



PEGYLATION – A REVIEW

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Abstract

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The paper discusses general problems in using PEG for conjugation to high or low molecular weight molecules. Poly (ethylene glycol) (PEG) is a highly investigated polymer for the covalent modification of biological macromolecules and surfaces for many pharmaceutical and biotechnical applications. PEGylation is the process of covalent attachment of polyethylene glycol (PEG) polymer chains to another molecule, normally a drug or therapeutic protein. Advantages of PEGylation (i.e. the covalent attachment of PEG) of peptides and proteins are numerous and include shielding of antigenic, shielding receptor-mediated uptake by the reticuloendothelial system (RES), and preventing recognition and degradation by photolytic enzymes. PEG conjugation also increases the apparent size of the polypeptide, thus reducing the renal filtration and altering bio distribution. An important aspect of PEGylation is the incorporation of various PEG functional groups that are used to attach the PEG to the peptide or protein. In this paper, we review PEG chemistry and methods of preparation with a particular focus on new (second-generation) PEG derivatives, reversible conjugation and PEG structures.

INTRODUCTION

A number of novel drug-delivery mechanisms have been developed to increase the utility of drugs that are otherwise limited by suboptimal pharmacokinetic properties, such as poor absorption, distribution, and elimination. These include continuous-release injectable and liposomal systems, which alter the formulation of the drug, and PEGylation, which alters the drug molecule.¹

The use of proteins and peptides as human therapeutics has expanded in recent years due to: (1) discovery of novel peptides and proteins, (2) a better understanding of the mechanism of action in vivo, (3) Improvements in expression or synthesis of proteins and peptides that closely resemble fully human proteins and peptides, and (4) improvements in formulation or molecule-altering technologies that have the ability to deliver polypeptides in vivo with improved pharmacokinetic and pharmacodynamic properties. It was estimated that in the year 2000, as many as 500 biopharmaceutical products were under-going clinical trials, and the estimated annual growth rates of

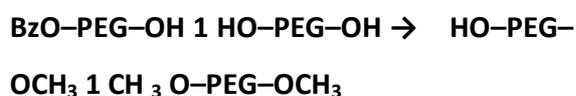
protein products (glycoprotein and antibodies) will range from 10 to 35%.

Although more biopharmaceuticals are in development than ever before, many of these have problems that are typical of polypeptide therapeutics, including short circulating half-life, immunogenicity, proteolytic degradation, and low solubility. Several strategies have emerged as ways to improve the pharmacokinetic and pharmacodynamic properties of biopharmaceuticals, including: (1) manipulation of amino acid sequence to decrease immunogenicity and proteolytic cleavage, (2) fusion or conjugation to immunoglobulin and serum proteins, such as albumin, (3) incorporation into drug delivery vehicles for protection and slow release, and (4) conjugating to natural or synthetic polymers²⁻⁶

Properties of PEG

In Monomethoxy PEG is synthesized by anionic ring opening polymerization initiated with methoxide ions. Commercially available mPEG contains a considerable amount of diol PEG due to the presence of trace amounts of water during

polymerization. This diol PEG is also of relatively high molecular weight due to polymerization at both ends of the polymer. The amount of diol PEG can exceed 15% of the composition of mPEG. A solution to the problem of diol contamination has been developed in our laboratories¹². In this work, a crude benzylox-y-PEG, containing diol impurity, is methylated and then hydrogenated to remove the benzyl group. Thus diol is converted to the inert dimethyl ether, which can be subsequently removed after activation and polypeptide attachment.



Another common route to remove diol is to convert the PEGs to PEG-carboxylic acids that can then be purified by ion-exchange chromatography¹³. PEG with various end groups can be prepared by use of suitable initiator and / or termination re-agents. Numerous functionalities can be introduced as end groups on PEG in this manner, including heterobifunctional products. For instance, Kataoka et al. synthesized a heterobifunctional PEG derivative containing aldehyde and thiol end groups¹⁴.

Polymerization was initiated with 3, 3-diethoxy-1-propanol, which forms a propionaldehyde after acid hydrolysis, and the polymerization was terminated with methansulfonyl chloride with successive conversion to ethyldithiocarbonate and a free thiol.

Compared with other polymers, PEG has a relatively narrow polydispersity (M_w/M_n) in the range of 1.01 for low molecular weight PEGs (5 kDa) to 1.1 for high molecular weight PEGs (>50 kDa). The unique ability of PEG to be soluble in both aqueous solutions and organic solvents makes it suitable for end group derivatization and chemical conjugation to biological molecules under mild physiological conditions. Studies of PEG in solution have shown that PEG typically binds 2–3 water molecules per ethylene oxide unit. Due to both the high flexibility of the backbone chain and the binding of water molecules, the PEG molecule acts as if it were five to 10 times as large as a soluble protein of comparable molecular weight. These factors have been suggested as the reason that PEG exhibits the ability to precipitate proteins¹⁵, exclude proteins and cells from surfaces¹⁶, reduce immunogenicity and antigenicity¹⁷ and

prevent degradation by mammalian cells and enzymes¹⁸.

Low molecular weight oligomers of PEG (,400 Da) have been shown to be degraded in vivo by alcohol dehydrogenase to toxic metabolites, however the lack of toxicity of PEGs with a molecular weight above 1000 Da has been revealed over many years of use in foods, cosmetics and pharmaceuticals¹⁸.

PEG is rapidly cleared in vivo without structural change and clearance is dependent on molecular weight. Below a molecular weight of about 20 kDa the molecule is cleared in the urine, and higher molecular weight PEGs are cleared more slowly in the urine and feces. PEG is only weakly immunogenic even at high molecular weights. Antibodies to PEG have been generated when attached to a highly immunogenic molecule under an immunization protocol with Freund's adjuvant¹⁹⁻²¹. There are no known situations in which anti-PEG antibodies have been generated under 'normal' clinical administration of a PEG-modified protein.

Chemistry of PEGylation

To couple PEG to a molecule (i.e.

polypeptides, polysaccharides, polynucleotides and small organic molecules) it is necessary to activate the PEG by preparing a derivative of the PEG having a functional group at one or both termini. The functional group is chosen based on the type of available reactive group on the molecule that will be coupled to the PEG. For proteins, typical reactive amino acids include lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group and the C-terminal carboxylic acid. In the case of glycoprotein, vicinal hydroxyls groups can be oxidized with periodate to form two reactive formyl moieties.

The most common route for PEG conjugation of proteins has been to activate the PEG with functional groups suitable for reaction with lysine and N-terminal amino based on the type of available reactive group on the molecule that will be coupled to the PEG. For proteins, typical reactive amino acids include lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group and the C-terminal carboxylic acid. In the case of glycoprotein, vicinal

hydroxyls groups can be oxidized with periodate to form two reactive formyl moieties.

Each positional isomer of the heterogeneous mixture is likely to have an influence on whether the conjugate is active or whether an antibody will bind an antigenic epitope. The heterogeneity in lysine substitution and in PEG molecular weights is of some concern for PEG-protein pharmaceuticals, and it is generally necessary to demonstrate that the pattern for a particular pharmaceutical can be measured and is reproducible. Many of the important benefits of PEGylation can be controlled by proper conjugation of various molecular weight PEGs to the protein at specific locations on the protein's surface.

The mono functionality of methoxy PEG makes it particularly suitable for protein and peptide modification because it yields reactive PEGs that do not produce cross linked polypeptides, as long as diol PEG has been removed. As we will see in the discussion of second generation PEGylation, it is also possible in some instances to reduce or eliminate heterogeneity in the position of substitution.

First-generation PEG chemistry

PEG chemistry for amine conjugation

Since most applications of PEG conjugation involve labile molecules, the coupling reactions require mild chemical conditions. In the case of polypeptides, the most common reactive groups involved in coupling are the alpha or epsilon amino groups of lysine. In Fig. 1 is listed a wide range of first generation PEG derivatives used for protein PEGylation of either the alpha or epsilon amino groups. First-generation chemistries are generally plagued by PEG impurities, restriction to low molecular weights, unstable linkages, and lack of selectivity in modification. Examples of first-generation PEG derivatives include: (a) PEG dichlorotriazine, (b) PEG tresylate, (c) PEG succinimidyl carbonate, (d) PEG benzotriazole carbonate, (e) PEG *p*-nitrophenyl carbonate, (f) PEG trichlorophenyl carbonate, (g) PEG carbonylimidazole and (h) PEG succinimidyl succinate.

The initial work of Davis et al. used cyanuric chloride to prepare activated PEG for attachment to proteins^{6, 17}. The PEG dichlorotriazine (Fig. 1a) derivative can react with multiple nucleophilic functional

groups such as lysine, serine, tyrosine, cysteine, and histidine, which results in displacement of one of the chlorides and produces a conjugate with retained charge in the form of a secondary amine linkage²³. The remaining chloride is less susceptible to reactions with nucleophilic residues. Unfortunately, the reactivity is sufficient to allow cross linking of protein molecules containing additional nucleophilic residues. To solve this problem, Inada et al. synthesized 2, 4-bis (methoxypolyethylene glycol)-6-chloro-s-triazine (mPEG₂-chlorotriazine) as shown in Fig. 2²⁴. The lower reactivity of the remaining chlorine translates into a more selective modification of lysine and cysteine residues without further side reactions.

Another alkylation reagent used to nonspecifically modify multiple amino groups to form secondary amine linkages to proteins, viruses and liposome's is PEG tresylate (Fig. 1b)²⁵.

Although more specific to amino groups than PEG dichlorotriazine, the chemistry of conjugation and the conjugation products are not unique and well defined. For example, Gais et al. have shown that PEG-

tresylate conjugation to small molecule amines can produce a product that contains a degradable sulfa mate linkage²⁶.

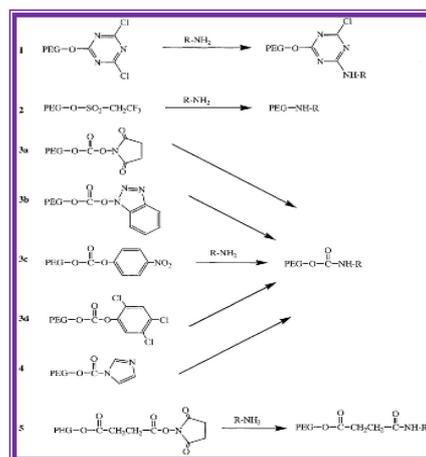
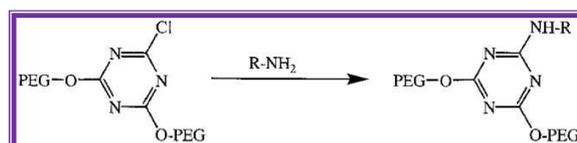


Figure 1. First generation amine reactive PEG derivatives

Therefore, a heterogeneous mixture that results from,



Attaching PEG-tresylate to proteins may contain a population of conjugates with degradable linkages.

Most first-generation PEG chemistries are those that produce conjugates through acylation. Two widely used first-generation activated mPEGs are succinimidyl carbonate (SC-PEG in Fig. 1c)^{27, 28} and benzotriazole

carbonate (BTC-PEG in Fig. 1d)²⁹.

SC-PEG and BTC-PEG react preferentially with lysine residues to form a carbamate linkage, but are also known to react with histidine and tyrosine residues. SC-PEG is slightly more stable to hydrolysis than BTC-PEG with a half-life of 20.4 min at pH 8 and 25 °C compared to the 13.5 min hydrolysis half-life of BTC-PEG under the same conditions³⁰. It has recently been observed that SC-PEG and BTC-PEG couple to histidine residues of α -interferon at slightly acidic conditions to form a hydrolytically unstable imidazolecarbamate linkage³¹. The weak linkage could be used to advantage in preparation of controlled-release formulation, or it could be a disadvantage if conjugate instability were not desired. Other PEG acylating reagents which produce urethane linked proteins include p-nitrophenyl carbonate (pNPC-PEG in Fig. 1e), trichlorophenyl carbonate (TCP-PEG in Fig. 1f) and carbonylimidazole (CDI-PEG in Fig. 1g)^{32, 33}. These reagents are prepared by reacting chloroformates or carbonylimidazole with the terminal hydroxyl group on mPEG, and these have much lower reactivity than either the SC-PEG or BTC-PEG. Generally, the slower the

reaction the more specific the reagent is to certain amino acid groups of the protein. In this way, some selectivity is achieved. The extent and rate of modification can easily be followed in the case of pNPC-PEG and TCP-PEG by monitoring the phenolate-ion leaving-group by colorimetric analysis.

The remaining first-generation PEG reagent is succinimidyl succinate (SS-PEG in Fig. 1h)³⁴. SS-PEG is prepared by reaction of mPEG with succinic anhydride, followed by activation of the carboxylic acid to the succinimidyl ester. The polymer backbone contains a second ester linkage that remains after the conjugation reaction with a protein.

This linkage is highly susceptible to hydrolysis after the polymer has been attached to the protein. Not only does this hydrolysis lead to loss of the benefits of PEG attachment, but the succinate tag that remains on the protein after hydrolysis can act as a hapten and lead to immunogenicity of the remaining protein³⁵.

Techniques used to form first generation PEG derivatives are generally straightforward and involve reacting the

PEG polymer with a group that is reactive with hydroxyl groups, typically anhydrides, chlorides, chloroformates and carbonates. With the exception of the work by Bentley et al., these techniques lack the ability to produce pure mono functional PEG derivatives of high molecular weight¹².

Since the diol content of high molecular weight PEGs can reach 15%, high-molecular-weight, first-generation PEG chemistry is inefficient for protein conjugation. The ability to generate an intermediate that can be purified from deactivated species renders second-generation chemistry a valuable tool for protein modification.

3.2. Second-generation PEGylation chemistry

3.2.1. PEG chemistry for amine conjugation

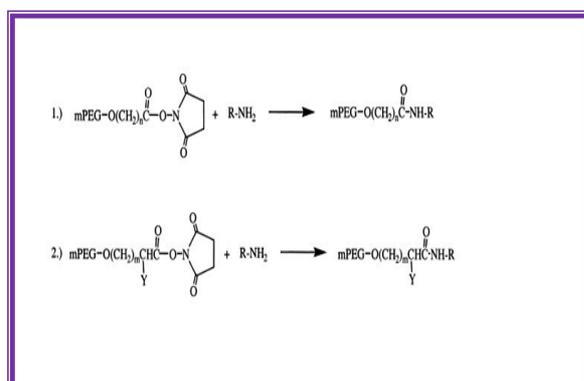
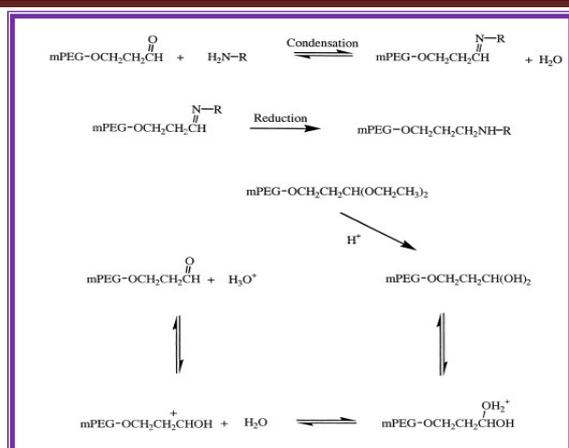
Second-generation PEGylation chemistry has been designed to avoid the above noted problems of diol contamination, restriction to low molecular weight mPEG, unstable linkages, side reactions and lack of selectivity in substitution. One of the first examples of second-generation chemistry is mPEG-propionaldehyde³⁶. mPEG-propionaldehyde is easier to prepare and use than PEG-acetaldehyde because the acetaldehyde is very susceptible to

dimerization via aldol condensation. A key property of mPEG-propionaldehyde, as disclosed by Kinstler et al. in work on PEGylation of G-CSF, sTNF-R1, and consensus IFN, is that under acidic conditions (approximately pH 5), aldehyde is largely selective for the N-terminal α -amine because of the lower pKa of the α -amine compared to other nucleophiles³⁷⁻³⁹.

The conjugation of electrophilic PEGs to amino acid residues on proteins is highly dependent on the nucleophilicity of each amino acid residue.

Nucleophilic attack will only take place when the pH of the protein solution is near or above the residue's pKa. Therefore the reactivity of each residue also depends on neighboring amino acid residues. Although complete selectivity is not observed, the extensive heterogeneity frequently seen with lysine chemistry is greatly reduced. Coupling of aldehydes to primary amines proceeds through a Schiff base,

This is reduced in situ to give a stable secondary amine linkage as shown in Figure 3.



An alternative approach to using PEG-aldehyde is to use the acetal derivative of PEG-propionaldehyde or PEG-acetaldehyde 40. The aldehyde hydrate of the acetal derivatives can be generated in situ by acid hydrolysis (Fig. 4). The pH of the solution can then be adjusted to values sufficient for protein modification with the same mechanism as the free aldehyde derivative in Fig. 3. The benefit of using the acetal derivative over the free propionaldehyde or acetaldehyde is longer storage stability and higher purity. Active esters of PEG

carboxylic acids are the most used acylating agents for protein modification. Active esters react with primary amines near physiological conditions to form stable amides as shown in Figure 5. Generating the carboxylic acid intermediate allows the PEG to be purified from unsubstituted or disubstituted impurities by ion-exchange chromatography 41. Purities of greater than 97% are routinely obtainable by this method. Activation of PEG-carboxylic acids to the succinimidyl active esters is accomplished by reacting the PEG-carboxylic acid with N-hydroxysuccinimide (NHS or HOSu) and a carbodiimide. The first carboxylic acid derivative of PEG not containing a degradable linkage to the PEG back(CM-PEG) 42. The succinimidyl ester of this compound (SCM-PEG) is extremely reactive (hydrolysis $t_{1/2}$ of 0.75 min at pH 8 and 25 °C) and is therefore difficult to use. To take advantage of the intermediate purification step and have an active ester that had more favorable kinetics for protein modification, Harris et al. prepared propionic acid (PEG-O-CH₂CH₂-COOH) and butanoic acid (PEG-O-CH₂CH₂CH₂-COOH) derivatives of PEG 13.

Changing the distance between the active ester and the PEG backbone by the addition of methylene units had a profound influence on the reactivity towards amines and water. For example, SBA-PEG, which has two additional methylene groups, has a longer hydrolysis half-life of 23 min at pH 8 and 25 °C. SPA-PEG, which has one additional methylene group, has a hydrolysis half-life of 16.5 min at pH 8 and 25 °C. Reactivity of PEG active esters towards amines propionic and butonic acid PEG chemistry for cysteine modification PEGylation of free cysteine residues in proteins is the main approach for site-specific modification because reagents that specifically react with cysteines have been synthesized, and the number of free cysteines on the surface of a protein is much less than that of lysine residues. In the absence of a free cysteine in a native protein, one or more free cysteines can be added by genetic engineering⁴³.

The advantage of this approach is that it makes possible site-specific PEGylation at areas on the protein that will minimize a loss in biological activity but decrease immunogenicity. This strategy is not without its shortcomings. The addition of

free cysteines by genetic engineering increases the possibility of incorrect disulfide formation and protein dimerization. PEG derivatives such as PEG-maleimide (Fig.6(1)), vinylsulfone (Fig. 6(2)), iodoacetamide (Fig6(3)), and orthopyridyl disulfide (Fig. 6(4)) have been developed for PEGylation of cysteine residues, with each derivative having its own advantages and disadvantages⁴³⁻⁴⁶. PEG-vinylsulfone (PEG-VS) reacts slowly with thiols to form a stable thioether linkage to the protein at slightly basic conditions (pH7-8) but will proceed faster if the pH is increased.

Although PEG-VS is stable in aqueous solutions, it may react with lysine residues at elevated pH. Unlike PEG-VS, PEG-maleimide (PEG-MAL) is more reactive to thiols even under acidic conditions (pH 6-7), but it is not stable in water and can undergo ring opening or addition of water across the double bond.

PEG-iodoacetamide (PEG-IA) reacts slowly with free thiols by nucleophilic substitution, creating a stable thioether linkage. The reaction should be done in slight molar excess of PEG-IA in a dark container to limit the generation of free iodine that may react

with other amino acids. The thioether linkage between the PEG-MAL and protein is stable, but slow cleavage of one of the amide linkages can occur by hydrolysis. Orthopyridyl disulfide-PEG (PEG-OPSS) reacts specifically with sulfhydryl groups under both acidic and basic conditions (pH 3–10) to form a disulfide bond with the protein. Disulfide linkages are also stable, except in a reducing environment when the linkage is converted to thiols.

Scientists in our laboratories recently prepared a highly active, long circulating and stable conjugate of IFN- β using a two-step method with PEG-OPSS⁴⁷. The tertiary structure of IFN- β was determined by Karpusas et al. who showed that the free cysteine residue at position 17 was proximal to the surface but hidden⁴⁸. In this case, the available thiol was not accessible to high molecular weight PEG that would be needed for improved pharmacokinetics.

The approach that was ultimately adopted was to couple a low molecular weight di-OPSS PEG (Mw2000) to the interferon and then couple a PEG thiol to the remaining terminal OPSS group. The disulfide linkage between PEG and the protein was found to

be stable in plasma circulation (unpublished data).

PEG chemistry for oxidized carbohydrates or N-terminus Oxidation of carbohydrate residues or N-terminal serine or threonine is an alternative method for site-directed PEGylation of proteins. Carbohydrates can be oxidized with enzymes, such as glucose oxidase, or chemically with sodium periodate. Oxidation of the carbohydrate residues generates multiple reactive aldehyde groups, which can be reacted with either PEG-hydrazide to produce a hydrazone linkage or with PEG-amine to produce a reversible Schiff base.

The hydrazone linkage may be reduced with sodium cyanoborohydride to a more stable alkyl hydrazide and the Schiff's base may be reduced to form a secondary amine. Reductive alkylation with PEG-amine is problematic because the amino groups of a protein possess similar reactivity to PEG-amines and thus may form cross linked aggregates. PEG-hydrazides are more useful in these situations. Under acidic conditions (approx. pH 5), amino groups of the protein are predominantly protonated, but because the PEG-hydrazide is a weaker

base (pKa approx. 3) than primary amines (pKa approx. 10), the reaction is selective to the PEG-hydrazone formation. Multiple attachment sites are generated using this method, but the modification site is specific to the carbohydrate. Another approach to site-specific conjugation is to take advantage of the presence of an N-terminal serine or threonine, which can be converted by periodate oxidation to a glyoxylyl derivative. Gaertner et al. oxidized the N-terminal serine of IL-8 to form a glyoxylyl derivative, which they conjugated to aminoxy and hydrazide PEG derivatives.

PEG chemistry for reversible PEGylation

Most PEGylation chemistry is designed to create a conjugate that contains a stable linkage to the protein. In most cases having a stable linkage to the protein is beneficial because of the suitability for long-term storage, easier purification and availability of prefilled syringes. It is also generally observed that stable linkages to a protein can reduce the activity, possibly due to the presence of the PEG chain at the active or binding site of the protein or steric crowding at the active or binding site. Also the PEG molecular weight has a direct

impact on the activity; higher molecular weight PEG conjugates tend to have lower in vitro activity but have higher in vivo activity due to the improved pharmacokinetic profile.

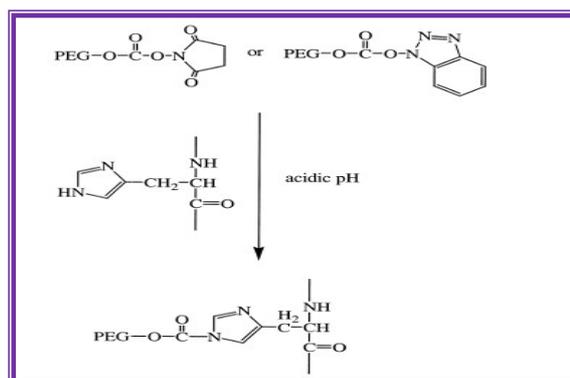
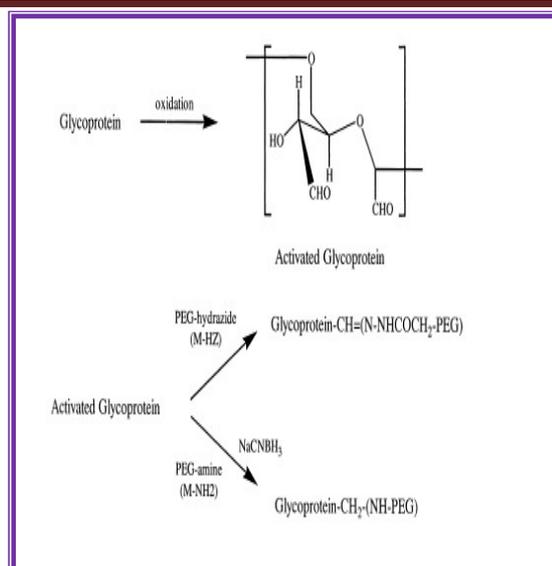
The objective of most PEG conjugation techniques is to increase the circulation half-life without altering activity. In the development of PEG Intron, Enzon used a degradable linkage between the PEG and protein to improve the pharmacokinetic half-life but minimize loss of activity by releasing native interferon alpha-2b conjugates coupled to His 34.

In this case, the PEG is coupled to the N position of the imidazole ring in histidine to form a carbamate linkage and the PEG was found to be released from the protein over time. Note should be taken when comparing PEG-Intron to the branched PEG 40 kDa -interferon alpha-2a conjugate (Pegasys) that the PEG-Intron product has a higher in vitro activity compared to Pegasys, but the in vivo activity of the Pegasys product is higher due to the superior pharmacokinetic profile 53,54.

An approach to regaining protein activity lost by PEGylation is the use of PEG

chemistry that releases the native protein over time through enzymatic degradation, hydrolytic cleavage or reduction. The first such PEG reagent was PEG-succinimidyl succinate, described above. Other 'double ester' PEG reagents have been investigated by Roberts et al. to help control the release rates of the protein and regain activity over a period of time 55. In this case, hydroxy acids are attached to carboxylic acids of PEG (carboxymethyl, propionic, or butanoic) to create a PEG acid that has an ester linkage between the hydroxy acid and PEG acid.

The terminal acid of the PEG derivative can then be activated and attached to α - and ϵ -amino groups of proteins. Regeneration of at least 60% of the native activity of lysozyme was recovered at physiological conditions of completely inactivated protein after release of the PEG from the protein. The problem with the double ester PEG reagents is that they release a protein that contains a 'tag' that could lead to immunogenicity of the protein.



To circumvent the loss of activity associated with some PEG modifications and the potential immunogenic nature of proteins released from PEG with a 'tag' as described above, reagents that release the native protein without 'tags' seem to be a better choice for protein modification. The first such reagent was PEG maleic anhydride used by Garman et al. to attach PEG to tissue plasminogen activator and urokinase (Fig. 10) ⁵⁶. Both of the conjugates

regenerated the native protein under physiological conditions and had a 5–103 slower clearance rate than the native protein in the guinea pig.

Another example of a releasable PEG reagent was prepared by Bentley et al.⁵⁷. In this work, mPEG phenyl ether succinimidyl carbonates and mPEG benzamide succinimidyl carbonates (Fig. 11) are used to conjugate to amino groups on lysozyme. Both conjugates regenerated the native protein under physiological conditions and the rate of release was controlled by the substitution position on the phenyl. Greenwald et al. also synthesized a releasable PEG reagent that released native protein by a 1,6 elimination mechanism (Fig. 12)⁵⁸. Again, the native protein was regenerated with nearly 100% of its bioactivity.

A further example of a releasable PEG was proposed by Zalipsky et al., which released the native protein by a mechanism other than hydrolysis⁵⁹. The linkage as shown in Fig. 13 employs a p or o-disulfide of a benzyl urethane. When subjected to mild reducing environments, such as that present in endosomal compartments of a cell, the

original amine component is regenerated.

PEG structures

In addition to the linear structure of the PEG molecule shown above, branched structures have proven useful for protein and peptide modification

The first branched PEG structure, 2,4-bis(methoxypolyethylene glycol)-6-chloro-s-triazine (mPEG chlorotriazine), was based on a triazine core and synthesized by Inada et al.²⁴.

Yamasaki et al. first synthesized a more useful branched PEG structure, based on a lysine core⁶⁴. A highly purified branched PEG or PEG2 (Fig. 14A) was constructed by Veronese et al. using two linear PEG-BTC (or the related PEG-SC) chains linked to the α - and ϵ -amino groups of lysine⁶⁵. This construct allows for a large molecular weight (upward of 60 kDa) and highly pure PEG to be synthesized with a single reactive end group. The standard preparation of PEG2 intermediate acid contains impurities. These impurities consist of unreacted PEG-BTC, a lysine residue that has only one PEG chain attached to one of the amino groups ('PEG1'), and 'PEG3', which is a linear PEG

having a lysine at each end of a deactivated PEG impurity and two mPEG-BTC molecules coupled to the remaining two amino groups (thus PEG3 is a diacid).

During aqueous work-up, the unreacted mPEG-BTC is converted back to mPEG-OH. Thus the reaction mixture contains a diacid, a zwitterion, a neutral mPEG, and the desired monoacid, which can be purified by careful ion-exchange chromatography. The acid of the lysine linker can be converted into a range of other derivatives including NHS esters, aldehydes, thiols and maleimide. PEG2 turns out to be a very exciting protein EGYlation reagent because of its unique characteristics when compared to linear PEGs. For example, PEG2 attached to proteins 'acts' much larger than a corresponding linear mPEG of the same M_w ⁶⁴.

This structure of PEG also has the advantage of adding two PEG chains per attachment site on the protein, therefore reducing the chance of protein inactivation. Furthermore, the PEG2 structure is more effective in protecting proteins from proteolysis, reducing antigenicity and reducing immunogenicity⁶⁶.

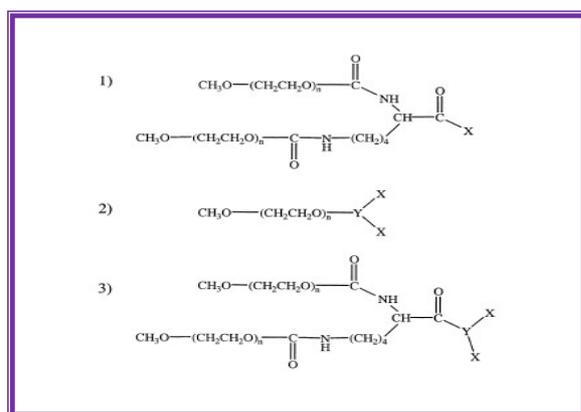
Another branched PEG is the forked PEG. Instead of having a single functional group at the end of two PEG chains, as with PEG2, forked PEG has two reactive groups at one end of a single PEG chain or branched PEG (Fig. 14B, C). Harris et al. first synthesized a forked PEG by attaching to the terminus of a polymer backbone a single functional group of a trifunctional linker, such as serinol or β -glutamic acid⁶⁷.

The remaining proximal functional groups, which are linked to a central carbon atom, are able to react with two other molecules, which can be the same or different, to produce a PEG molecule that contains two molecules at a single terminus of the PEG chain. Arnold et al. synthesized PEG compounds having terminal metal chelating groups, which consisted of two free carboxylic acid or amino groups linked to a central nitrogen atom⁶⁸.

The PEG compounds were used to extract and precipitate proteins from solutions with the carboxylic acid or amino groups together with the nitrogen atom capable of forming ionic complexes with metal ions. Similar PEG compounds were synthesized by Martinez et al. to link hydroxyl containing

moieties to the two terminal carboxylic acid groups to create a PEG-linked prodrug⁶⁹.

Forked PEG is useful for conjugating molecules where the benefit would be to bring two moieties in close proximity to one another, for example, dimerization of cell surface receptors to activate cellular mechanisms. Also, of interest is conjugating a F (ab) 9 fragment of an antibody to the proximal reactive groups to produce a conjugate that closely resembles the structure of the full-length antibody. Another useful attribute of forked PEG is the increased loading capacity of small molecule pharmaceuticals.



CONCLUSION

The array of PEG chemistries reviewed here are among dozens being used for clinical development of PEGylated peptides and proteins. The transition from first-

generation chemistries to second-generation chemistries is taking place at a rapid pace and future demands for PEG reagents will lead to new reagents for novel applications in the biopharmaceutical industry. Novel PEG chemistry for site-specific modification, as well as control of PK / PD parameters, will be synthesized when the needs arise. The importance of chemistry and quality of PEG reagents for peptide and protein modification has only been realized in the last several years as more and more PEG-conjugates make it to late phase clinical trials.

Clearly, the scientific community is eagerly awaiting the results of the ongoing clinical trials to determine future product candidates. We expect that the approval of PEG-Intron for Hepatitis C and the FDA filing of Pegasys (PEG-IFN-alpha 2a), PEG-Neupogen (PEG-G-CSF) and PEGVisomant (PEG-hGHra) will bring new life to a seasoned technology. What was thought to be a failing technology is now fulfilling its long recognized potential.

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