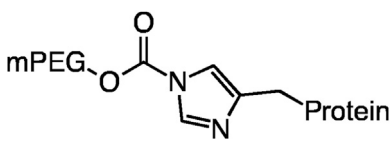

Figure 14. PEGylated histidine residues which are labile under basic conditions.

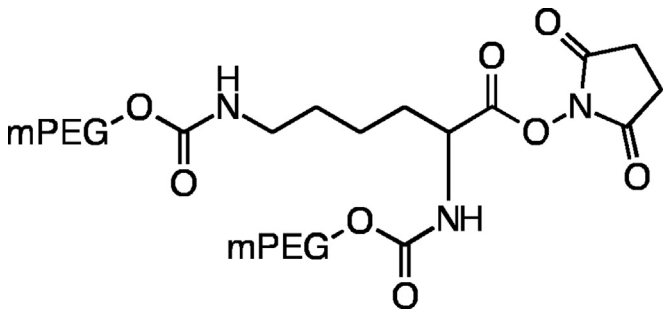

Figure 15. Branched PEG reagent used for synthesis of Pegasys®.

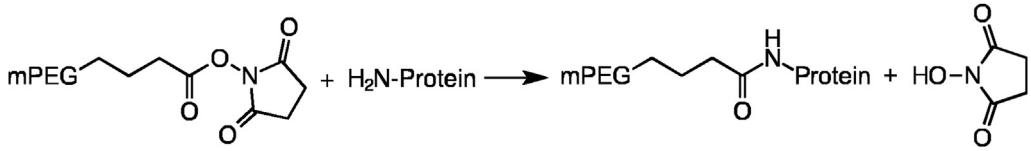

Figure 16. PEGylation reaction used for synthesis of Mircera® with an mPEG succinimidyl butanoatereagent attaching primarily lysine 52 and 56.

Somavert® (Pegvisomant)

The third marketed product using multiple 5-kDa PEGs is pegvisomant (Somavert®),[108,109] a recombinant analog of human growth hormone (GH) that is first in the class of GH receptor antagonists.

Both GH and the analog protein used in pegvisomant (B-2036) are 22-kDa proteins comprising a single 191 amino acid chain[110] and both have half-lives of less than 20 min. Addition of PEG to achieve a total molecular weight of more than 40 kDa extends the half-life to longer than 70 h, but the lysine-conjugated PEG interferes with receptor binding. A total of 8 additional amino acid substitutions in binding site 1 were made to increase the affinity for the GH receptor. The amino acid substitutions, coupled with an average of 4-6 stable 5-kDa PEGs that are not subject to esterase hydrolysis, resulted in the marketed drug pegvisomant.[111] The three predominant PEGmers of pegvisomant are ~42 kDa, 47 kDa, and 52 kDa species resulting from the protein component (B-2036) with 4, 5, or 6 PEGs bound. The package insert indicates the most probable sites of PEG attachment are the N terminus Phe^{1} and $Lys^{38,120,140,158}$. However Lys^{145} is a preferred site over $Lys^{120,112}$ in other sources.

IV and/or SC nonclinical toxicology studies up to 13-weeks' duration were conducted in mice and Rhesus monkeys. A 2-year carcinogenicity study was conducted after approval. As seen from the publicly available information (Somavert EMA EPAR, FDA SBA), cellular vacuolation was reported in macrophages at the injection sites in mice, but not in monkeys, and was attributed to PEG. Vacuolation in rats was also observed in submandibular and mesenteric lymph nodes, in splenic reticuloendothelial cells, and in a few animals in both ovarian interstitial cells and uterine cells. These findings were not attributed to PEG despite the fact that they were not observed in controls. Some of these effects might reflect the presence of PEG, but the inconsistency of the findings and the nonspecific nature of vacuoles observed by light microscopy means that they cannot be definitely attributed to PEG.

Mircera® (Methoxy Polyethylene Glycol-Epoetin Beta)

Mircera® is an erythropoiesis-stimulating agent with extended half-life, approved in 2007 for IV and SC treatment of anemia associated with chronic renal failure.

Erythropoietin is a highly glycosylated 165–amino acid protein that stimulates erythropoiesis. Mircera® is a mono-PEGylated extended half-life version using mPEG succinimidyl butanoate 30K (30-kDa PEG)[113-115] (Fig. 16). PEGylation occurs primarily at Lys 52 and Lys 56.

Toxicology studies were conducted in rats with a duration up to 26 weeks (0, 0.3, 1.0, and 3.0 μg/kg) and in dogs up to 13 weeks (0, 0.5, 1.5, and 15 μg/kg) once weekly IV or SC. No PEG-related changes were observed in these toxicity studies. In rats, both the parent protein and the 30-kDa PEG were shown to be excreted in urine (Mircera® FDA SBA, EMA EPAR). Overall, the doses in the toxicology studies and also the clinical doses are relatively low when compared to some other PEGylated products.

Omontys® (Peginesitide, Formerly Hematide™; Voluntary Recall Reported 2/24/2013)

Another erythropoiesis-stimulating agent, Omontys®, is a PEGylated peptide that has no sequence homology to erythropoietin.[116] Omontys® consists of 2 identical 21 amino acid chains peptides, covalently bound via a linker to a 40-kDa lysine branched PEG for a 45 kDa overall size (Fig. 17). The structure of Omontys® is unique in that a linker comprised iminodiacetic acid and β alanine is used to connect the 2 peptides creating a peptide dimer. The peptide dimer is then connected by a single coupling point to the branches.[117] The construction of the linker makes only one positional isomer possible.

Toxicology studies were conducted by SC or IV dosing in rats for up to 6 months (dosed every third week SC or IV in separate studies at 0, 0.1, 1.0, and 10 mg/kg) and in Cynomolgus monkey for up to 9 months (dosed every third week at 0, 0.2, 2.0, 20.0 mg/kg). Vacuolation was observed in the choroid plexus (described as mild cytoplasmic vacuolation) in a 4-week monkey study in 3 of 6 animals of the high dose of 50 mg/kg. In the 9-month monkey study at 20 mg/kg "brain, infiltration of mononuclear cells" was observed at the 3-, 6-, and 9-month necropsies and at 2.0 mg/kg at the 6- and 9-month necropsies (Omontys FDA SBA). It is not clear from the SBA whether this represents the same finding as observed in the 4-week study.

Macugen® (Pegaptanib)

Macugen® is a PEGylated 28 nucleotide aptamer for intravitreal treatment of wet age-related macular degeneration in the eye developed using SELEX technology. Final iterations using SELEX to improve affinity resulted in 3 new potent aptamers, which were not substantially differentiated on the basis of *in vitro* activity alone.[118] Aptamers generally have a short half-life *in vivo* as they are subject to cleavage by nucleases. This requires modification, for example, by PEG, to make them applicable as a pharmaceutical product. The candidate which was most effective at inhibiting *in vivo* vascular leakage (guinea pig model) was chosen for PEGylation with a single 40-kDa lysine branched PEG attached to an amine at the 5′ end. Attachment occurs via a phosphodiester bond.

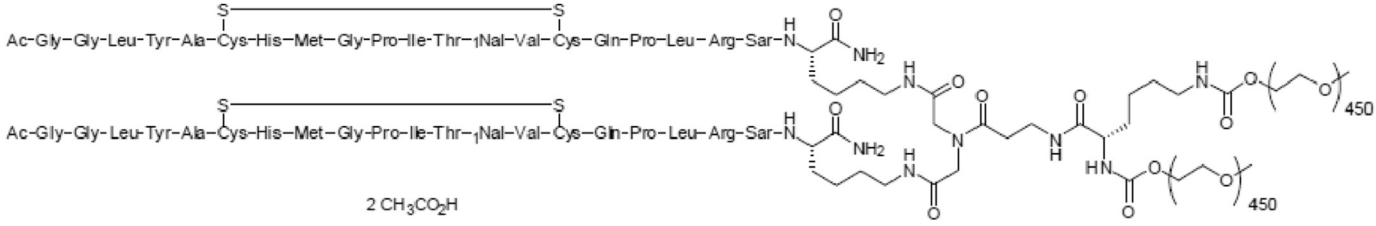

Figure 17. Structure of Omontys®, a PEGylated peptide dimer. A branched linker connects the 2 peptides to a branched 40-kDa PEG.

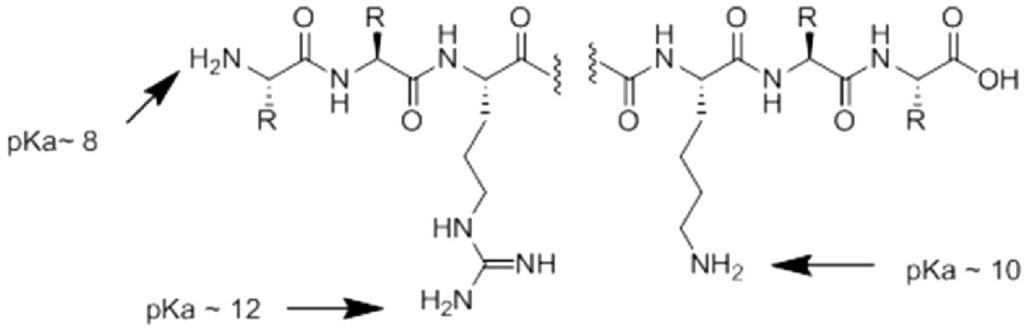

Figure 18. Differences in pKa of N terminal amine versus the ε amino acid side chains allows site-directed PEGylation controlled by pH.

The structure has been described in detail.[119] The 5′ terminus of the selected aptamer was converted to a primary amine using trifluoroacetyl-protected pentylamine phosphoramidite. Coupling with 40-kDa PEG2 N-hydroxysuccinimide ester yielded a conjugate, which had an affinity for vascular endothelial growth factor of 200 pM and inhibited vascular endothelial growth factor–induced permeability[118] with a greater efficacy than the non-PEGylated aptamer.

In monkeys, the elimination half-life of pegaptanib was 9.3 h when given by a single IV injection. SC injection provided a somewhat improved half-life of 12 h.[120] Additional studies examined the clearance in monkeys when injected into the vitreous humor of the eye. Pegaptanib was found to clear from the eye through plasma clearance. At a dose of 0.5 mg, the drug cleared from the eye with a half-life of ~94 h with no observed toxicity or antibody responses. Active drug could still be detected in the eye for at least 28 d.[121] Thus, the thick viscous vitreous environment of the eye contributed to the long effectiveness of the drug.

Various intraocular studies and one additional systemic IV 13-week toxicity study in the rat were conducted. No drug-related toxicity was seen systemically or in the eye after intraocular administration. Vacuolation was seen at 1.0 mg/kg IV and above in macrophages in the bone marrow, kidney, hepatic Kupffer cells, ovaries, salivary glands, pancreas, and testis (Macugen EMA EPAR, FDA SBA) without tissue damage.

Alkylating Agents: Marketed PEGylated Biopharmaceuticals Modified With Alkylating PEG Reagents

Neulasta® (Pegfilgrastim)

Neulasta® is a PEGylated form of recombinant human GCSF and was approved for the treatment of neutropenia. Neulasta® is made with 20-kDa PEG and application of aldehyde chemistry to GCSF using selective N terminal amine conjugation at low pH.[51,122-126]

Coupling chemistries using alkylating PEG reagents yield stable amine linkages to the protein without reducing the charge on the protein. Alkylating agents such as PEG aldehydes may be used to generate “selective” PEGylation at the N-terminus of proteins. In general, the pKa of the N-terminal amine is lower than the pKa for other amine side chains[3,63] (Fig. 18).

Acidic pH favors PEGylation of the N-terminus if accessible. Higher pH allows more PEGylation to occur at the lysine side chains while still retaining the charge on the nitrogen where the protein is conjugated. A second advantage of the aldehyde reagent is stability in water. The reaction proceeds through reversible formation of a Schiff base. Addition of a reducing agent such as cyanoborohydride stabilizes the linkage and a stable secondary amine is formed (Fig. 19).

Initial work with 6-kDa PEG tested the effects of positional isomers. PEGylation at the N-terminus provided both the best retention of functional activity and prolongation of *in vivo* activity. Conjugates created by alkylation (secondary amine; Fig. 20a) and acylation (amide linkage; Fig. 20b) had similar *in vitro* and *in vivo* activities, but the conjugate formed using the aldehyde reagent was more stable and less prone to aggregation.[127]

With Neulasta®, no cellular vacuolation was observed (Neulasta EMA EPAR, FDA SBA) in SC or IV rat studies up to 6 months with once weekly dosing (highest SC dose, 1.0 mg/kg; IV dose, 0.3 mg/kg) or in monkey studies of 4-weeks' duration (highest dose, 0.75 mg/kg).

Plegridy® (Peginterferon Beta-1, BIIB017)

Plegridy® is a PEGylated form of INF beta-1a (IFN β-1a), approved for the treatment of relapsing multiple sclerosis. The non-PEGylated IFN β-1a is also marketed under the name Avonex® (IFN β-1a) for the same indication. Although Avonex® is recommended for once weekly IM administration, the dosing frequency for Plegridy® is once every 14 days with SC administration. Plegridy® (peginterferon beta-1a) is an INF beta-1a to which a single, linear 20 kDa methoxy poly(ethyleneglycol)-O-2-methylpropionaldehyde molecule is covalently attached to the alpha amino group of the N-terminal amino acid residue via

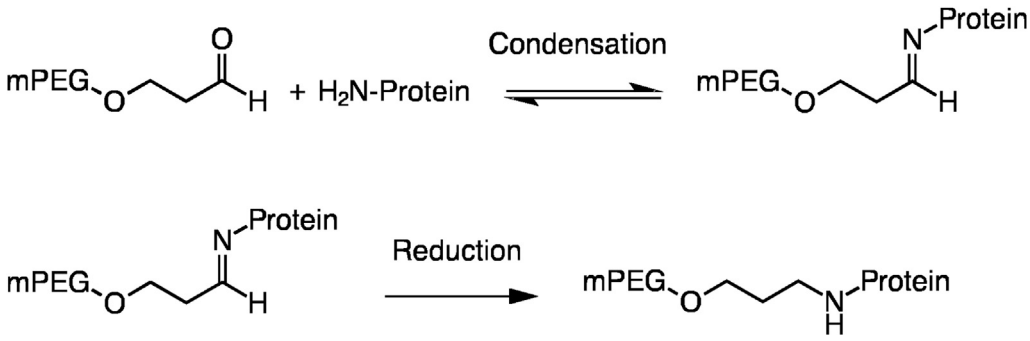

Figure 19. Two-step PEGylation by aldehyde chemistry: first step: formation of a labile Schiff base; second step: reduction of Schiff base to a stable secondary amine.

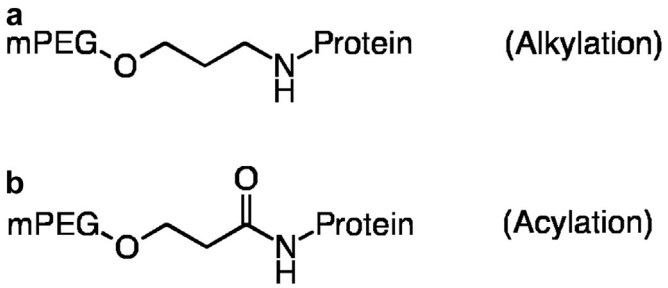

Figure 20. Schematic structures of PEGylated proteins generated by (a) alkylation and (b) acylation.

reductive amination with sodium cyanoborohydride as the reductant (Fig. 21).[128] The PEGylation process was similar to the one used for Neulasta®. The IFN β-1a portion of Plegridy® is produced as a glycosylateted protein using genetically engineered Chinese hamster ovary cells into which the human INF beta gene has been introduced. The amino acid sequence of the recombinant INF beta-1a is identical to that of human INF beta. The molecular mass of Plegridy® is approximately 44 kDa. The increase in apparent mass of Plegridy® because of PEG compared with INF beta-1a has been shown to contribute to the reduced clearance *in vivo*.

Toxicity studies were conducted in Rhesus monkeys. The ability to assess peginterferon beta-1 in nonclinical studies in other species was limited by a lack of biological activity in mice and rats and rapid development of neutralizing antidrug antibodies in the biologically relevant species (Rhesus monkey). In the pivotal 5-week study, peginterferon beta-1 was administered at doses of 0, 2, 10, and 100 μg/kg/week SC and 100 μg/kg/week IM for 5 weeks with additional animals included to demonstrate reversibility in a 4-week recovery period. Findings were consistent with the expected pharmacologic effects of an IFN β-1a, for example, increased body temperature and reduced circulating lymphocytes. No cellular vacuolation was reported in these studies. No reproductive or developmental toxicity studies were conducted with Plegridy®. The embryo–fetal study conducted in Rhesus monkeys with the non-PEGylated product (Avonex) was considered relevant to Plegridy®. Drug substance of Plegridy® was tested for potential genetic toxicity in an Ames test and in an *in vitro* chromosomal aberration test with human peripheral blood lymphocytes. The result of both tests was negative. In-silico evaluation of the organic linker (mPEG-O-2 methylpropionaldehyde) resulted in a structural alert for potential genotoxicity, but this signal was not followed up by further genetic toxicity studies with the linker molecule in particular.

Thiol Modifying Agent: Marketed PEGylated Biopharmaceutical Modified With a Thiol Reactive PEG Reagent

Thiol reactive PEG reagents in the form of a maleimide are used to modify either naturally occurring thiol groups or site-specific engineered cysteines.

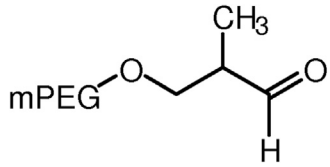

Figure 21. Structure of the linear PEG reagent used to modify interferon beta-1a in Plegridy®.

Cimzia® (Certolizumab Pegol, CDP879)

Cimzia® is a PEGylated anti-TNF recombinant antibody Fab fragment approved for treatment of chronic, moderate-to-severe rheumatoid arthritis, Crohn's disease, and axial spondyloarthritis. CIMZIA® has an engineered thiol for PEGylation.[129-132] The antibody fragment produced in a microbial *E coli* system is monovalent and then bound to a 40-kDa PEG. A PEG2MAL 40-kDa PEG reagent is bound covalently through an inserted cysteine moiety, located 3 amino acids from the C terminus of the heavy chain antibody fragment.[133] The 40-kDa branched PEG has a lysine linkage between the 2 chains and is attached to the Fab with the following reaction scheme (Fig. 22). Coupling chemistries that bind PEG to free thiol groups in the protein yield a disulfide linked conjugate. Proteins naturally have a limited number of “free” cysteines, and these may be buried or located at an active site making them unsuitable for PEGylation. Thiol-reactive agents may provide site-selective PEGylation limiting the number of positional isomers. A cysteine may be added and/or engineered into a site not involved with activity, allowing for site-selective PEGylation. The most common active group used for cysteine coupling is a maleimide linkage shown in Figure 22, where L can be a linker group between the PEG and active head group. Cimzia® is the only thiol-engineered PEG conjugate currently marketed (Fig. 23).[134]

Acute and repeated-dose toxicology studies (acute, 4, 13, 26, and 52 weeks with either IV or SC dosing once weekly) were conducted in Cynomolgus monkeys. PEG-related histologic changes were observed mainly in the reticuloendothelial system. Macrophage vacuolation (foamy macrophages) in several organs (lymph nodes, injections sites, red pulp of spleen, adrenal cortex, uterus, cervix, and choroid plexus of the brain) was seen after 26 weeks at 100 mg/kg and after 52 weeks at 50 and 100 mg/kg Cimzia®. These changes did not lead to functional deficits and were reversible within 13 weeks, except at the high dose of 100 mg/kg in the 52-week study (Cimzia® EMA EPAR, FDA SBA). Similar macrophage changes were seen in the rat, where Cimzia® is not pharmacologically active. Therefore, it can be concluded that the macrophage changes were caused by PEG and not by exaggerated pharmacologic action of the drug. Cimzia® was cleared from the circulation via proteolysis (of the protein component Fab') and renal excretion of PEG polymers.[135] *In vitro* macrophage function assays with Cimzia® indicated that the PEG moiety alone reduced phagocytosis of bacteria and fungi at high concentrations above the intended pharmacologic use (NOEL 1.0 mg/mL); some inhibition of T-cell proliferation

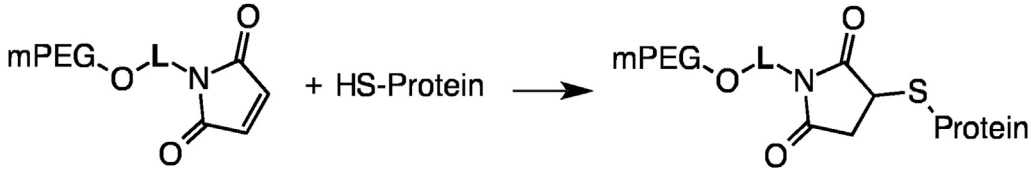

Figure 22. PEGylation reaction used for selective thiol PEGylation with a PEG maleimide reagent (L: linker group between the PEG and active head group).

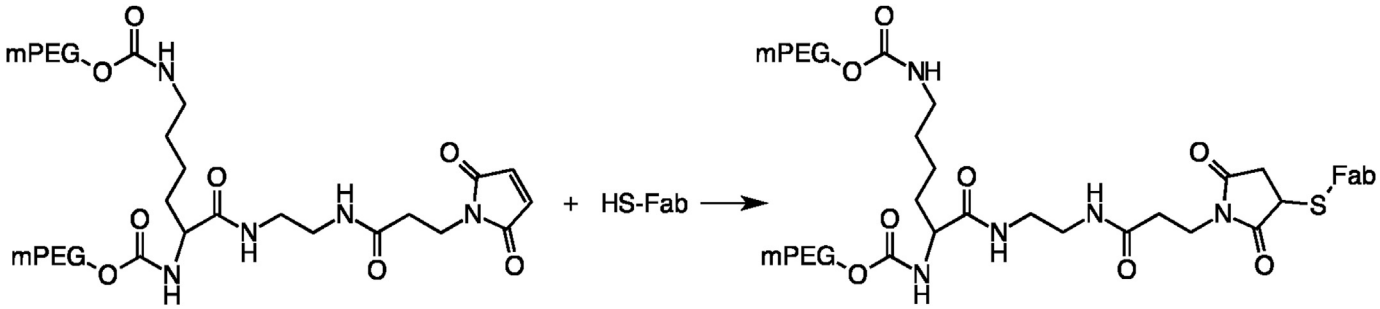

Figure 23. Synthesis and structure of Cimzia® a thiol-engineered PEGylated antibody fragment conjugate.

to a toxoid challenge in a human cell system was also seen (NOEL 1.0 mg/mL; EMA EPAR). Although vacuole formation at high doses was seen *in vivo*, no adverse effects were attributable to the vacuoles.

Conclusions

PEGylation has led to 12 approved biopharmaceuticals. Although initial PEGylation was mainly random attachment of relatively small PEG molecules (5 or 10 kDa), recently larger chain PEG molecules have been used that are targeted via a linker to distinct and specific sites in a protein or aptamer. Even more recently, cleavable linkers have been used giving the PEGylated protein a "prodrug" characteristic as the PEG is cleaved off the drug molecule during metabolism resulting in a biologically active drug.

The review of publicly available toxicology information for the 12 approved PEGylated biopharmaceuticals indicates that their toxicologic effects were derived from the active part of the drug substance rather than the PEG moiety.[21-24] The only effect attributed to PEG seen in nonclinical toxicology studies was cellular vacuolation observed with 5 of the 12 approved PEGylated biopharmaceuticals (for 2 drugs no toxicology information is publicly available). Two of the 5 products where vacuolation was seen, Somavert® and Krystexxa®, are linked to several small PEG molecules (5 and 10 kDa, respectively), whereas 3 have a single 40-kDa PEG molecule (Omontys®, Macugen®, Cimzia®) covalently bound. Vacuolation is seen mainly in phagocytic but sometimes also in nonphagocytic cells. Macrophages likely contribute to the clearance of larger PEG conjugates after phagocytosis and cellular vacuolation as an adaptive change because of uptake and removal of PEGylated biopharmaceuticals or PEG alone.[136] No functional impact of vacuolation was reported in toxicology studies and no indication of PEG-related effects have been reported from clinical trials or postmarketing surveillance with any of the approved drugs.[21] Despite uptake of large PEG protein conjugates by macrophages little is known about the molecular mechanisms by which PEGylation influences clearance mechanisms. Most proteins are retained in the vasculature as they are not filtered by the kidney. Smaller molecules and peptides may be simply hindered from renal filtration by increase in size, which would represent a rather unspecific principle. Some studies indicate specific clearance receptor interference upon PEGylation as was, for example, recently shown for human coagulation factor VIII.[137,138]

There is a clear need to improve pharmacologic and pharmaceutical properties of biopharmaceuticals. PEGylation technology has proven to be a valuable tool by chemically modifying biological molecules. Thus, it has a long history and several different PEGylation reagents and linker and coupling chemistries have been described and new ones are still being developed. PEGylation of biopharmaceuticals has been used safely and effectively for over 20 years and has led to 12 marketed compounds. Currently, this technology is very actively used, and more PEGylated drug candidates with improved linker and purification methods are in preclinical and clinical development.

Acknowledgments

The authors are grateful to Juergen Siekmann and Antoni Kozlowski for creating the chemicals structures and for critical review of the manuscript.

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