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Chemistry for peptide and protein PEGylation

M.J. Roberts*, M.D. Bentley, J.M. Harris

Shearwater Corporation, 490 Discovery Drive, Huntsville, AL 35806, USA

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Abstract

Poly(ethylene glycol) (PEG) is a highly investigated polymer for the covalent modification of biological macromolecules and surfaces for many pharmaceutical and biotechnical applications. In the modification of biological macromolecules, peptides and proteins are of extreme importance. Reasons for PEGylation (i.e. the covalent attachment of PEG) of peptides and proteins are numerous and include shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the reticuloendothelial system (RES), and preventing recognition and degradation by proteolytic enzymes. PEG conjugation also increases the apparent size of the polypeptide, thus reducing the renal filtration and altering biodistribution. 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.

Keywords: PEGylation; PEG-protein; PEG conjugation; PEG chemistry

Contents

*Corresponding author. Tel.: +1-256-704-7524; fax: +1-256-533-4201.
E-mail address: mroberts@shearwatercorp.com (M.J. Roberts).

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1. Introduction

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 undergoing clinical trials, and the estimated annual growth rates of protein products (glycoproteins, unglycosylated proteins and antibodies) will range from 10 to 35% [1].

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 immunoglobulins and serum proteins, such as albumin, (3) incorporation into drug delivery vehicles for protection and slow release, and (4) conjugating to natural or synthetic polymers [2–6].

Those in the biomedical, biotechnical and pharmaceutical communities have become quite familiar with the improved pharmacological and biological properties that are associated with the covalent attachment of poly(ethylene glycol) or PEG to therapeutically useful polypeptides. For instance, PEG conjugation can shield antigenic epitopes of the polypeptide, thus reducing reticuloendothelial (RES) clearance and recognition by the immune system and also reducing degradation by proteolytic enzymes. PEG conjugation also increases the apparent size of the polypeptide, thus reducing renal filtration and altering biodistribution. Contributing factors that affect the foregoing properties are: (1) the number of PEG chains attached to the polypeptide, (2) the molecular weight and structure of PEG chains attached to the polypeptide, (3) the location of the PEG sites on the polypeptide and (4) the chemistry used to attach the PEG to the polypeptide.

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 have reached late phase clinical trials. The first few PEG-protein products, now on the market (Adagen®, Oncospar®, and PEG-Intron®), were developed using first generation PEG chemistry. One characteristic of first generation PEG chemistry is the use of low molecular weight linear PEGs (≤ 12 kDa) with chemistry that may result in side reactions or weak linkages upon conjugation with polypeptides.

The next generation of PEG-protein therapeutics, which will come to market in the next several years, uses second-generation PEG chemistries. Second-generation PEGylation was designed to avoid the problems of first generation chemistry, notably diactivated PEG impurities, restriction to low molecular weight mPEG, unstable linkages and lack of selectivity in modification. Readers are referred to several detailed reviews on different aspects of PEGylation [7–11]. In this paper, we review chemistries of both first- and second-generation, with an emphasis on newer PEGylation technologies, in order to provide an introduction to those chemistries that will be used in the following reviews.

2. Properties of PEG

In its most common form poly(ethylene glycol), PEG, is a linear or branched polyether terminated with hydroxyl groups and having the general structure:

$$HO–(CH_2CH_2O)_n–CH_2CH_2–OH$$

PEG is synthesized by anionic ring opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring. Most useful for polypeptide modification is monomethoxy PEG, mPEG, having the general structure:

$$CH_3O–(CH_2CH_2O)_n–CH_2CH_2–OH$$

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 laboratories [12]. In this work, a crude benzyloxy-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.

$$BzO–PEG–OH + HO–PEG–OH \rightarrow \rightarrow HO–PEG–OCH_3 + CH_3O–PEG–OCH_3$$

Another common route to remove diol is to convert the PEGs to PEG-carboxylic acids that can then be purified by ion-exchange chromatography [13]. PEG with various end groups can be prepared by use of suitable initiator and/or termination reagents. 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 groups [14]. 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 proteins [15], exclude proteins and cells from surfaces [16], reduce immunogenicity and antigenicity [17] and prevent degradation by mammalian cells and enzymes [18].

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 pharmaceuticals [18].

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 adjuvant [19–21]. There are no known situations in which anti-PEG antibodies have been generated under 'normal' clinical administration of a PEG-modified protein.

3. 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 glycoproteins, vicinal hydroxyl 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 acid groups. Lysine is one of the most

prevalent amino acids in proteins and can be upwards of 10% of the overall amino acid sequence. In reactions between electrophilically activated PEG and nucleophilic amino acids, it is typical that several amines are substituted. When multiple lysines have been modified, a heterogeneous mixture is produced that is composed of a population of several polyethylene glycol molecules attached per protein molecule ('PEGmers') ranging from zero to the number of ε- and α-amine groups in the protein. For a protein molecule that has a single PEG attached by this nonspecific modification method, the polyethylene glycol moiety may be attached at a number of different amine sites. Therefore there is the potential for a large number of positional isomers (P) as the degree of modification increases:

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

where N is the number of possible sites and k is the number of sites modified. The extent of modification is important in determining the pharmacological properties of the bioconjugate. Typically, a higher degree of modification will extend the circulation half-life and reduce the likelihood of antigenicity [22]. 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 monofunctionality of methoxyPEG makes it particularly suitable for protein and peptide modification because it yields reactive PEGs that do not produce crosslinked 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.

3.1. First-generation PEG chemistry

3.1.1. 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 linkage [23]. The remaining chloride is less susceptible to reactions with nucleophilic residues. Unfortunately, the reactivity is sufficient to allow crosslinking of protein molecules containing additional nucleophilic residues. To solve this problem, Inada et al. synthesized 2,4-bis(methoxypolyethylene glycol)-6-chloro-s-triazine ($mPEG_2$-chlorotriazine) as shown in Fig. 2 [24]. The lower reactivity of the remaining chlorine translates into a more selective modification of lysine and cysteine residues without further side reactions.

Another alkylating reagent used to nonspecifically modify multiple amino groups to form secondary amine linkages to proteins, viruses and liposomes 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,

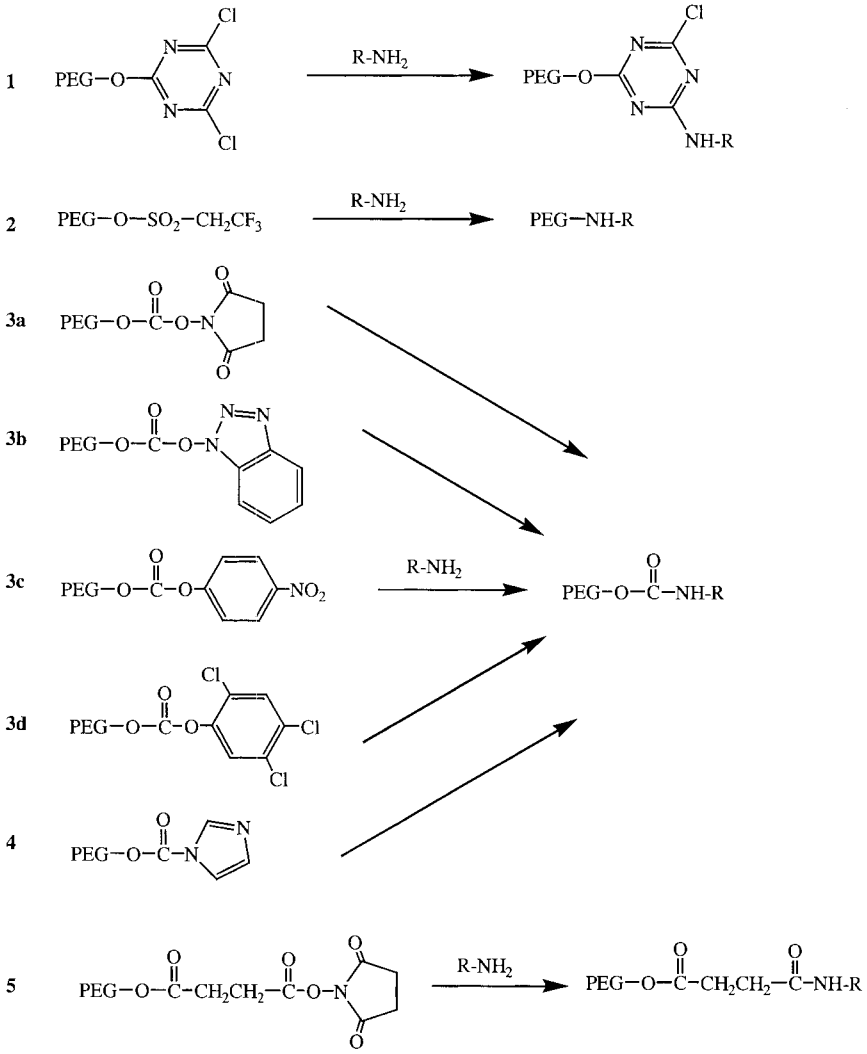

Fig. 1. First-generation amine reactive PEG derivatives.

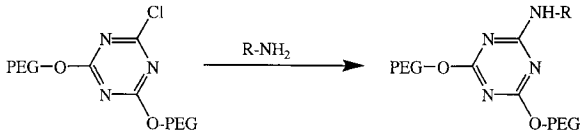

Fig. 2. Branched PEG (PEG2) based on PEG-triazine.

Gais et al. have shown that PEG-tresylate conjugation to small molecule amines can produce a product that contains a degradable sulfamate linkage [26]. 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 hy-

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