



ELSEVIER

Advanced Drug Delivery Reviews 54 (2002) 459–476

Advanced
DRUG DELIVERY
Reviews

www.elsevier.com/locate/drugdeliv

Chemistry for peptide and protein PEGylation

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

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

Received 17 December 2001; accepted 22 January 2002

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. © 2002 Elsevier Science B.V. All rights reserved.

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

Contents

1. Introduction	460
2. Properties of PEG	460
3. Chemistry of pegylation.....	461
3.1. First-generation PEG chemistry.....	462
3.1.1. PEG chemistry for amine conjugation.....	462
3.2. Second-generation PEGylation chemistry.....	464
3.2.1. PEG chemistry for amine conjugation.....	464
3.2.2. PEG chemistry for cysteine modification	466
3.2.3. PEG chemistry for oxidized carbohydrates or N-terminus	467
3.2.4. PEG chemistry for reversible PEGylation	467
3.2.5. Heterobifunctional PEG chemistry	469
3.3. PEG structures	473
4. Conclusions	474
References	474

*Corresponding author. Tel.: +1-256-704-7524; fax: +1-256-533-4201.

E-mail address: mroberts@shearwatercorp.com (M.J. Roberts).

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 at-

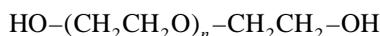
tached to the polypeptide, (3) the location of the PEG sites on the polypeptide and (4) the chemistry used to attach the PEG to the polypeptide.

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 deactivated 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

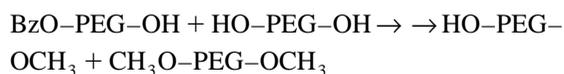
In its most common form poly(ethylene glycol), PEG, is a linear or branched polyether terminated with hydroxyl groups and having the general structure:



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:



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 benzyl-*oxy*-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 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 (mPEG₂-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,

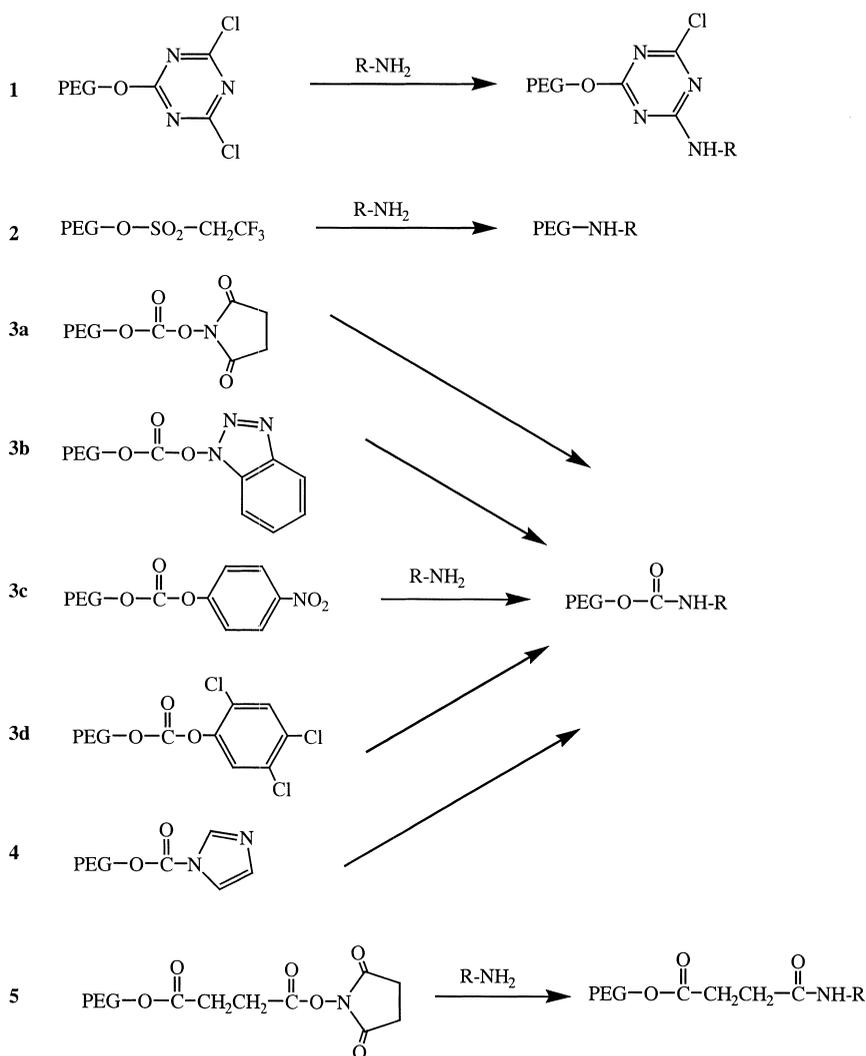


Fig. 1. First-generation amine reactive PEG derivatives.

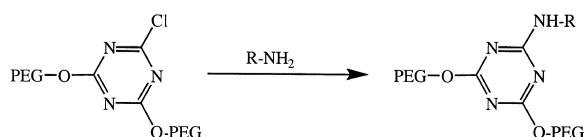


Fig. 2. Branched PEG (PEG2) based on PEG-triazine.

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-

drololysis 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 conditions [30]. 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 linkage [31]. 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 protein [35].

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 weight

[12]. 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-propionaldehyde [36]. 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-RI, 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 pK_a of the α -amine compared to other nucleophiles [37–39]. 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 pK_a . 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, which is reduced in situ to give a stable secondary amine linkage as shown in Fig. 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

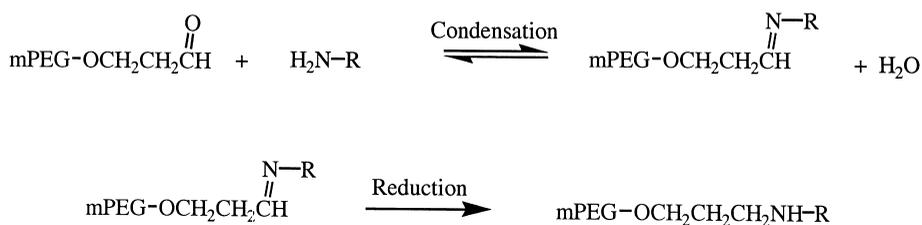


Fig. 3. Reductive amination using PEG-propionaldehyde.

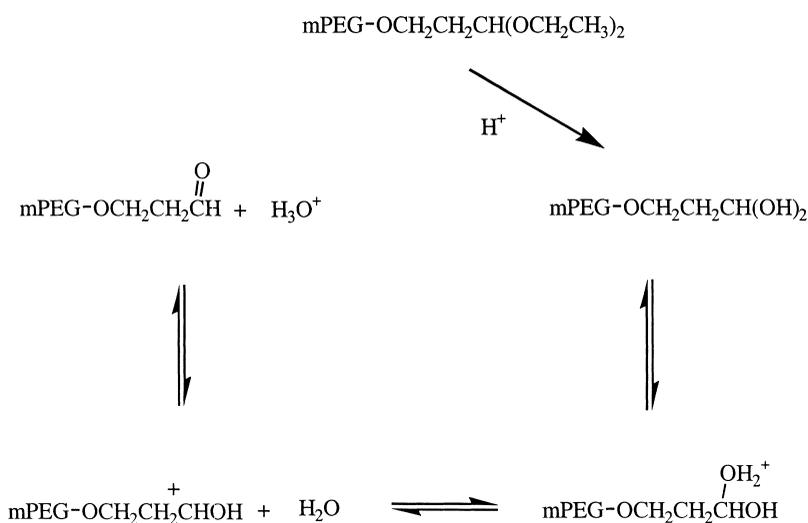


Fig. 4. In situ generation of PEG-aldehyde hydrate for use in reductive amination.

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 Fig. 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 backbone, as in SS-PEG, was carboxymethylated PEG

(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 (Fig. 5(1)) [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

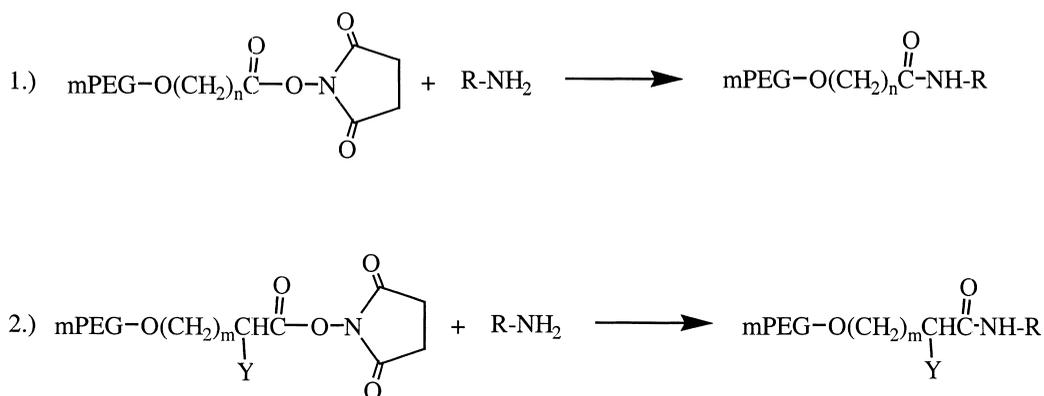


Fig. 5. PEG NHS esters. (1) PEG NHS esters based on propionic and butanoic acids and (2) α -branched PEG NHS esters based on propionic and butanoic acids.

and water can be further decreased by introducing an α -branching moiety to the carboxylic acid as shown in Fig. 5(2). An α -methyl branched PA-PEG derivative has a hydrolysis half-life of 33 min at pH 8 and 25 °C.

3.2.2. 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 engineering [43]. 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 (Fig. 6(3)), and orthopyridyl disulfide (Fig. 6(4)) have been developed for PEGylation of cysteine residues, with each derivative having its own advantages and disadvantages [43–46]. PEG-vinylsulfone (PEG-VS) reacts slowly with thiols to form a stable thioether linkage to the protein at slightly basic conditions (pH 7–8) 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-OPSS [47]. 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 hidden [48]. 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 (M_w 2000) to the interferon and then couple a PEG thiol

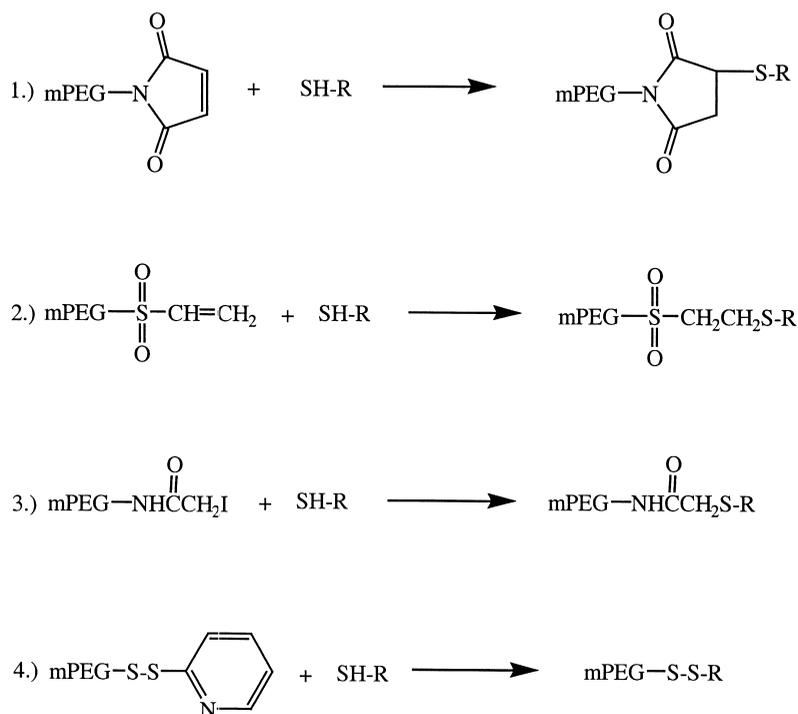


Fig. 6. Thiol reactive PEGs. (1) PEG maleimide, (2) PEG vinyl sulfone, (3) PEG iodoacetamide, and (4) PEG orthopyridyl disulfide.

to the remaining terminal OPSS group. The disulfide linkage between PEG and the protein was found to be stable in plasma circulation (unpublished data).

3.2.3. 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 (Fig. 7) [49]. 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 (pK_a approx. 3) than primary amines (pK_a 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 a 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 [50].

3.2.4. 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

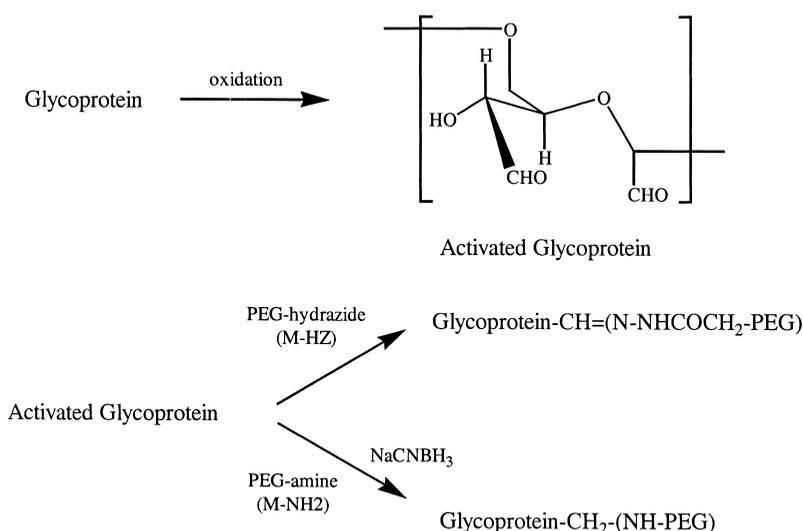


Fig. 7. Attachment of poly(ethylene glycol) to oxidized carbohydrates of glycoproteins.

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 [51]. 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 [52]. PEG-Intron is formed by conjugation of PEG-SC to interferon alpha-2b at low pH (around pH 5). The conjugation leads to a population of PEG conjugates coupled to His³⁴ (Fig. 8). In this case, the PEG is coupled to the N^{δ1} 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

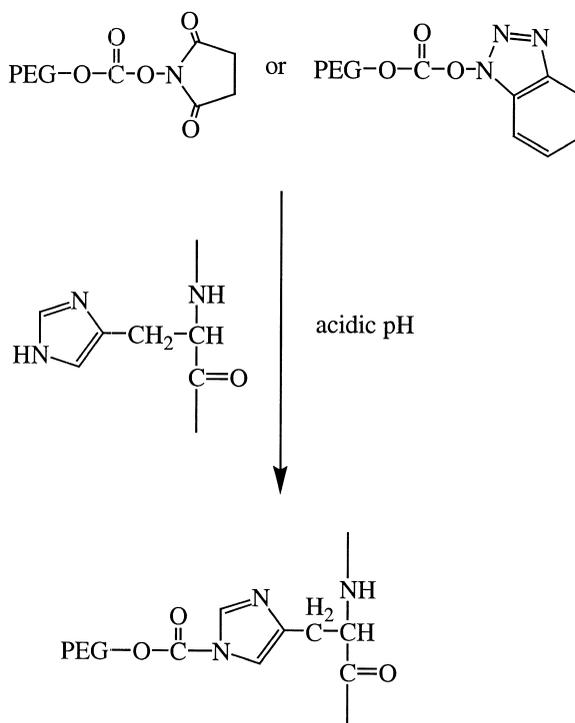


Fig. 8. Attachment of PEG-benzotriazole or PEG-succinimidyl carbonate to histidine residues on proteins.

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 (Fig. 9). 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.

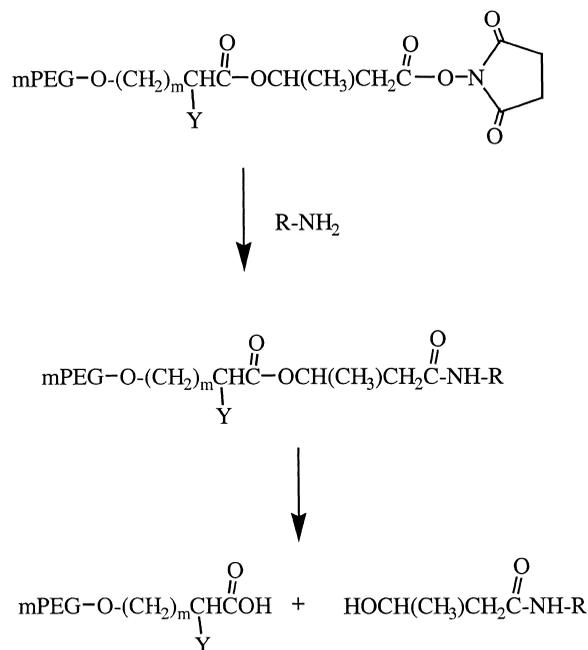


Fig. 9. Attachment and release of PEG-double esters from proteins (Y is an aliphatic or aromatic moiety).

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 5–10 \times 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 hydrolysis [59]. 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.

3.2.5. Heterobifunctional PEG chemistry

As applications of PEG chemistry have become more sophisticated, there has been an increasing need for heterobifunctional PEGs, which are PEGs bearing dissimilar terminal groups. Such heterobifunctional PEGs bearing appropriate functional groups may be used to link two entities where a hydrophilic, flexible, and biocompatible spacer is needed. Heterobifunctional PEG can be used in a variety of ways that includes linking macromolecules to surfaces (for immunoassays, biosensors or various probe applications), targeting of drugs, liposomes and viruses to specific tissues, liquid phase peptide synthesis and many others.

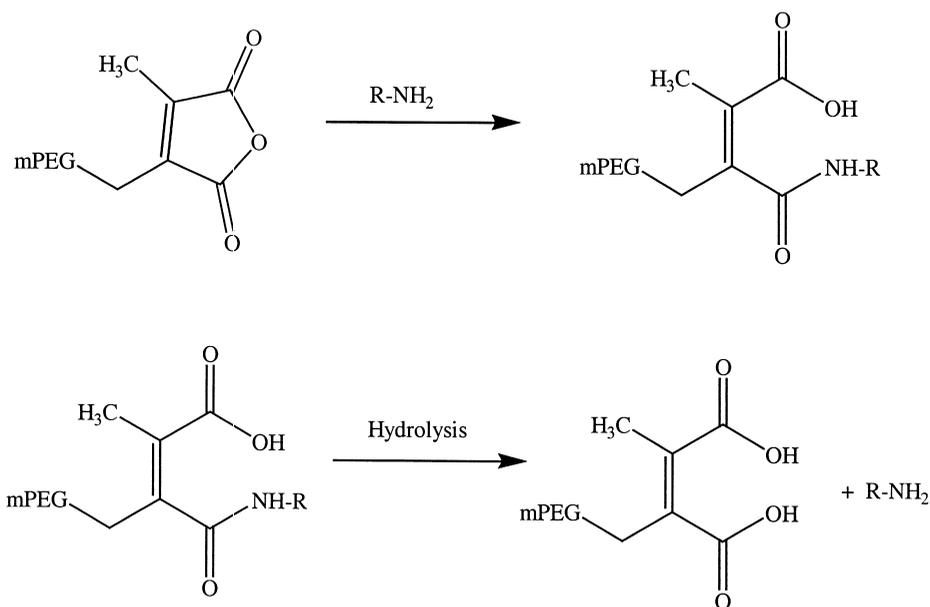


Fig. 10. Attachment and release of PEG-methylmaleic anhydride from amine containing drugs.

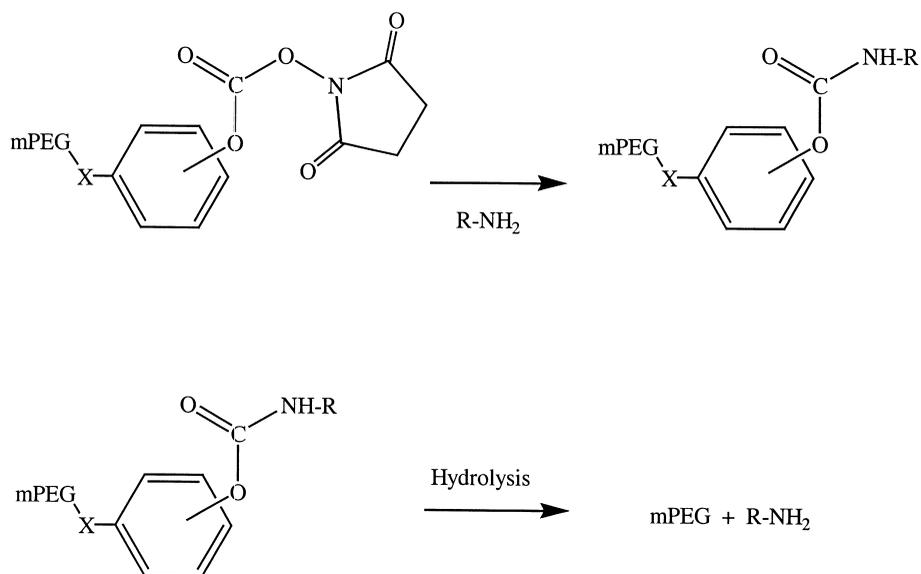


Fig. 11. Attachment and release of PEG-phenyl NHS carbonates from amine containing drugs (hydrolysis mechanism) [X is O or -CONH-].

Several methods have been developed to synthesize heterobifunctional PEGs, each having its own advantages and disadvantages. Preferred end groups

for heterobifunctional PEGs are NHS esters, maleimide, vinyl sulfone, pyridyl disulfide, amine, and carboxylic acids. The first such synthetic method

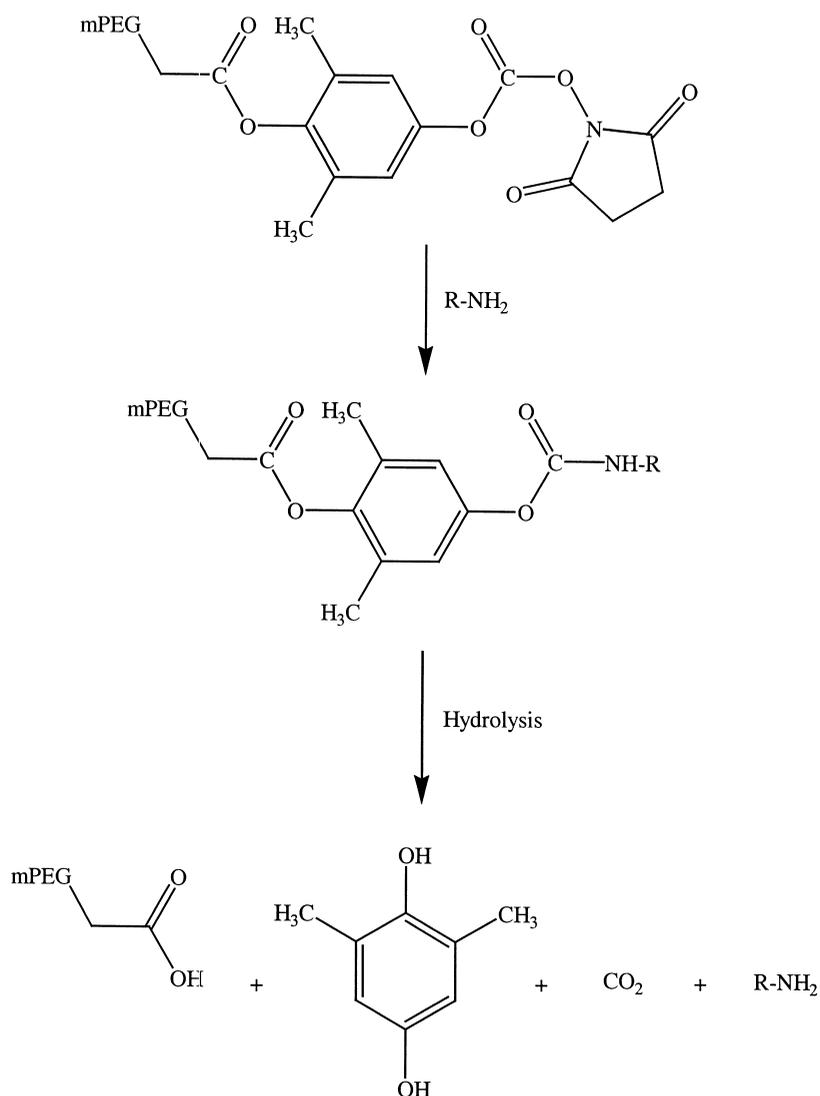


Fig. 12. Attachment and release of PEG-phenyl NHS carbonates from amine containing drugs (1,6-elimination mechanism).

is to simply limit molar equivalents of each reagent. However, a distribution of substitution will result as shown below.

HO-PEG-OH

HO-PEG-COOH

HOOC-PEG-COOH

A chromatographic approach was used by Zalipsky

et al. to purify a PEG acid that was synthesized by reacting glycine with a PEG succinimidyl carbonate derivative [40]. Ion exchange chromatography was shown to be a very effective purification method to separate monoacid from diacid and unsubstituted PEG.

Bentley et al. prepared heterobifunctional PEGs by first initiating PEG polymerization with benzyl alcohol to produce benzyl PEG (Bz-PEG-OH) [60]. The hydroxyl group of the intermediate polymer is

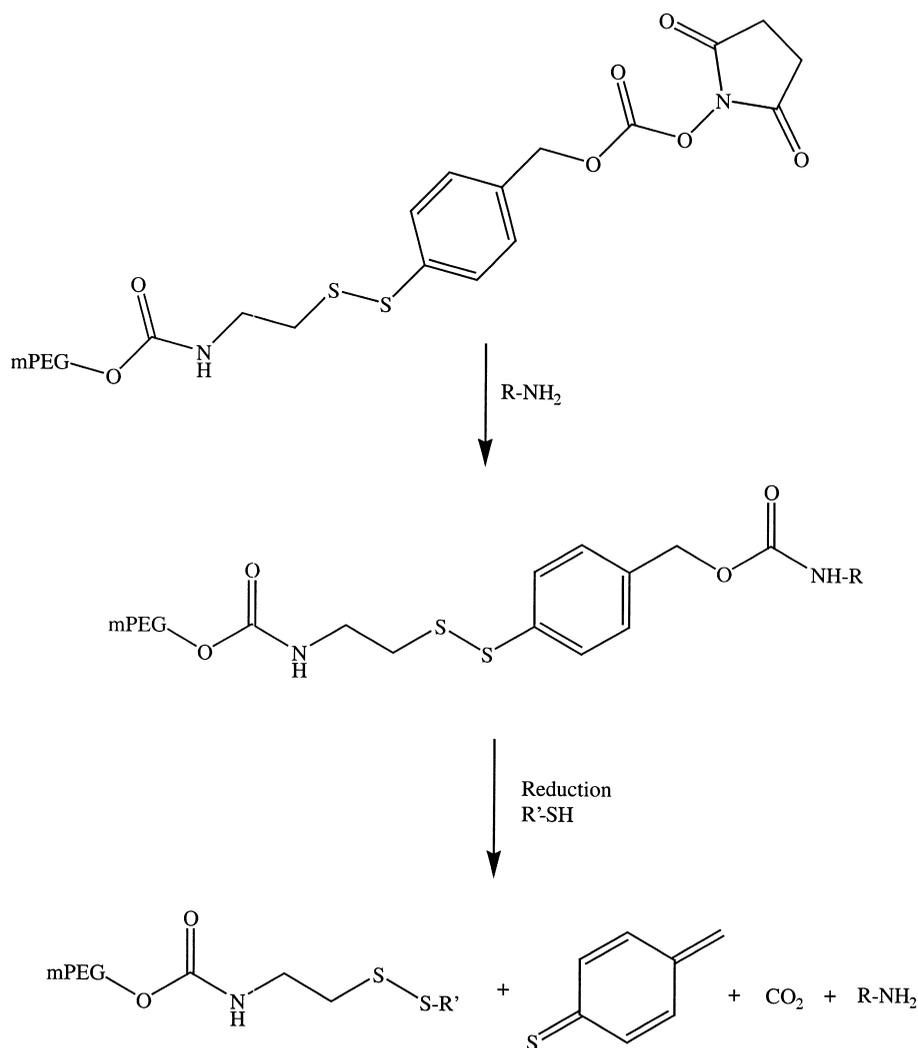


Fig. 13. Attachment and release of PEG phenyl NHS carbonates from amine containing drugs (reduction mechanism).

converted to a first reactive functional group. The benzyl group can then be removed by hydrogenolysis or hydrolysis, without chemically affecting the first functional group, thus making available a new terminal hydroxyl group for the addition of a second functional group.

A promising strategy for generating heterobifunctional PEGs is the polymerization approach. Initiating polymerization of ethylene oxide with an anion that ultimately becomes the end-group of PEG is the most direct route for heterobifunctional synthesis. Terminating the reaction with water generates a

hydroxyl group at the second terminal end that can undergo activation by chemical methods. For example, this method has been used by Yokoyama et al. to prepare a PEG with a hydroxyl group at one terminus and an amino group at the other [61]. Cammas et al. have also used this method to prepare PEGs with an amino group on one terminus and a hydroxyl or methoxy group on the other [62]. It has also been used by Nagasaki et al. to prepare a PEG having a formyl group at one terminus and a hydroxyl group at the other [63]. This strategy also has its limits. Only those anions that are desirable as

end groups and suitable for initiating polymerization are useful for synthesis of heterobifunctional PEG by this route. This method is also limited by the fact that rigorous exclusion of water is necessary to prevent the formation of diol. This problem becomes more severe as PEG molecular weight increases. If these limits are resolved, generating heterobifunctional PEG reagents by anionic polymerization may be the most efficient and cost effective method.

3.3. 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(methoxy polyethylene glycol)-6-chloro-s-triazine (mPEG₂-chlorotriazine), was based on a triazine core and synthesized by Inada et al. as shown in Fig. 2 [24].

Yamasaki et al. first synthesized a more useful branched PEG structure, based on a lysine core [64]. 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 lysine [65]. 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 diactivated 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 PEGylation 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 immunogenicity [66].

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 acid [67]. 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

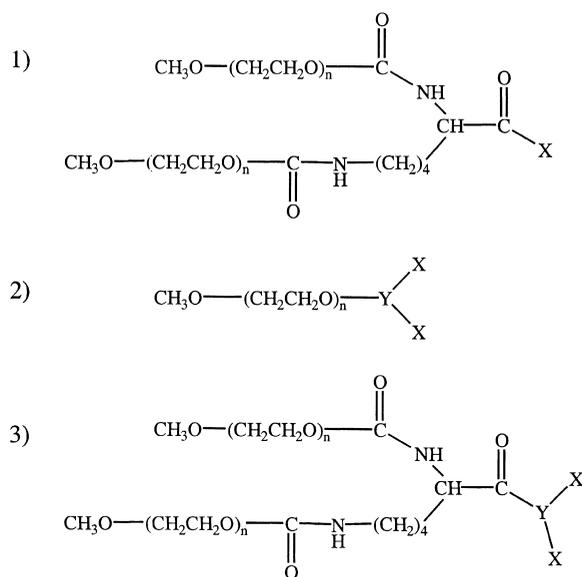


Fig. 14. Various PEG structures. (1) Branched PEG (PEG2), (2) linear forked PEG, and (3) branched forked PEG [Y is a group having a carbon branching moiety and X is a molecule, linker and/or a functional group].

two free carboxylic acid or amino groups linked to a central nitrogen atom [68]. 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 pro-drug [69].

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)' 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.

4. Conclusions

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- α 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.

References

- [1] G. Walsh, Biopharmaceutical benchmarks, *Nat. Biotechnol.* 18 (2000) 831–833.
- [2] C. Mateo, J. Lombardero, E. Moreno, A. Morales, G. Bombino, J. Coloma, L. Wims, S.L. Morrison, R. Perez, Removal of amphipathic epitopes from genetically engineered antibodies: production of modified immunoglobulins with reduced immunogenicity, *Hybridoma* 19 (2000) 463–471.
- [3] J.B. Lyczak, S.L. Morrison, Biological and pharmacokinetic properties of a novel immunoglobulin-CD4 fusion protein, *Arch. Virol.* 139 (1994) 189–196.
- [4] S. Syed, P.D. Schuyler, M. Kulczycky, W.P. Sheffield, Potent antithrombin activity and delayed clearance from the circulation characterize recombinant hirudin genetically fused to albumin, *Blood* 89 (1997) 3243–3252.
- [5] S. Cohen, T. Yoshioka, M. Lucarelli, L.H. Hwang, R. Langer, Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres, *Pharm. Res.* 8 (1991) 713–720.
- [6] A. Abuchowski, J.R. McCoy, N.C. Palczuk, T. van Es, F.F. Davis, Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase, *J. Biol. Chem.* 252 (1977) 3582–3586.
- [7] J.M. Harris (Ed.), *Polyethylene Glycol Chemistry, Biotechnical and Biomedical Applications*, Plenum, New York, 1992.
- [8] J.M. Harris, Synthesis of polyethylene glycol derivatives, *J. Macromol. Sci. Rev. Macromol. Chem. Phys. C25* (1985) 325–373.
- [9] S. Zalipsky, Chemistry of polyethylene glycol conjugates with biologically active molecules, *Adv. Drug Deliv. Rev.* 16 (1995) 157–182.
- [10] F.M. Veronese, Peptide and protein PEGylation: a review of problems and solutions, *Biomaterials* 22 (2001) 405–417.
- [11] G. Hooftman, S. Herman, E. Schacht, Review: Poly(ethylene glycol)s with reactive endgroups. II. Practical consideration for the preparation of protein–PEG conjugates, *J. Bioact. Compat. Polym.* 11 (1996) 135–159.
- [12] M.D. Bentley, J.M. Harris, A. Kozlowski, Heterobifunctional poly(ethylene glycol) derivatives and methods for their preparation, P.C.T. US99/23536 (1999).
- [13] J.M. Harris, A. Kozlowski, Polyethylene glycol and related polymers monosubstituted with propionic or butanoic acids and functional derivatives thereof for biotechnical applications, US Patent 5,672,662 (1997).
- [14] Y. Akiyama, H. Otsuka, Y. Nagasaki, M. Kato, K. Katoaka, Selective synthesis of heterobifunctional poly(ethylene glycol) derivatives containing both mercapto and acetal terminals, *Bioconjug. Chem.* 11 (2000) 947–950.
- [15] A. Polson, A theory for the displacement of proteins and viruses with polyethylene glycol, *Prep. Biochem.* 7 (1977) 129–154.
- [16] W.R. Gombotz, W. Guanghai, T.A. Horbett, A.S. Hoffman, Protein adsorption to and elution from polyether surfaces, in: J.M. Harris, S. Zalipsky (Eds.), *Polyethylene Glycol Chemis-*

- try, *Biotechnical and Biomedical Applications*, Plenum, New York, 1992, pp. 247–261.
- [17] A. Abuchowski, T. van Es, N.C. Palczuk, F. F. Davis, Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol, *J. Biol. Chem.* 252 (1977) 3578–3581.
- [18] P.K. Working, M.S. Newman, J. Johnson et al., Safety of poly(ethylene glycol) and poly(ethylene glycol) derivatives, in: J.M. Harris, S. Zalipsky (Eds.), *Poly(ethylene glycol) Chemistry and Biological Applications*, ACS Books, Washington, DC, 1997, pp. 45–57.
- [19] A.W. Richter, E. Akerblom, Antibodies against polyethylene glycol produced in animals by immunization with monomethoxy polyethylene glycol modified proteins, *Int. Arch. Allergy Appl. Immunol.* 70 (1983) 124–131.
- [20] A.W. Richter, E. Akerblom, Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood donors, *Int. Arch. Allergy Appl. Immunol.* 74 (1984) 36–39.
- [21] T.L. Cheng, P.Y. Wu, M.F. Wu, J.W. Chern, S.R. Roffler, Accelerated clearance of polyethylene glycol-modified proteins by anti-polyethylene glycol IgM, *Bioconjug. Chem.* 10 (1999) 520–528.
- [22] R. Clark, K. Olson, G. Fuh, M. Marian, D. Mortensen, G. Teshima, S. Chang, H. Chu, V. Mukku, E. Canova-Davis, T. Somers, M. Cronin, M. Winkler, J.A. Wells, Long-acting growth hormones produced by conjugation with polyethylene glycol, *J. Biol. Chem.* 271 (1996) 21969–21977.
- [23] S. Zalipsky, C. Lee, Use of functionalized poly(ethylene glycol)s for modification of polypeptides, in: J.M. Harris, S. Zalipsky (Eds.), *Polyethylene Glycol Chemistry, Biotechnical and Biomedical Applications*, Plenum, New York, 1992, pp. 347–370.
- [24] A. Matsushima, H. Nishimura, Y. Ashihara, Y. Yakata, Y. Inada, Modification of *E. Coli* asparaginase with 2,4-bis(o-methoxypolyethylene glycol)-6-chloro-s-triazine (activated PEG2); disappearance of binding ability towards anti-serum and retention of enzymatic activity, *Chem. Lett.* (1980) 773–776.
- [25] G.E. Francis, D. Fisher, C. Delgado, F. Malik, A. Gardiner, D. Neale, PEGylation of cytokines and other therapeutic proteins and peptides: the importance of biological optimization of coupling techniques, *Int. J. Hematol.* 68 (1998) 1–18.
- [26] H.J. Gais, S. Ruppert, Modification and immobilization of proteins with polyethylene glycol tresylates and polysaccharide tresylates: evidence suggesting a revision of the coupling mechanism and the structure of the polymer–polymer linkage, *Tetrahedron Lett.* 36 (1995) 3837–3838.
- [27] S. Zalipsky, R. Seltzer, S. Menon-Rudolph, Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins, *Biotechnol. Appl. Biochem.* 15 (1992) 100–114.
- [28] T. Miron, M. Wilchek, A simplified method for the preparation of succinimidyl carbonate polyethylene glycol for coupling to proteins, *Bioconjug. Chem.* 4 (1993) 568–569.
- [29] E.K. Dolence, C. Hu, R. Tsang, C.G. Sanders, S. Osaki, Electrophilic polyethylene oxides for the modification of polysaccharides, polypeptides (proteins) and surfaces, US Patent 5,650,234 (1997).
- [30] Shearwater Corporation Catalog, 2001.
- [31] S. Lee, C. McNemar, Substantially pure histidine-linked protein polymer conjugates, US Patent 5,985,263 (1999).
- [32] F.M. Veronese, R. Largajolli, E. Bocca, C.A. Benassi, O. Schiavon, Activation of monomethoxy poly(ethylene glycol) by phenylchloroformate and modification of ribonuclease and superoxide dismutase, *Appl. Biochem. Biotechnol.* 11 (1985) 141–152.
- [33] C.O. Beauchamp, S.L. Gonias, D.P. Menapace, S.V. Pizzo, A new procedure for the synthesis of polyethylene glycol-protein adducts, effects on function, receptor recognition and clearance of superoxide dismutase, lactoferrin and α 2-macroglobulin, *Anal. Biochem.* 131 (1983) 25–33.
- [34] A. Abuchowski, G.M. Kazo, C.R. Verhoest et al., Cancer therapy with chemically modified enzymes. I. Antitumor properties of polyethylene glycol-asparaginase conjugates, *Cancer Biochem. Biophys.* 7 (1984) 175–186.
- [35] M.C. Carter, M.E. Meyerhoff, Instability of succinyl ester linkages in O²-monosuccinyl cyclic AMP-protein conjugates at neutral pH, *J. Immunol. Methods* 81 (1985) 245–257.
- [36] J.M. Harris, R.M. Herati, Preparation and use of polyethylene glycol propionaldehyde, US Patent 5,252,714 (1993).
- [37] O.B. Kinstler, D.N. Brems, S.L. Lauren, Characterization and stability of N-terminally PEGylated rhG-CSF, *Pharm. Res.* 13 (1996) 996–1002.
- [38] O.B. Kinstler, N.E. Gabriel, C.E. Farrar, R.B. DePrince, N-terminally chemically modified protein compositions and methods, US Patent 5,985,265 (1999).
- [39] C.K. Edwards, PEGylated recombinant human soluble tumor necrosis factor receptor type I (rHu-sTNF-RI): A novel high-affinity TNF receptor designed for chronic inflammatory diseases, *Ann. Rheum. Dis.* 58 (1999) I73–I81.
- [40] M.D. Bentley, J.M. Harris, Poly(ethylene glycol) aldehyde hydrates and related polymers and applications in modifying, US Patent 5,990,237 (1999).
- [41] S. Zalipsky, G. Barany, Preparation of polyethylene glycol derivatives with two different functional groups at the termini, *Polym. Preprints* 27 (1986) 1–2.
- [42] S. Zalipsky, G. Barany, Facile synthesis of α -hydroxy- ω -carboxymethylpolyethylene oxide, *J. Bioact. Compat. Polym.* 5 (1990) 227–231.
- [43] R.J. Goodson, N.V. Katre, Site-directed pegylation of recombinant interleukin-2 at its glycosylation site, *Biotechnology* 8 (1990) 343–346.
- [44] T.P. Kogan, The synthesis of substituted methoxy-poly(ethylene glycol) derivatives suitable for selective protein modification, *Synth. Commun.* 22 (1992) 2417–2424.
- [45] M. Morpurgo, F.M. Veronese, D. Kachensky, J.M. Harris, Preparation and characterization of poly(ethylene glycol) vinyl sulfone, *Bioconjug. Chem.* 7 (1996) 363–368.
- [46] C. Woghiren, B. Sharma, S. Stein, Protected thiol-polyethylene glycol: a new activated polymer for reversible protein modification, *Bioconjug. Chem.* 4 (1993) 314–318.

- [47] N. El Tayar, M.J. Roberts, J.M. Harris, W. Sawlivich, Polyol-IFN- β conjugates, WO99/55377 (1999).
- [48] M. Karpusas, M. Nolte, C.B. Benton, W. Meier, W.N. Lipscomb, S. Goelz, The crystal structure of human interferon beta at 2.2-Å resolution, *Proc. Natl. Acad. Sci. USA* 94 (1997) 11813–11818.
- [49] S. Zalipsky, S. Menon-Rudolph, Hydrazide derivatives of poly(ethylene glycol) and their bioconjugates, in: J.M. Harris, S. Zalipsky (Eds.), *Poly(ethylene glycol) Chemistry and Biological Applications*, ACS Books, Washington, DC, 1997, pp. 318–340.
- [50] H.F. Gaertner, R.E. Offord, Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins, *Bioconjug. Chem.* 7 (1996) 38–44.
- [51] P. Bailon, A. Palleroni, C.A. Schaffer, C.L. Spence, W.J. Fung, J.E. Porter, G.K. Erlich, W. Pen, Z.X. Xu, M.W. Modi, A. Farid, W. Berthold, M. Graves, Rational design of a potent, long-lasting form of interferon: A 40 kDa branched polyethylene glycol-conjugated interferon α -2a for the treatment of hepatitis C, *Bioconjug. Chem.* 12 (2001) 195–202.
- [52] J.M. Harris, A. Kozlowski, Improvements in protein PEGylation: pegylated interferons for treatment of hepatitis C, *J. Controlled Release* 72 (2001) 217–224.
- [53] P. Glue, R. Rouzier-Panis, C. Raffanel et al., PEG-interferon- α 2b: pharmacokinetics, pharmacodynamics, safety and preliminary efficacy data, *Hepatology* 30 (1999) 189AA.
- [54] N.E. Algranati, S. Sy, M. Modi, A branched methoxy 40 kDa polyethyleneglycol (PEG) moiety optimizes the pharmacokinetics (PK) of PEG-interferon α 2a (PEG-IFN) and may explain its enhanced efficacy in chronic hepatitis C (CHC), *Hepatology* 40 (Suppl.) (1999) 190A.
- [55] M.J. Roberts, J.M. Harris, Attachment of degradable poly(ethylene glycol) to proteins has the potential to increase therapeutic efficacy, *J. Pharm. Sci.* 87 (1998) 1440–1445.
- [56] A.J. Garman, S.B. Kalindjian, The preparation and properties of novel reversible polymer–protein conjugates, *FEBS Lett.* 223 (1987) 361–365.
- [57] X. Zhao, M.D. Bentley, A hydrolyzable linkage for PEG-proteins, in: *Ninth International Symposium on Recent Advances in Drug Delivery System*, 1999, pp. 144–146.
- [58] S. Lee, R.B. Greenwald, J. McGuire, K. Yang, C. Shi, Drug delivery systems employing 1,6-elimination: Releasable poly(ethylene glycol) conjugates of proteins, *Bioconjug. Chem.* 12 (2001) 163–169.
- [59] S. Zalipsky, M. Qazen, J.A. Walker II, N. Mullah, Y.P. Quinn, S.K. Huang, New detachable poly(ethylene glycol) conjugates: Cysteine-cleavable lipopolymers regenerating natural phospholipid, diacyl phosphatidylethanolamine, *Bioconjug. Chem.* 10 (1999) 703–707.
- [60] M.D. Bentley, J.M. Harris, A. Kozlowski, Heterobifunctional poly(ethylene glycol) derivatives and methods for their preparation, WO 126692A1 (2001).
- [61] M. Yokoyama, T. Okano, Y. Sakurai, A. Kikuchi, N. Ohsako, Y. Nagasaki, K. Kataoka, Synthesis of poly(ethylene oxide) with heterobifunctional reactive groups at its terminals by an anionic initiator, *Bioconjug. Chem.* 3 (1992) 275–276.
- [62] S. Cammas, Y. Nagasaki, K. Kataoka, Heterobifunctional poly(ethylene oxide): synthesis of alpha-methoxy-omega-amino and alpha-hydroxy-omega-amino PEOs with the same molecular weights, *Bioconjug. Chem.* 6 (1995) 226–230.
- [63] Y. Nagasaki, T. Kutsuna, M. Iijima, M. Kato, K. Kataoka, S. Kitano, Y. Kadoma, Formyl-ended heterobifunctional poly(ethylene oxide): synthesis of poly(ethylene oxide) with a formyl group at one end and a hydroxyl group at the other end, *Bioconjug. Chem.* 6 (1995) 231–233.
- [64] N. Yamasaki, A. Matsuo, H. Isobe, Novel polyethylene glycol derivatives for modification of proteins, *Agric. Biol. Chem.* 52 (1988) 2125–2127.
- [65] C. Monfardini, O. Schiavon, P. Caliceti, M. Morpurgo, J.M. Harris, F.M. Veronese, A branched monomethoxy-poly(ethylene glycol) for protein modification, *Bioconjug. Chem.* 6 (1995) 62–69.
- [66] F.M. Veronese, P. Caliceti, O. Schiavon, Branched and linear poly(ethylene glycol): influence of the polymer structure on enzymological, pharmacokinetic and immunological properties of protein conjugates, *J. Bioact. Compat. Polym.* 12 (1997) 196–207.
- [67] J.M. Harris, A. Kozlowski, Poly(ethylene glycol) derivatives with proximal reactive groups, WO 99/45964 (1999).
- [68] F.H. Arnold, G.E. Wuenschell, Immobilized metal aqueous two-phase extraction and precipitation, US Patent 5,283,339 (1994).
- [69] A.J. Martinez, A. Pendri, R.B. Greenwald, Y.H. Choe, Terminally-branched polymeric linkers and polymeric conjugates containing the same, US Patent 6,153,655 (2000).