Evolution of Reactive mPEG Polymers for the Conjugation of Peptides and Proteins

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Abstract: The covalent attachment of methoxy-poly(ethylene glycol) (mPEG) is a well established strategy used to improve the pharmaceutical properties of several biomolecules. Since the pioneering work of Abuchovsky, PEGylation has emerged as a powerful technology of significant relevance, not only for the development of new and better drugs, but also for application in material science. Peptides and proteins are the most traditional targets for PEGylation due to their intense and diverse biotechnological applications. The terminal amino group, as well as the ε-amino group of lysine and the thiol group of cysteine, are all well known nucleophilic sites that have traditionally been used to couple peptides and proteins to mPEG derivatives. Advances in the methods for preparation of the mPEG starting materials, together with a careful selection of new mPEG functional end-groups have allowed new reactive mPEGs to emerge, which show narrow polydispersity and controlled reactivity, providing more homogeneous conjugates. In the last few years the trend has moved towards site-selective, reversible and enzymatic PEGylation using a new generation of tailor-made reagents and strategies. The main goal of this article is to present some of the most relevant achievements obtained in the PEGylation of peptides and proteins. The chemistry underlying the current methods used for the preparation of mPEG reagents, as well as the chemistry involved in the PEGylation reactions are presented in detail, in order of stimulating the synthetic and polymer chemist to turn their attention in this fascinating multi and interdisciplinary field of research.

Keywords: PEGylation, poly(ethylene glycol), PEG reagents, Conjugation, Protein PEGylation, Controlled drug release, Activated PEG.

1. INTRODUCTION

In the last 30 years the attachment polyethylene glycol (PEG) has been used extensively to improve the physicochemical, biomedical and pharmacological properties of several therapeutic molecules, such as peptides, proteins, antibodies, antibody fragments, oligonucleotides, and small drugs [1]. PEG is an FDA approved diol compound that has historically been used as an excipient in pharmaceutical and cosmetic formulations. Moreover, PEGs of different molecular weights are well known solvents, widely used in organic synthesis. PEGylation strategies using PEG diol derivatives have been rather limited to the conjugation of small molecules, which require increased drug loads per polymer chain. Indeed, PEG dendrimers and dendromers are preferred to linear PEG diol derivatives for small drug delivery, owing to the fact that higher drug ratios can be obtained. In the PEGylation of biomacromolecules such as peptides and proteins, the usefulness of PEG diol reagents for conjugation is relatively low, as they often lead to cross-linking of the target molecules, leading to the formation of aggregates with decreased bioactivity. On other hand, methoxy-poly(ethylene glycol) (mPEG) is a protected version of PEG, from which monofunctional reactive polymers can be prepared. These are ideal for the conjugation of proteins as they do not lead to the cross-linking of the macromolecules. mPEG is a biocompatible and non-biodegradable class of polymer that has distinctive properties that make it particularly attractive for protein-polymer conjugation: i) mPEG is soluble in water and also in many typical organic solvents. As a distinctive feature, mPEG is soluble in methylene chloride, chloroform, toluene, acetonitrile and other solvents; while it is not soluble in ethyl ether, *iso*-propanol and aliphatic hydrocarbon solvents. This unique solubility behaviour allows the purification of mPEG and mPEG derivatives from low molecular weight organic impurities via a simple precipitation-centrifugation work-up. ii) mPEG is not antigenic, immunogenic or toxic [2,3]. However, the occurrence of antibodies against PEG (anti-PEG) in healthy blood donors has been reported when high polymer loads were employed [4,5]. iii) mPEG has been approved by the FDA for human use in intravenous, oral and dermal applications. iv) mPEG chains are flexible, and in aqueous solution coordinate 2-3 water molecules per oxyethylene unit. The flexibility of the polymer chain, together with the ability of coordinating several water molecules, means that mPEGs have an apparent molecular weight that is 5 to 10 times higher than that of globular proteins of comparable molecular weights. v) Nowadays, commercially available mPEG polymers have narrow polydispersities, typically ranging from 1.01 for low molecular weight mPEGs (≅5000 Da) to 1.1 for mPEG of up to 50 kDa, which are adequate for pharmacological applications.

The covalent attachment of mPEG to bioactive polypeptides and proteins increases the plasmatic half-life of the biomacromolecules by increasing the hydrodynamic ratio, which in turn reduces excretion rate. Furthermore, the PEGylation of proteins: i) decreases the immunogenicity by shielding antigenic epitopes, a major drawback with heterologous proteins; ii) reduces the degradation by proteolytic enzymes and antibody recognition by covering the recognition sites [6]; iii) increases water solubility, which can be can be extremely important for low molecular weight drugs [7,8]; iv) modifies the biodistribution; promoting the accumulation of the conjugated bio-drugs into tumors [9,10]. Additionally PEG can promote accumulation in specific organs, tissues or cells by the EPR effect [11,12]; v) enhances bioavailability and plasmatic half-life, owed to the increment of hydrodynamic volume, which re-

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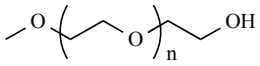
duces renal clearance; vi) stabilizes labile drugs from chemical degradation.

The hydroxyl group of mPEG is not reactive enough to allow direct coupling of the polymer to biomacromolecules. Thus, to make mPEG useful for the conjugation of proteins, the terminal hydroxyl group should be converted into a group that can react with some of the functional groups present in the biomacromolecule. In this regard, most of the functional groups present in the polypeptidic chains that are amenable for PEGylation are nucleophilic groups, namely, primary amino groups (such as terminal amino group and the ε amino group of lysine), the secondary amino group of histidine, the thiol group of cysteine, and, to a lesser extent, the terminal carboxyl, the carboxyl group of aspartic and glutamic acid and the hydroxyl group of tyrosine, serine and threonine. Hence, and as will be shown later, the vast majority of the reactive mPEGs used in bioconjugation are electrophilic derivatives. From a synthetic chemistry standpoint, proteins could be considered as sensitive compounds whose bioactivity would easily be altered even under relatively mild reaction or purification conditions. Thus, the reactive mPEGs should be able to react with the macromolecules under mild and usually aqueous conditions, in a relatively narrow *p*H range. Furthermore, the conjugate and the unreacted protein should be purified from the unreacted mPEG derivatives, the poly-PEGylated protein and other reaction by-products under mild and strictly controlled purification conditions.

2. GENERAL AND PRACTICAL CONSIDERATIONS ABOUT FUNCTIONAL PEGs REAGENTS

mPEG **1** is a neutral and linear polyether alcohol with the general structure shown below.



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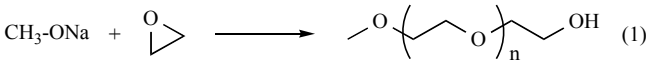
mPEG is obtained by anionic polymerization of ethylene oxide using a methoxide to initiate nucleophilic attack of the epoxide ring (equation 1) [13,14].



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Today, mPEGs of different molecular weights and of variable quality are commercially available. The diol content of the starting mPEG raw materials, which arises from trace amounts of water, oxygen or other impurities during polymer synthesis, is a key factor that determines the mPEG quality as upon synthetic manipulation, mPEG diols give rise to bifunctional reactive polymers, ultimately affording undesirable high degrees of cross-linking [15]. Usually, the diol content increases with the molecular weight of the polymer and, generally, the molecular weight of the diol polymer is twice than that of mPEG (as propagation occurs at both ends of the polymer chain). Diol contents as high as 10-15% have been observed in high molecular weights mPEGs [16,17]. It has been shown that diol-free mPEG can be obtained by methylation of crude benzyloxy-PEG, followed by hydrogenation. This reaction renders mPEG and inert dimethoxy-PEG that can ultimately be removed after biomacromolecule conjugation [18].

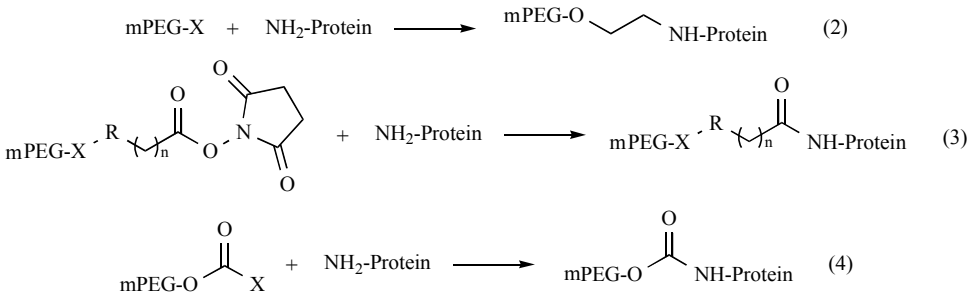
The polydispersity index (PDI, a measure of distibution of molecular masses, defined as weight average molecular weight (M_w) divided by number average molecular weight (M_n)) is another important parameter of the starting mPEG raw material, since it can critically influence the homogeneity of the conjugates. Nowadays, mPEG raw materials with PDI of nearly 1.01 for PEGs of 2-10 kDa and up to 1.1 for PEGs with higher molecular weight (>20 kDa) are commercially available.

The free hydroxyl group of mPEG is not reactive enough to allow conjugation with bioactive molecules and materials; as result, mPEG has to be converted to more reactive species, most usually to electrophilic derivatives, for protein and peptide PEGylation. In high molecular weight mPEGs, the structural changes introduced upon modification of the terminal hydroxyl groups can be negligible when compared with the structure of the repeat oxyethylene unit. With the aim of avoiding demanding purification techniques, great efforts have been made in order of providing synthetic methodologies that affords the mPEG derivatives with conversions that are as high as possible. As mentioned earlier, mPEG and mPEG derivatives can be purified via precipitation from diethyl ether or other suitable solvents. Purification and spectroscopic characterization issues of PEG derivatives have all been reviewed in detail, however it is worth mentioning that most chromatographic and spectroscopic methods used within organic chemistry are also well suited for PEGylation chemistry [19-21]. Particularly useful techniques include ^{13}C and ^{1}H NMR as well as chromatographic methods (gel permeation, reverse phase and ion exchange). Moreover, polyacrylamide gel electrophoretic methods, capillary electrophoresis and HPLC with ELSD detection can be used for the characterization of branched mPEGs and mPEGs conjugates [22].

Reactive mPEG derivatives should be able to react with peptides and proteins under mild and buffered aqueous conditions. Hence, the reactivity of mPEG derivatives should be high enough to give acceptable yields of conjugated products, but without affording undesirable high degrees of poly-PEGylated bioconjugates, compromising the protein activity, or reacting too quickly with the (aqueous) reaction solvent. As expected, most commercially available reactive mPEGs are not completely stable and it is advisable to store them in an environment protected from moisture, light and air. A summary of advantages and drawbacks associated with use of mPEG in bioconjugation are presented in Table **1**.

Table 1. Advantages and Drawbacks Associated with the Use of mPEG.

Advantages	Drawbacks
• Non-toxic. • Broad molecular weight availability. • Stable if stored properly. • Solubility in organic solvents and water; precipitation with ethyl ether.	• High diol content (cross-linking). • Spectroscopic, chromatographic and purification issues would be difficult to resolve. • Reactive mPEGs are not stable.



3. GENERAL FEATURES OF THE CHEMISTRY OF PEGYLATION

In a series of papers from 1977, Davies and co-workers demonstrated that not only was it possible to covalently attach mPEG to bovine serum albumin and bovine liver catalase, but that the enzyme conserved 95% of its activity, and that both proteins showed altered immunogenicity and prolonged circulation half-lives [23,24]. The chemistry of these pioneering PEGylations involved the reaction of the nucleophilic ε-amino group of lysine amino acids with electrophilic cyanuric chloride-mPEG derivatives. Very high levels of amino group conjugation were achieved with these extremely reactive mPEG reagents.

From the 20 proteinogenic amino acids, alanine, valine, leucine, isoleucine, proline and phenylalanine do not have side chains that could react with activated mPEG reagents, and hence, are not amenable for PEGylation. In the same fashion, glycine does not have a functional side chain, and asparagine and glutamine have relatively non-reactive amide side chains. All the remaining amino acids have side chains with pendant functional groups that could be manipulated for chemical conjugation with adequately substituted mPEG reagents. In this regard, it has been well documented in the last thirty years that the most suitable amino acids for PEGylation are (in order of suitability): Lys>Cys>Tyr>His>Asp>Glu>Arg>Trp> Ser>Thr>Met. The terminal amino group and ε-amino group of lysine amino acids of peptides and proteins have traditionally been exploited for the attachment of mPEG using different electrophilic reagents. In fact, and as will be shown later, a good part the chemistry of PEGylation involves the reaction of these nucleophilic amino groups to yield stable amide, amine and urethane bonds. The primary ε-amino group of lysine is good nucleophile at *p*H above 8.5 (*p*Ka 9.4), while terminal amino groups are less reactive, since they are close to the electrophilic carboxyl group (*p*Ka ≈ 1 to 2 *p*H units lower than the ε amino group of lysine), but they are able to react with electrophiles at *p*H of around 7. This *p*Ka difference has been exploited to achieve more site-selective PEGylation reactions. In addition, lysine is widely present in most proteins, making it likely for the conjugation of polypeptides from very different origins and structures. The free thiol group of cysteine is an excellent nucleophile, especially at high *p*H, that has intensively been used for the conjugation of proteins by mean of formation of stable thioether bonds. However, only a limited number of cysteine residues are usually available in most proteins and, for this reason, PEGylation of native thiol groups has been explored to a lesser extent in earlier developments.

The conjugation of carboxyl groups with mPEG derivatives has scarcely been used for bioconjugation. The conjugation of carboxyl groups with mPEG amines by diimide activation is difficult since this strategy can lead to the undesired cross-linking of the biomacromolecule. This problem has been surpassed using mPEG hydrazide under a specific set of reaction conditions [25]. Moreover, the conjugation at carboxylic groups has been mostly applied for the PEGylation of small drugs. The hydroxyl groups of serine, threonine and tyrosine, although nucleophilic, are not as reactive as amino group towards electrophilic mPEG derivatives. In fact, some of the reactions of these hydroxyl groups with electrophilic mPEG derivatives may lead to the formation of ester linkages, which are unstable under physiologic conditions. The guanidine moiety of arginine is a strongly nucleophilic group whose application in PEGylation with electrophilic mPEG reagents is limited, due to its high *p*Ka; *i.e.* the conjugation reaction should be performed at too high *p*H values in order of having significant amounts of the unprotonated nucleophilic group. However, a site-selective method for the conjugation of arginine has been recently accomplished (see section 7 of this article).

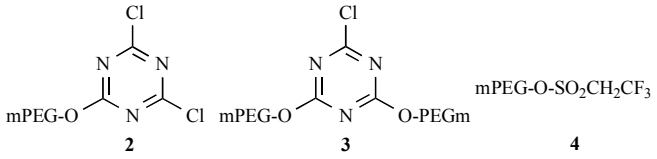
In the forthcoming pages we present some of the historically most important reactive mPEGs that, owing to their intense or relevant application, are considered nowadays as standard reagents for PEGylation chemistry. Key references on laboratory synthesis of mPEG reagents are provided, together with the chemistry supporting the PEGylation reaction, as well as exemplary applications. Actual and future trends on PEGylation are also discussed.

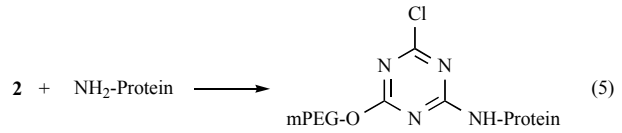
4. FIRST GENERATION MPEG REAGENTS

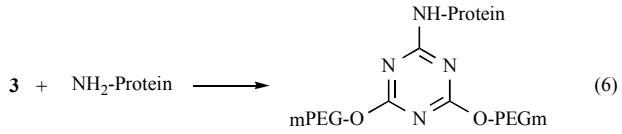
The first generation of PEGylation reagents were restricted to commercially available low molecular weight PEGs. Furthermore, the PDI of the starting mPEG raw materials were high, yielding ultimately heterogeneous conjugates. The diol content was also high, which afforded homobifunctional reagents that led to the cross-linking of biomacromolecules, and to the formation of inactive protein aggregates. Although novel, the chemistry of the PEGylation reactions was simple, and the high reactivity mPEG derivatives made the conjugation reactions essentially non-selective, affording heterogeneous and multi-PEGylated conjugates, many of which involved the formation of hydrolytically unstable bonds. In spite of these difficulties, the emerging technology quickly allowed to two PEGylated products to reach the market, namely, Adagen® and Oncaspar®. Adagen® is a PEGylated form of bovine adenosine deaminase approved by the FDA in 1990 for the treatment of severe combined immunodeficiency. This conjugate is obtained by random PEGylation with mPEG-succininmidyl carbonate (5 kDa). In the same fashion, Oncaspar® is a PEGylated form of asparaginase obtained by random PEGylation with the same reactive mPEG, which is used for the treatment acute lymphoblastic leukemia [26].

The first generation of reactive mPEG reagents for protein and peptide modification were directed towards the attachment of the polymer chain at nucleophilic amino groups. In this regard, the PEGylation reactions involved most often alkylation reactions to form stable secondary amine (equation 2) and acylation reactions to form amide (equation 3) or urethane (equation 4) bonds.

In the PEGylation of albumin and bovine liver catalase, mPEG-dichlorotriazine **2** was used as an electrophilic mPEG reagent [23,24]. Compound **2**, which can be obtained by the reactions of mPEG or mPEG alkoxide with cyanuric chloride, reacts in a non-selective fashion with the nucleophilic groups present in proteins, such as lysine, tyrosine, histidine, cysteine and serine, yielding linkages that retain the same charge of the native polypeptide (equation 5). PEGylation reactions with **2** and related compound **3** are performed at neutral or basic *p*H (7.4-10.0).







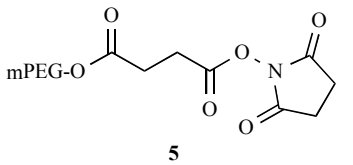
Although the remaining chloride of **2** is less reactive than the first for a second substitution, the reactivity is high enough to obtain undesirable cross-linking of the proteins. Examples of proteins that have been PEGylated using reactive mPEG **2** includes ovoalbumin [27,28], bovine serum albumin [23], superoxide dismutase [29], elastase [30], acyl-plasmin-streptokinase complex [31], trypsin [32], silk fibroin [33] and immunoglobulin [34] amongst others. The Y-shaped, branched mPEG **3** is a more convenient reagent than **2** as the decreased reactivity obtained upon replacement of two chlorine atoms yields more selective PEGylation reagents [35]. Reactive mPEG **3** has two polymer chains on one reagent molecule, allowing the attachment of two mPEG chains on each conjugation site, decreasing the possibility of inactivation of the biomacromolecule activity (equation 6). Branched polymer **3** was initially prepared by reaction of mPEG with cyanuric chloride in benzene using Na_2CO_3 as base. The activated polymer was successfully used in the conjugation of *E. coli* asparaginase [36]. It was later shown that this method affords a mixture of linear and branched polymers **2** and **3**. An improved method that affords **3** as the only product has also been provided [37]. Some further examples of use of branched mPEG **3** includes the PEGylation of ovoalbumin and phenylalanine ammonia-lyase [38,39].

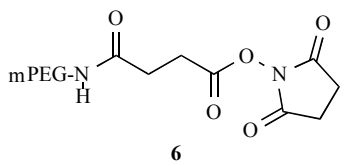
mPEG tresylate (mPEG-2,2,2-trifluroethanesulfonate) **4** has successfully been employed as an electrophilic mPEG reagent for the conjugation of proteins by mean of alkylation reactions (equation 2). Activated mPEG **4** can easily be prepared by the reaction of mPEG with tresyl chloride in the presence of pyridine [40]. The alkyation of the amino groups of peptides and proteins proceed under mild conditions, rendering conjugates that retain the net charge of the native biomacromolecule [41]. A detailed kinetic study on the coupled hydrolysis-nucleophilic substitution reactions of PEG *bis*-tresylate with amines was performed by Griffith and coworkers [42]. In this work it was indicated that low temperatures and *p*H around 8.0 are advisable for secondary amino group PEGylation, while the *p*H should be rised to around 8.8 for the conjugation to the ε amino group of lysine. Although these PEGylation reactions yield stable secondary amine bonds, it has been indicated that **4** reacts in non-specific fashion, yielding a mixture of products that can involve unstable bonds, originating non-well defined mixture of conjugated adducts [43]. For this reason, the use of mPEG **4** has been almost abandoned. Representative proteins that have been conjugated to mPEG by mean of tresylate **4** include lipases [44], alkaline phosphatase [45], albumin [40], granulocyte-macrophage colony stimulating factor [46], insulin [47] and B-deleted recombinant coagulation factor VIII [48]. Related mPEG tosylates are well known activated polymers that have been used, for example, in the PEGylation of trypsin [49].

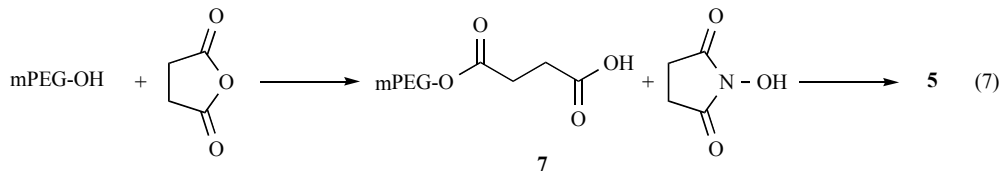
mPEG hydroxysuccinimidyl succinate **5** is a reagent that has widely been used for the conjugation of several different proteins. Reactive mPEG **5** is more selective than derivatives **2**-**4** and it reacts selectively with primary amino groups yielding stable amide bonds (equation 3). A half-life of about 20 minutes at *p*H 8 for **5** has been reported [50].

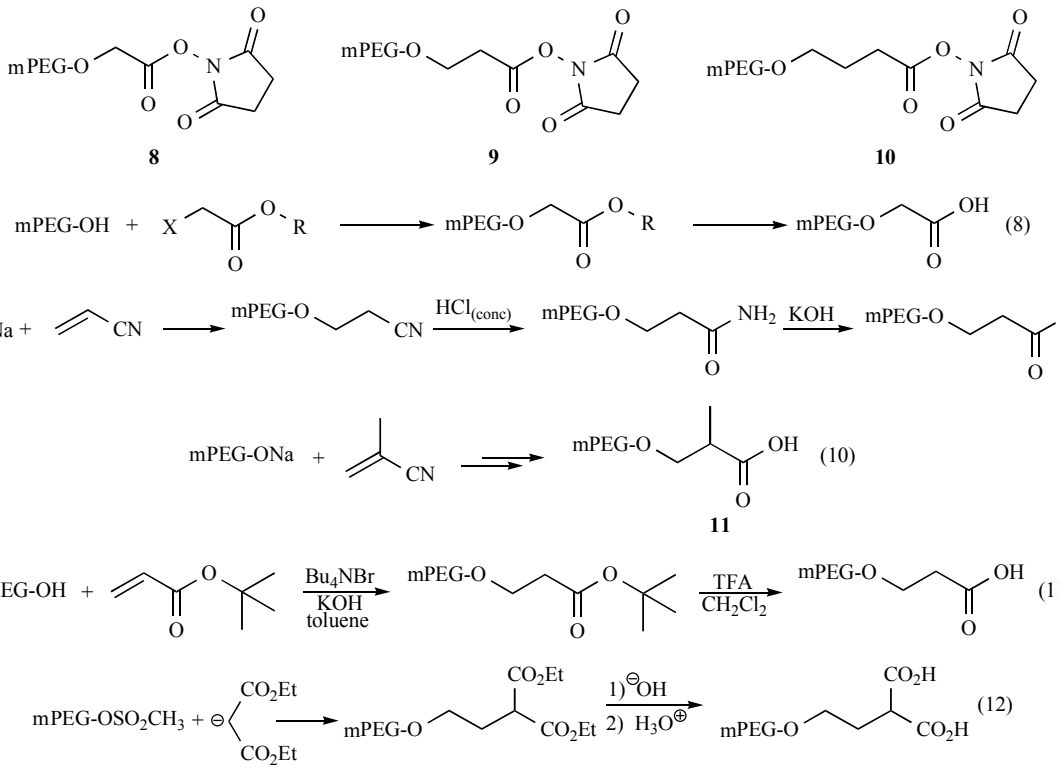
Succinimidyl succinate **5** can easily be obtained by nucleophilic ring opening of succinic anhydride to give succinic acid derivative **7**, followed by diimide mediated esterification with *N*-hydroxysuccinimide (equation 7) [51]. The nucleophilic ring opening reaction can be accelerated using DMAP as nucleophilic acylation catalyst. A melt synthetic process for the preparation of this reagent has also been reported [52].

The ester linkage between the polymer chain and the succinate moiety is very sensitive to hydrolysis under physiological conditions, and the beneficial effect obtained by PEGylation can be lost due to hydrolysis of the ester bond. In fact, the conjugation of activated **5** with proteins is nowadays recognized as an early development in releasable PEGylation. In addition, it has been indicated that the succinate moiety remains linked after hydrolysis of the polymer chain, and that it can behave as a hapten, increasing the immunogenicity of the protein [53]. Succinimidylation of mPEG-NH_2 affords reactive mPEG **6**, in which the polymer chain is at-









tached to the succinic acid residue by hydrolytically stable amide bonds [20]. In another approach, mPEG was attached to different amino acids, usually by means of stable urethane bonds, and an appropriately activated carboxyl group was employed to link the polymer (with spacer) to the macromolecule [16]. In this regard, the activated mPEG bearing a norleucine moiety is particularly important since it can be used to study the degree of PEGylation by amino acid analysis of the mPEG-protein conjugates [54]. Other amino acids, including ^{14}C or tritium labelled glycine, tryptophan and β-alanine, have also been employed in bioconjugation for different applications. In addition, Met-NLe and Met-βAla dipeptides were used for the identification of the PEGylation sites [55].

The ease of the preparation of reactive mPEG **5** stimulated its evaluation for conjugation to several different proteins. To date, the most relevant application of **5** is its use in the preparation of commercially available Adagen® (adenosine deaminase [56]) and Oncaspar® (asparaginase [57]). Further examples include the PEGylation of peptides [58], alkaline phosphatase [45], rHuG-CSF [59], arginine deiminase [60] and other proteins [61].

Activated polymers **8**-**10** are a related family of mPEG derivatives to **5**, on which the polymers are attached to the spacers by hydrolytically stable ether bonds. Compounds **8**-**10** react with primary amino groups, such as the ε amino group of lysine, yielding stable amide bonds (equation 3). Reactive mPEGs **8**-**10** are obtained using diimide activated esterification of the corresponding mPEG-acetic, propionic and butyric acid derivatives with *N*-hydroxysuccinimide (Steglich type esterifications). The syntheses of these useful derivatives are not easy and, given their utility for the PEGylation of therapeutically relevant proteins, most of the methods for their preparation have been published in the patent literature. mPEG-acetic acid has early been employed in PEGylation technology. Although mPEG-propionic and butyric acid derivatives are usually considered as second generation reagents, they are included here for comparison purposes.

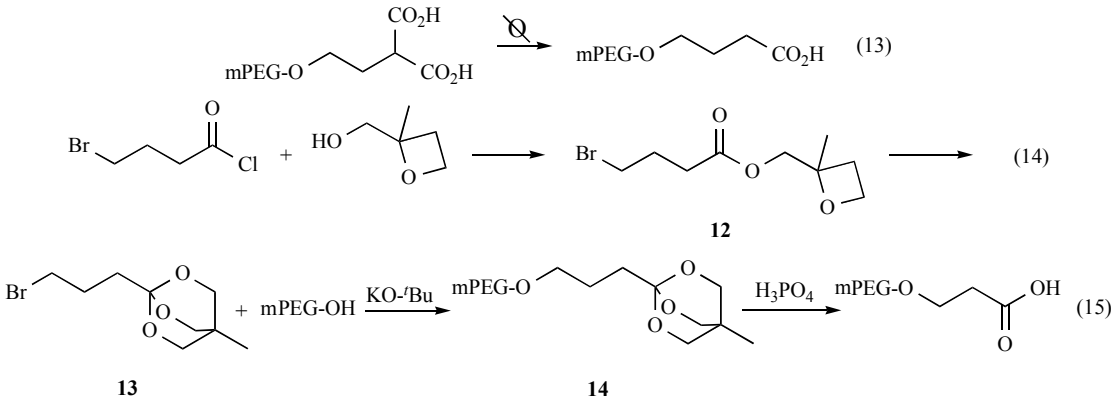
The direct oxidation of mPEG with $KMnO_4$, as well as the more selective stepwise oxidation with MnO_2, followed by H_2O_2, have both been proposed as synthetic methods for the preparation of mPEG-acetic acid **8**. However, a straightforward and more convenient synthesis of mPEG-acetic acid involves the reaction of mPEG alkoxide with alkyl 2-haloacetate, followed by hydrolysis (equation 8). mPEG akoxides can be obtained by reaction of the polymer with sodium or potassium *tert*-butoxide, sodium naphthalenide or butyllithium [20]. Most often, mPEG alkoxides are obtained by an acid-base reaction with *tert*-butoxide in *tert*-butanol, and then reacted with *tert*-butyl 2-bromoacetate (equation 8, where X: Br and R: ^{t}Bu). Although the hydrolysis of intermediate mPEG-ester can be done with trifluoroacetic acid, it has been indicated that the reaction is best performed under standard basic conditions that prevent the degradation of the polymer chain. For this method, yields of up 100% have been reported [62].

mPEG propionic acid has been prepared by conjugate addition of mPEG alkoxide to acrylonitrile, followed by hydrolysis in strongly and basic conditions [63,64]. Using mPEG of 20 kDa it was shown that this method affords 68% of the required polymer derivative (equation 9) [65].

Following a similar strategy, but employing α-methylacrylonitrile as acceptor, α-methylpropionic mPEG acid derivative **11** can be obtained (equation 10) [66,67].

A related method involves the use of *tert*-butyl acrylate as electrophilic acceptor (equation 11). The method works well with mPEGs of 20 and 30 kDa (68%) and it has been indicated that the reaction does not proceed in the absence of the quaternary ammonium salt as phase transfer catalyst (equation 11) [65].

mPEG-butyric acid has been prepared by nucleophilic substitution reaction using diethylmalonate and mPEG-methanesulfonate as substrate (equation 12). Hydrolysis in alkaline media followed by decarboxylation rendered the target mPEG-acid derivative (equation 13) [63].



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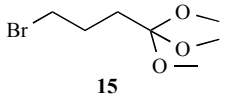
13 **14**

Other interesting syntheses of mPEG butyric acid have also been published in the patent literature [68]. The esterification reaction of 4-bromobutanoyl chloride with 3-methyl-3-oxetanemethanol affords ester **12**, which was converted to the bicyclic compound **13** via $BF_3.Et_2O$ complex. Nucleophilic displacement with an mPEG alkoxide yielded protected acid **14** that was converted to mPEG-butyric acid under acidic conditions (equations 14-15).

Commercially available trimethyl 4-bromo*ortho*butyrate **15** was used as electrophilic starting material in a similar substitution reaction. Halides **13** and **15** are synthetically equivalent to 4-bromobutyrate esters, but they lack acidic protons in the α position that may compete through acid-base reaction with the nucleophilic substitution



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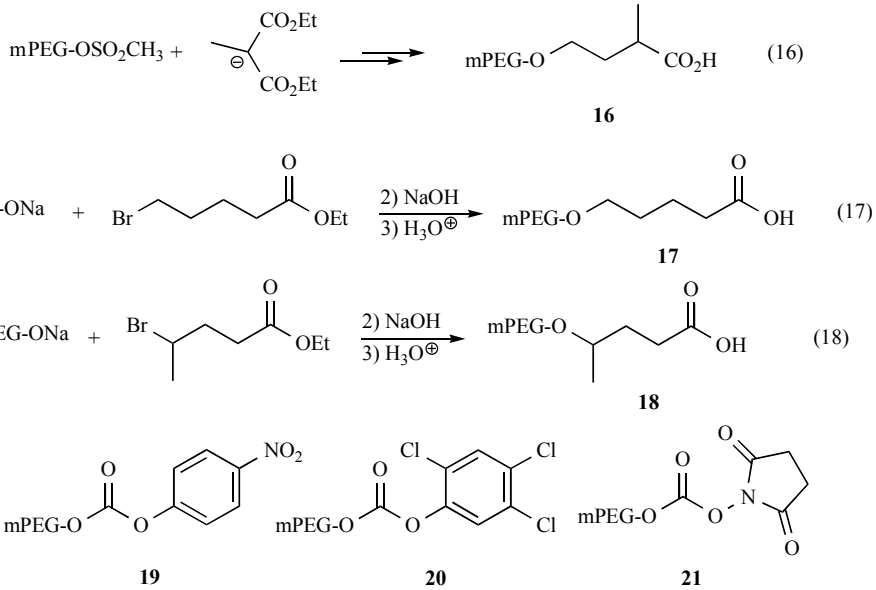
It has been indicated that related mPEG-butyric acid derivatives bearing a methyl group in the α-position (**16**) can be prepared following the reaction sequence presented in equations 12-13 (equation 16).

In addition, it has been shown that mPEG-valeric acid (**17**, equation 17) and mPEG-butyric acid derivative **18** (equation 18) can be obtained by nucleophilic substitution reactions using mPEG alkoxides with ethyl 5-bromovalerate and ethyl 3-bromobutyrate as substrates [69].

The reactivity of activated mPEG acids **8**-**10** towards the amino groups of peptides and proteins depends on the length of the hydrocarbon spacer moiety. Thus, the longer the the hydrocarbon chain between the polymer and the reactive center, the lower the reactivity towards the amino group and hence, the higher the selectivity of the PEGylation reaction. The hydrolysis rates of derivatives **8**-**10** and of *N*-hydroxysuccinimdyl esters of **11** and **18** and related compounds correlates well with this reactivity tenet. For example, the half-lives of compounds **8**-**10** and of *N*-hydroxysuccinimdyl esters of **11** and **18** are 0.75, 16.5, 23.3, 33 and 44 minutes respectively (25ºC, *p*H 8) [63,65].

mPEG-acetic acid **8** and mPEG-propionic acid derivative **9** have extensively been employed in bioconjugation of relevant proteins, such as methioninase [70], insulin [71], murine GM-CSF [72], glucagon-like peptide 1 [73], protamine [74], salmon calcitonin [75], α-lactalbumin [76], recombinant human arginase I [77], concanavalin A [78], pretreatment of allografts [79], adenovirus vectors [80,81], platelet CD42a [82], red blood cells [83], formate dehydrogenase [84] and antibodies against glutamic acid decarboxylase [85-87].

An example of the use activated mPEG-butyric acid **10** includes the PEGylation of erythropoietin [88]. This conjugate, commercially available as Mircera®, is used for the treatment of anaemia associated with chronic kidney disease.

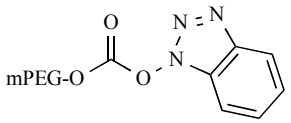


16

17

18

19 **20** **21**



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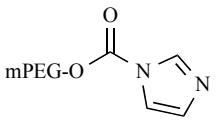
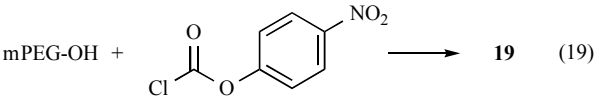


23

mPEG carbonates **19-22** and mPEG-carbonylimidazole **23** belong to a closely related family of activated polymer derivatives that react with primary amino groups of peptides and proteins forming stable urethane bonds (equation 4). The reactivity of these compounds towards the amino groups depends on nature of the leaving group, and this can affect not only the selectivity, but also the degree of conjugation. Kinetic data for hydrolysis rates of some of these derivatives have been published. For example, the half-live of compounds **21**-**23** are as follow: **21**, 20.4 min (*p*H 8, 25ºC), **22**, 13.5 min (*p*H 8, 25ºC), and of around 10 h for **23** (*p*H 8, 25 ºC). From a practical point of view, the reactivity order of derivatives **19**-**24** is **22**>**21**>**19**>**20**>**23**. Although all these mPEG reagents have intensively been used in early developments, the actual trend in PEGylation reactions involving urethane bond formation has moved towards derivatives **22** and **21**, since they show a good balance between the reactivity and selectivity, but lacking from the toxicity problems found with reagents **19** and **20**.

mPEG-4-nitrophenyl carbonate **19** has been prepared by the reaction of mPEG with 4-nitrophenylchloroformate under different conditions (equation 19). Veronese and co-workers initially prepared **19** in acetonitrile using pyridine as base [89]. Variations of this method include the reaction performed in dichloromethane with pyridine and triethylamine [90,91]. Although useful, it has been indicated that these methods afford an unknown mPEG-amine as impurity. It was suggested that pure **19** can be prepared upon reaction of mPEG with 4-nitrophenylchloroformate in toluene with tripentylamine [92] or with 4-dimethylaminopyridine in dioxane [93].

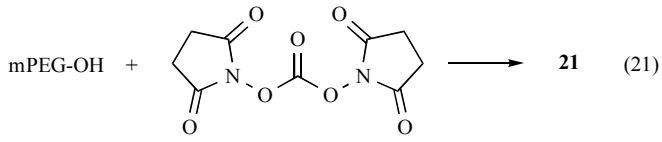


(19)

Representative applications of the use of **19** include the PEGylation of ribonuclease and superoxide dismutase [89], doxorubicin [94], recombinant mammalian urate oxidase [95], uricase [96] and lysozyme [97].

Reactive mPEG **20** can be prepared following the same method used for the synthesis **19**, but employing commercially available 2,4,5-trichlorophenylchloroformate [89].

mPEG-succinimidylcarbonate **21** is a well known activated mPEG that has been employed in the conjugation of several different peptide and proteins, such as asparaginase [98], trypsin [99], chymotrypsin [100], bacteriorhodopsin [101], salmon calcitonin [102], interferon α-2b [103], arginine deiminase [104] and recombinant human arginase I [77], among other bio-macromolecules [105]. Compound **21** reacts with primary amino groups yielding stable urethane linkages and with the hydroxyl group of tyrosine, threonine and serine to afford hydrolytically unstable carbonate bonds. Moreover, it has been indicated that, depending on the *p*H of the conjugation medium, **21** can react with the imidazole ring of histidine to yield unstable urethane linkages. For example, activated mPEG **21** reacts with interferon α2-b at *p*H 6.5 to give a conjugate that has 47% of PEGylation at histidine residues and 32% at lysine amino acids [106]. The bioconjugate obtained in this conditions and employing mPEG of 12 kDa is commercially available (PEGintron®) and is actually used for the treatment of hepatitis C and melanoma (Sylatron®). PEGylation at histidine residues to yield unstable urethane bond is also considered as a releasable or reversible conjugation method. Activated mPEG **21** is more reactive than derivatives **19** and **20** and it can be obtained by reaction of mPEG with phosgene followed by *N*-hydroxysuccinimide (equation 20) [56]. It has been proposed that this reaction is complete affording **21** in almost quantitative yields [107]. A variation of this method, in which toxic phosgene was replaced by more friendly triphosgene, has been published in the patent literature [108]. In addition, activated mPEG **21** can be prepared by the reaction of mPEG with commercially available *bis*-*N*-hydroxysuccinimidylcarbonate (equation 21) [109]. An alternative method, in which mPEG is reacted with *N*,*N*'-disuccinimidyl oxalate (**24**) in the presence of pyridine, has also been published. The same reaction, but using 1,1'-*bis*[6-(trifluromethyl)benzotriazolyl]oxalate (**25**), was used for the synthesis of an activated mPEG closely related to hydroxybezontriazolyl derivative **22**.



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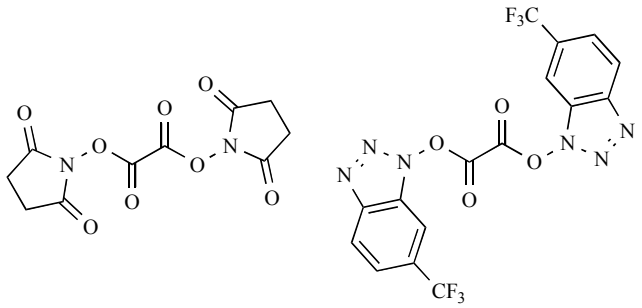
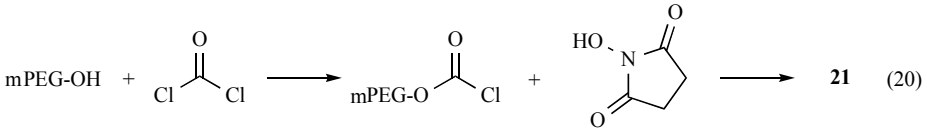


24 **25**

1-Benzotriazolyl derivative **22** is a reactive mPEG that has found application in the PEGylation of D-glucosamine, sodium heparin, human serum albumin [110], fibrin biomatrix [111], lysozyme [112] and peptides among other targets [113]. In similar fashion than **21**, activated mPEG **22** reacts with histidine and tyrosine residues to afford unstable urethane and carbonate linkages. mPEG-benzotriazolyl carbonate **22** can be prepared following similar strategies than those depicted in equations **20** and **21**, *i.e.* the reaction of mPEG with phosgene followed by reaction with 1-hydroxybenzotriazole [110], or the reaction with *bis*-1-bentriazole carbonate in presence of pyridine as base [112].

mPEG-carbonylimidazole **23** is a mild PEGylating reagent that has been used since the mid 1980's for the conjugation of proteins, amongst other targets. It has been claimed that, due to its low reac-



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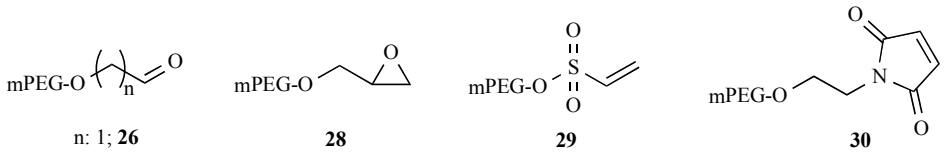
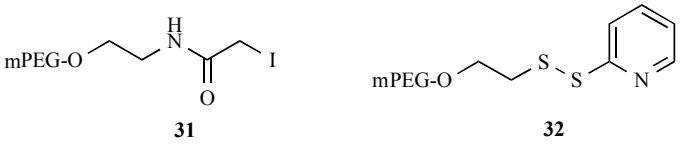
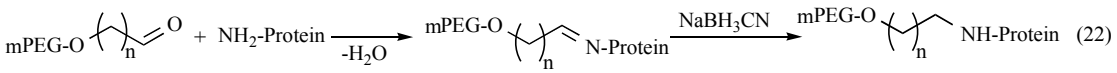


n: 1; **26**
n: 2; **27**

28 **29** **30**



31 **32**



n: 1; **26**
n: 1; **27**

tivity, activated mPEG **23** behave as more selective reagent than **21** and **22**. In agreement with this reactivity, usually low to moderate yields of conjugation are obtained with reagent **23**. Compound **23** was initially prepared by reaction of mPEG with *N,N'*-carbonyl-*bis*-imidazole (CDI) in dioxane at 37ºC [114]. It was later demonstrated that the yield of the activated polymer obtained following this method was around 42% [115]. An improved method that affords activated mPEG **23** with quantitative yields, which involves the use 4-dimethylaminopyridyne in toluene, has also been published [115]. Similarly, an excellent yield of activation was obtained for the reaction of mPEG (20 kDa) with CDI in THF at 60ºC [116].

Activated polymer **23** is a mild PEGylating reagent that has been applied in the conjugation of superoxide dismutase [114], α2-macroglobulin [117], lactoferrin [114], streptokinase [117], alkaline phosphatase [45], IgG [118] and urokinase [119], among others.

5. SECOND GENERATION mPEG REAGENTS

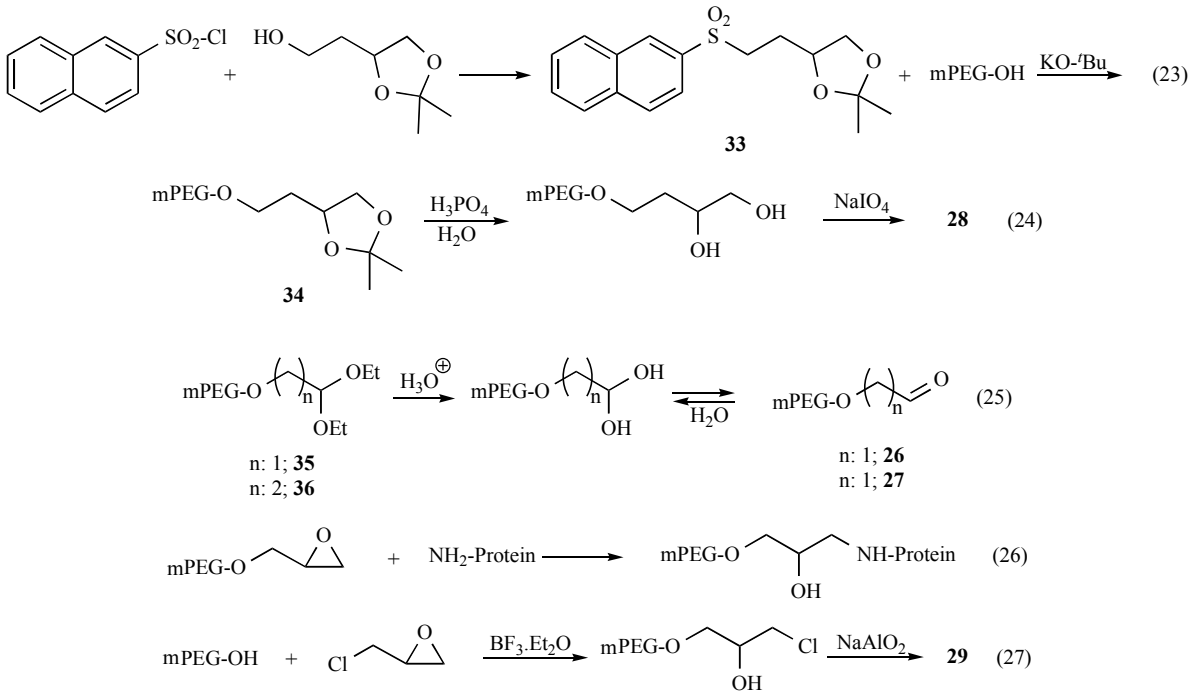
The syntheses of first generation mPEG reagents involve usually simple and straightforward reaction steps with very reactive reagents that were aimed to convert the relatively unreactive hydroxyl group of mPEG to electrophilic derivatives. However, it has been indicated that most of the first generation synthetic methodology are problematic with respect to high molecular weight mPEGs, affording heterogeneous mPEG reagents that contain variable amounts of unreacted polymer. In addition, the high diol content of the initially available mPEGs yielded high molecular weight bifunctional polymers that ultimately led to undesirable cross-linking of the proteins. The vast majority of first generation chemistries were directed towards the conjugation of electrophilic mPEG reagents with amino groups and, mainly, with the primary ε amino group of ubiquitously present lysine amino acids. Furthermore, the high reactivity of the first generation reagents conducted to unselective random PEGylation reactions, which, in addition, can afford poly-PEGylated conjugates with decreased biological activity, or to polymer conjugates bearing unstable links.

Nowadays, the improvement on the methods for the preparation of mPEGs allows for the easy commercial availability of high molecular weight mPEGs with adequate polydispersities and low diol contents. This fact, together with the huge efforts made in the development of more selective PEGylation reactions, allowed the emergence of a second generation of mPEG reagents that lack of some of the drawbacks associated to the first generation chemistries. Although nucleophilic amino groups remained as important targets for the attachment of electrophilic PEGs, some second generation mPEG derivatives were designed to anchor PEG to the thiol group of cysteine amino acids, which are amenable residues for site-selective PEGylation.

The reductive alkylation of primary amino groups is a well known two-step method used in organic synthesis for the preparation of amines without over-alkylation. Under slightly acidic conditions (*p*H 4.5-5.0) a carbonyl compound reacts with a nucleophilic amine to yield a Schiff base that can be isolated and converted to the amine by mean of an adequate reducing reagent, such a $NaBH_4$ or LAH. One-pot versions of the same reaction using $NaBH_3CN$ and $NaBH(OAc)_3$ are also well established methods. In PEGylation chemistry, the reductive alkylation reaction has successfully been used to achieve mild and quite site-selective conjugation reactions using mPEG-acetaldehyde **26** and mPEG-propanaldehye **27** with $NaBH_3CN$ as the reducing reagent (equation 22). mPEG-propanaldehyde **27** is preferred over acetaldehyde **26** since the later is unstable and dimerizes by aldol condensation reaction.

The reaction of electrophilic mPEG **26** and **27** with amino groups depends upon i) the nucleophilicty of the particular amino group involved in the condensation reaction, and ii) the *p*H at which the condensation reaction is performed. In this regard, the ε amino group of lysine is more nucleophilic and basic than most of terminal (and α carboxyl) amino groups. Under the slightly acidic conditions required for the condensation reaction, the more reactive ε amino groups are in the protonated and non-nucleophilic form, allowing for relatively high site-specific PEGylations at terminal amino group. As with previous alkylating reagent, the net charge of the protein remains unchanged after the conjugation reaction.

mPEG-acetaldehyde **26** has been prepared by the oxidation of mPEG with pydrinium chlorochromate in methylene chloride, albeit in low yield (30%) [120]. Aldehyde **26** can also be prepared by Moffat-type oxidation reaction or by the nucleophilic substitution reaction of mPEG potassium alkoxide with chloro- or bromoacetaldehyde diethylacetal followed by acid hydrolysis [121-123]. In a similar approach, aldehyde **27** can be obtained by nucleophilic substitution reaction of mPEG alkoxide with 3-chloropropanaldehyde diethyl acetal followed by deprotection (50%) [124]. In the patent literature it has been claimed that 2-naphthalenesulfonyl chloride reacts with 2,2-dimethyl-1,3-dioxolane-4-ethanol, yielding an intermediate sulfonate **33** (equation 23). Sulfonate **33** is then reacted with the mPEG alkoxide to afford a protected mPEG-diol derivative



34, which after hydrolysis in acid conditions, followed by oxidation with $NaIO_4$ gives mPEG-propionaldehyde **27** (equation 24) [125].

It has been indicated that acetals **35** and **36** are more stable and can be obtained with higher purities than the corresponding mPEG-aldehydes **26** and **27**. The diethylacetals **35-36** are converted to aldehyde hydrates under acidic conditions, and after adjusting the *p*H, they can directly be used for the conjugation reaction (equation 25) [126].

Examples of the use of aldehydes **26** and **27** includes the PEGylation of horseradish peroxidase [127], CD4 immunoadhesin [121], doxorubicin [128], recombinant human granulocyte colony-stimulating factor (Filgrastim, r-metHuG-CSF) [129,130], human megakaryocyte growth and development factor (MGDF) [131], lysozyme [132], tumor necrosis factor receptor type I [133], interferon β-1a [134], octreotide [135], recombinant human arginase I [77], recombinant erythropoietin [136], porcine follicle-stimulating hormone [137] and others [124,125].

mPEG-epoxide **28** is a mild PEGylating reagent that reacts with the amino groups of peptides and proteins, producing stable secondary amine bonds (equation 26). Due to the low reactivity of this electrophilic mPEG reagent, its application for the mPEGylation of biomacromolecules has been limited. It addition, it has been suggested that this reagent is not selective for amino group conjugation, since it reacts with hydroxyl, thiol and imidazole groups. However, mPEG-epoxides have found application in material and polymer science among other uses [138-142]. Proteins that have been PEGylated with this reagent include glutathione, bovine serum albumin, protein A, and monoclonal antibodies [143-145].

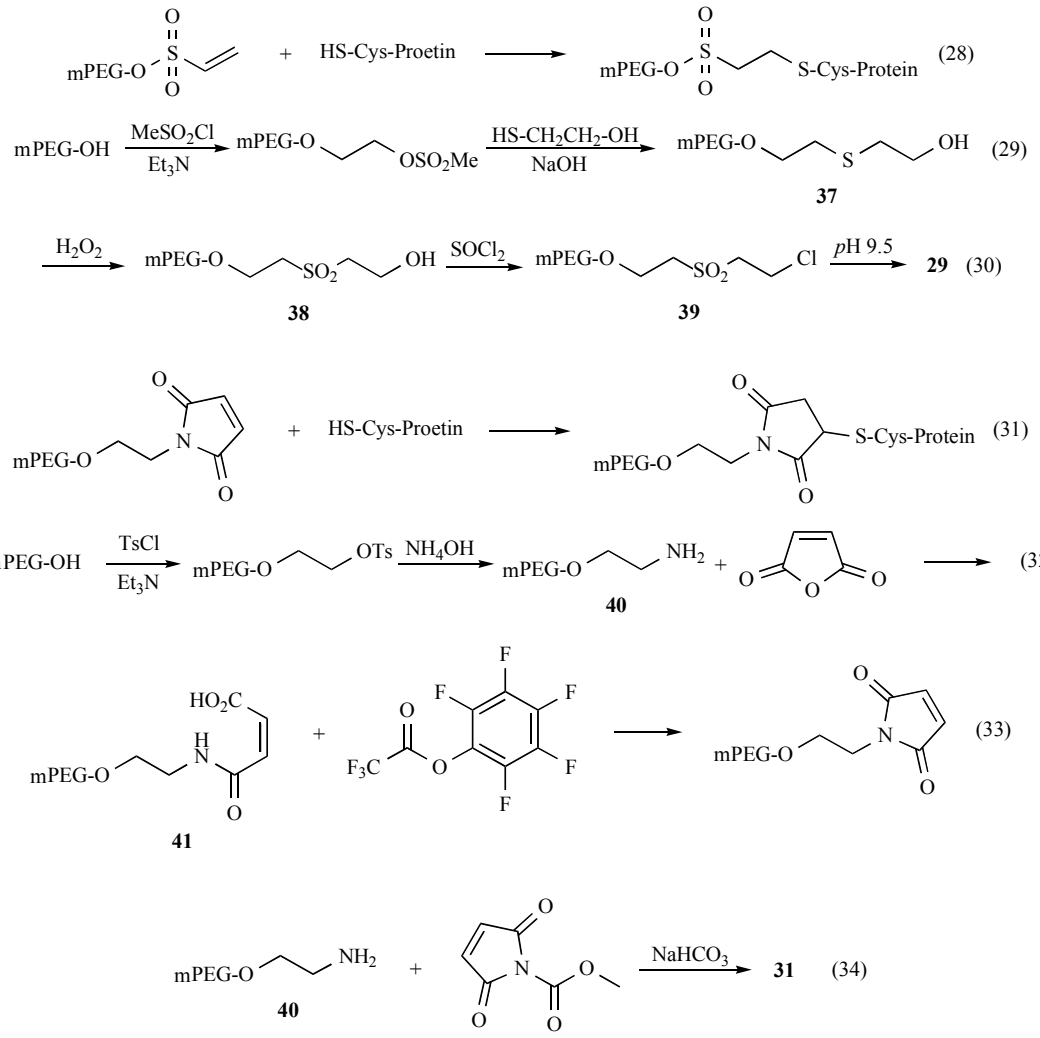
Epoxide **29** can be prepared by nucleophilic reaction of mPEG with the oxyrane ring of epichlorohydrin in presence of $BF_3.Et_2O$ complex. The intermediate alcohol product is then dehalogenated with sodium aluminate to afford the required mPEG reagent (equation 27) [146].

In the last 15 years, thiol group conjugation has evolved as a major approach for site-selective PEGylation. In this regard, cysteine amino acids are scarcely present in peptides and proteins and the availability of this nucleophilic group at the surface of the protein can be limited. In addition, the SH moiety is one of most reactive nucleophilic groups present in biomolecules, that can react with electrophilic mPEG reagents at slightly acidic or neutral *p*H (6-7). At these *p*H values, nucleophilic amino groups are present in the protonated form that cannot compete with thiol nucleophiles. Cysteine is a hydrophobic amino acid that is usually located in the internal part of the protein, limiting its accessibility for conjugation. On the other hand, thiol group PEGylations at cysteine residues located in the surface of the protein usually do not compromise the protein bioactivity, taking full advantage of the benefits of PEGylation. In the case of the absence of cysteine residues in the native protein, one or more of these amino acids can be added by genetic engineering methods [147,148]. Although useful, this approach can lead to the formation of unwanted disulfide linkages in addition to protein dimerization. Furthermore, another approach to increase the amount of available cysteine amino acids for conjugation involves the reduction of protein disulfide bridges [149]. This strategy proved to be particularly well suited for the site-selective PEGylation of antibodies, the activity of which can critically be decreased by terminal amino group conjugation, among other proteins. Of course, these conjugation methods are site-selective when the protein possesses only one disulfide bridge or when only one of two or more of the disulfides bridges can be reduced selectively.

Activated mPEGs **29-31** are commercially available reagents that have been designed to selectively react with the thiol group of cysteine.

Vinyl sulfone **29** reacts selectively with SH of cysteine in slightly basic conditions (*p*H 7-8) yielding a stable thioether linkage (equation 28). However, it has been indicated that lysine conjugation also occurs at *p*H of around 9.3 using large excess of the PEGylating reagent.

mPEG-vinylsulfone **29** can be prepared following the method presented in equations 29-30. Mesylation of the mPEG starting material under standard conditions renders the corresponding methanesulfonate, that is then reacted with 2-mercaptoethanol under basic conditions to give sulfide **37** (equation 29). Oxidation



with H_2O_2 yields sulfone **38** that by halogenation reaction with $SOCl_2$ produces halide **39**. Under very mild basic conditions chlorosulfone **39** is dehalogenated to the target reagent with quantitative yield (equation 30) [150].

Recently, an extremely simple one-step "click" method for the preparation of the vinyl sulfones of mPEG, among other hydroxyl-terminated polymers, has been published. This reaction involves the solubilization of mPEG in alkaline solution followed by Michael-type addition of the alkoxide ions using divinylsulfone as acceptor to afford directly the desired reactive polymer [151].

mPEG-maleimide derivative **30** is a well known reagent that has been widely used for selective thiol group conjugation. In neutral or slightly basic conditions (*p*H 7.5-8.5) mPEG-maleimide reacts with the thiol group in a Michael addition reaction, yielding hydrolitically stable thioether linkages (equation 31). As with mPEG-sulfone **29**, side-reactions that arise from the addition reaction of amino groups are observed when the *p*H is increased, although at very low rates. It has been suggested that the rate of amino conjugation also increases if an organic co-solvent is added [16].

Activated mPEG **30** can be prepared as depicted in equations 32-33. Tosylation of mPEG under standard conditions followed by nucleophilic displacement with aqueous ammonia solution gives mPEG-amine **40** (equation 32). Acylation of **40** with succinic anhydryde in a polar aprotic solvent yields acid derivative **41**, which under harsh reactions conditions, is ultimately converted to the desired maleimide (equation 33) [152].

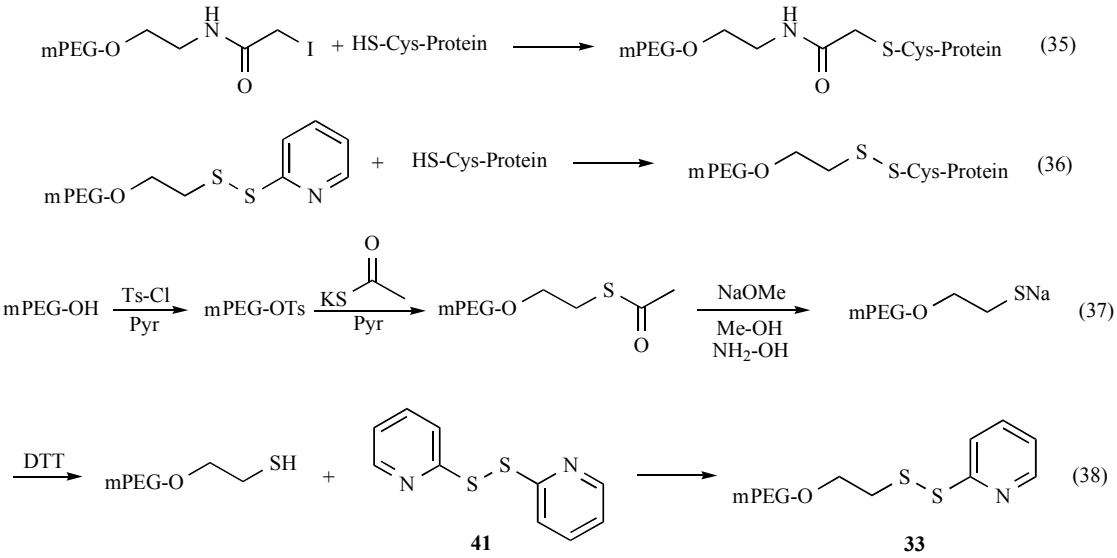
Although closely related methodology for synthesis of **30** has earlier been published, it has been indicated that these methods affords undesirable high amounts of side-products [153-155].

In the patent literature it has been claimed that mPEG-amine **40** reacts with *N*-methoxycarbonylmaleimide in aqueous basic solution to yield mPEG derivative **30** in one step (equation 34) [156].

Although **30** is, probably, one of the most widely used mPEG for thiol group conjugations, it has been shown that this compound is unstable in aqueous solution. Furthermore, it has been indicated that it cannot be stored for a prolonged period of time. Selected examples of PEGylation with **30** include the conjugation of interleukin-2, certolizumab pegol, staphylokinase, rHuGM-CSF, erythropoietin analogues, cyanovirin–N, prolidase, recombinant human arginase I, si-RNA, bone morphogenetic protein-2, exenatide analogues and aptamers, among many other examples [77,157-167].

Related to **29** and **30**, mPEG acrylate has also been used as Michael acceptor for selective cysteine PEGylation [168].

mPEG-iodoacetmide **31** is a mild PEGylating reagent that reacts with thiol group of cysteine aminoacids of peptides and proteins, yielding stable thioeter linkakes (equation 35). In the same fashion as shown for derivatives **30** and **31**, some loss in the selectivity of the conjugation reaction is observed as the *p*H is increased.



PEGylation reactions with derivative **31** are usually performed using excess of the reagent in a darkened flask in order to limit the formation of iodine, which can react with other amino acids. mPEG-iodoacetamide can be prepared by the reaction of mPEG-amine **40** with iodoacetic anhydryde in dioxane [154]. Among other examples, reagent **31** has been used for the PEGylation of acyl-coenzyme A and cholesterol acyltransferase [169].

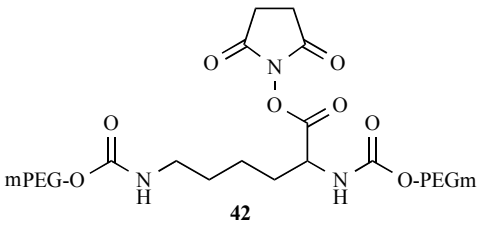
mPEG-*ortho*pyridyldisulfide **32** (mPEG-OPSS) is the most selective reagent for thiol group conjugation. This derivative reacts with SH groups in acid and alkaline media via a thiol exchange reaction to afford a disulfide linkage that is unstable under reducing conditions (equation 36).

Several other mPEG-OPSS polymers bearing different spacers between the polymer chain and the pyridyl-disulfide moiety have been published, mainly in the patent literature [170].

mPEG-OPSS **32** has been prepared by the tosylation of mPEG followed by nucleohilic displacement with potassium thioacetate to give an intermediate thioester (equation 37). After methanolysis and treatment with dithiothreitol, the free thiol group was converted to the final product by reaction with *ortho-bis*-dithiopyridine **41** (equation 38) [171].

Examples of the application of **32** includes the PEGylation of interferon β and staphilokinase [158,171,172].

Branched mPEGs have received particular attention due the well documented improvements in the biopharmaceutical properties of several therapeutically relevant proteins. In this regard, it has been showed that branched mPEGs behave as if they have higher molecular weights than linear mPEGs of identical molecular weight. In addition, when branched mPEGs are used, more than one polymer chain can be linked onto each attachment site. The net result of these two effects translates into a better shielding of the protein, while at the same time the possibility of deactivation of the active site of the protein is diminished. Among the several different branched mPEG structures that have been prepared and evaluated, a branched mPEG bearing a lysine linker (**42**) proved to be particularly well suited for the conjugation of several different proteins, including: ribonuclease, catalase, asparaginase, trypsin [173], interferon α-2a [174-176], interferon α-2b [177], interferon β-1b [178], TNYL-RAW - Fc portion of human IgG1 [179], peptides targeting the human neonatal Fc receptor [180], α-momorcharin [181,182], erythropoietin [136], organophosphorus hydrolase [183] and lactoferrin [184] among several others.



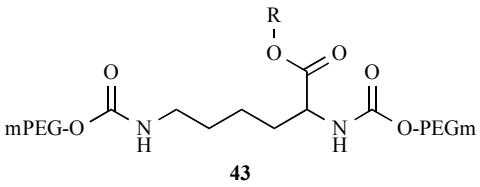
42

As with the *N*-hydrosuccinimidyl esters **8-10**, mPEG derivative **42** reacts with the amino group of peptides and proteins yielding stable amide bond (equation 3).

Several methods for the synthesis of **42** have been published or patented. mPEG-*p*-nitrophenylcarbonate **19** was reacted with lysine in buffered aqueous media to yield mPEG-monosubstituted lysine, that was then treated with the same reagent in methylene chloride to yield **43a** in a two-step procedure. Given that the ε amino group of lysine is more reactive than the α amino group, this two-step procedure allows the preparation of branched **43a** bearing mPEG chains of different molecular weight [173]. A more convenient method proved to be the reaction of mPEG-*N*-hydroxysuccinimidyl carbonate **21** with lysine in buffered aqueous solution [173]. In these methods **43a** was purified by ion exchange chromatography and converted to **42** by diimide mediated esterification with *N*-hydroxysuccinimide [185]. The related **43b** has been prepared by reation of lysine with **19** using pyridine as base [186].

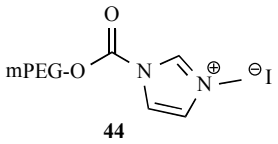
A straightforward method for the synthesis **43a** has recently been published. *N*-Carbamoylimidizaolium iodide **44,** which can easily be obtained by the methylation of mPEG-carbonylimidazole **23**, was reacted with an organic soluble sylilated lysine in DMSO/acetonitrile using di-*iso*proylethylamine as base, to yield **43c** in a single step. Compound **43c** was converted to **43a** during the aqueous workup of the reaction, and the product was purified by hydrophobic interaction chromatography [116].

An elaborate but useful method for the preparation and use of **42** in the PEGylation of proteins that avoids intermediate purification steps has been published in the patent literature [108]. mPEG was reacted with triphosgene to yield the activated mPEG derivative together with unreacted mPEG. The crude product mixture was



43

43a: R: H
43b: R: Et
43c: R: $SiMe_3$



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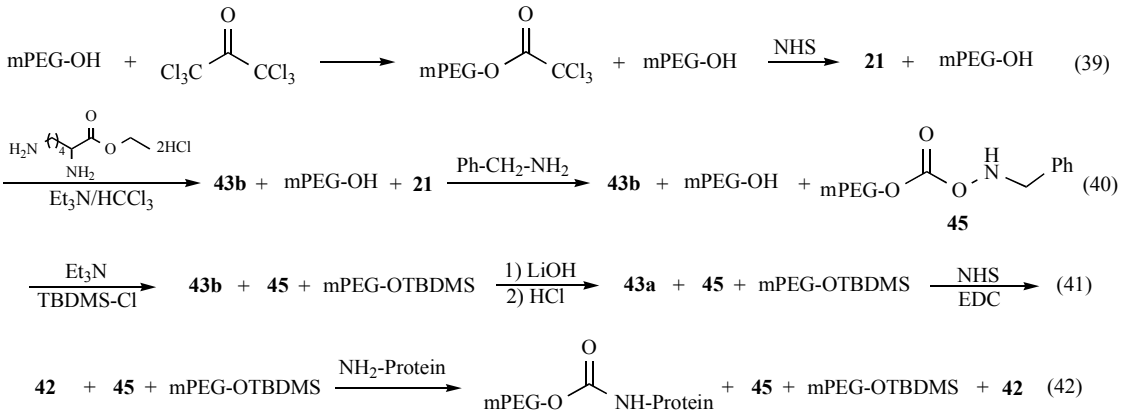


treated with *N*-hydroxysuccinimide (NHS) affording the required mPEG-NHS carbonate **21** (equation 39). The reaction mixture containing of **21** and unreacted mPEG was reacted with lysine ethyl ester di-hydrochloride to give a complex reaction mixture that contains protected branched mPEG **43b**, unreacted mPEG and activated **21** (equation 40). In order to deactivate **21**, the mixture was treated with benzylamine, which converted **21** into an unreactive carbamate **45** (equation 40). Upon reaction of this mixture with *tert*-butyl-dimethylsilane chloride (TBDMS-Cl), unreacted mPEG was transformed to the inert silyleter mPEG-OTBDMS (equation 41). Finally, saponification followed by esterification with NHS under diimide activation yields a mixture of products containing reagent **42**, which can directly be used for the PEGylation of proteins (equation 41 and 42). A noteworthy point is that only one purification step is performed after the conjugation reaction.

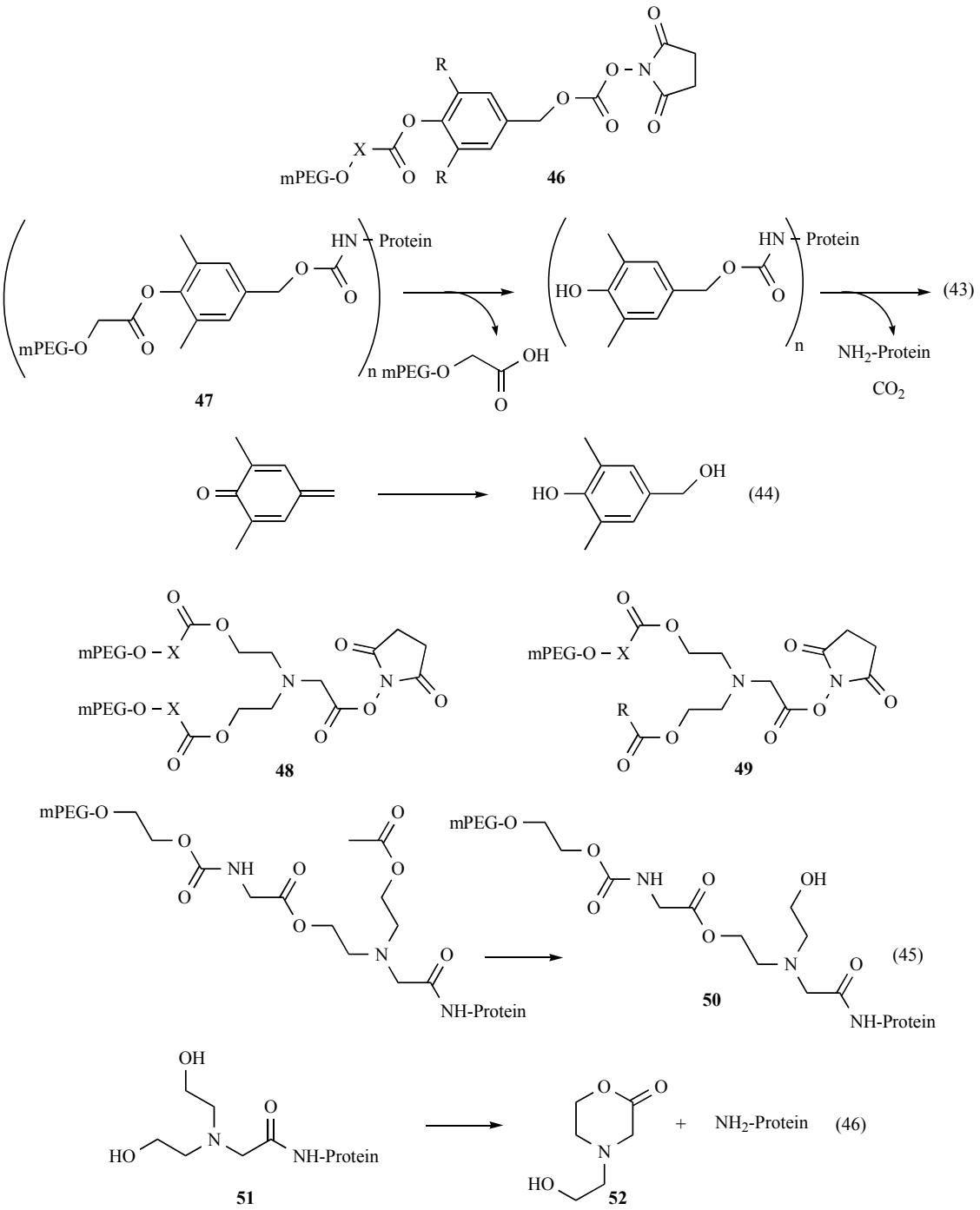
It is worth mentioning that four conjugates available in the marketplace are prepared using branched mPEG **42** (Pegasys® (interferon α-2a), Macugen® (aptanib), Cimzia® (anti-TNF fab') and Omontis® (dimeric synthetic peptide for the stimulation of erythropoiesis).

6. REVERSIBLE OR RELEASABLE PEGYLATION

As described earlier, one of the main goals of PEGylation is to covalently attach mPEG polymer chains to biomacromolecules. Such attachment should be stable under physiological conditions in order to increase the time of residence of the conjugate in the blood and, in this way, take full advantage of the benefits of PEGylation. On the other hand, the attachment of the polymer chain can decrease the activity of the protein by conjugation at the active site or by steric hindrance. These effects may be so profound that they can lead to drug inactivation. In order to overcome the drug inactivation by polymer conjugation, in the last few years different strategies for releasable or reversible PEGylation have been developed [187,188]. In releasable PEGylation, the polymer is bound to the drug or biomolecule through an unstable bond or a degradable linker which can be cleaved under specific conditions, releasing the native unmodified drug at a controlled rate. In fact, in releasable PEGylation the drug-polymer conjugate is a prodrug from which the unmodified active drug is delivered after polymer release. In the particular case of proteins, this conjugate prodrug may show reduced activity or be completely inactive. In addition, since PEG is water soluble, this approach can be used to deliver poorly soluble drugs. Moreover, the careful design and selection of specific linkers allows tissue or organ site-specific drug release.

Esters, carbonates, carbamates, hydrazones and amides have all been used as hydrolytically or enzymatically degradable linkages for reversible PEGylation. These approaches proved to be particularly useful for the reversible PEGylation of small drugs, such as paclitaxel, camptothecin and podophyllotoxin, amongst others [189-191]. It has been shown that the design of the linker which brings together the protein and the polymer chain, forming a tripartate prodrug, is a key issue to provide specific initiation and accurate rates of release. As a result, releasable prodrugs from daunorubicine, vancomycin, amphotericine B, and doxorubicine with bicine and linkers for 1,4- or 1,6- benzyl elimination (BE) have all been prepared [192-195].

Benzyl elimination has been found to be particularly attractive for reversible protein PEGylation. In this approach, the polymer is initially released by the cleavage of a hydrolytically unstable linkage, followed by a well known fast 1,4- or 1,6- elimination reaction that renders the unmodified native protein (equations 43-44) [196,197]. The chemistry of the PEGylation reaction with these linkers involves the formation of urethane bonds between free amino groups (preferably ε amino groups of lysine amino acids) of proteins and a reactive mPEG *N*-hydroxysuccinimidyl carbonate, such as **46**, in a similar fashion to that shown in equation 4. The kinetics of the release reaction can be finely tuned by the introduction of substituents in the *ortho* position to the hydroxyl group. For example, it has been shown that the presence of two methyl groups *ortho* to the hydroxyl functionality affords ester linkages that are relatively more stable than the unsubstituted counterparts. Furthermore, a wide variety of compounds related to **46** where the mPEG

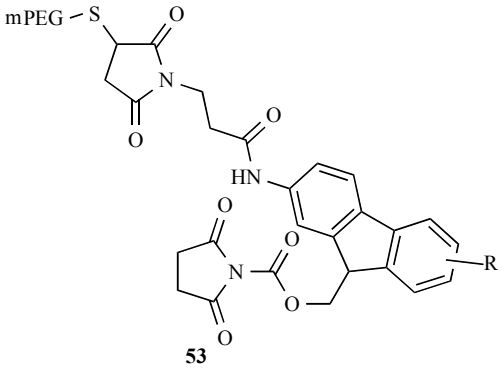


chains are linked by groups of different stability (trigger groups), and derivatives having different substitution patterns at the *ortho* position, have all been prepared and evaluated in reversible PEGylation. These trigger groups may influence the reactivity of the parent reactive mPEG with ε amino groups of the specific protein. The derivative bearing two *ortho* methyl groups and the mPEG chain linked by an ester moiety (**47**) has proven to be particularly attractive for releasable PEGylation, as the hydrolysis rate is slower than with unsubstituted compounds.

Releasable PEGylation with branched (**48**) and linear (**49**) bicine linkers has emerged in the last few years as powerful method for the controlled delivery of small drugs and proteins [198,199]. The chemistry of the delivery with these linkers, using a linear derivative **49**, is depicted in equations 45-46. The fast hydrolysis of the ester group affords the corresponding carboxylate and the hydroxyethyl derivative **50**, which further reacts with water at a controlled rate to yield *bis*-hydroxyehtyl intermediate **51**. Intermediate **51** undergoes cyclization affording bicine **52** and delivering the native protein (equation 46).

It has been shown that the rate of release can be modulated upon modification of the heteroatom of the trigger (X in compounds **48** and **49**), by introduction of additional side chains or by increasing the steric hindrance. Branched bicine linkers **48** have proven to be interesting structures due to the advantages that branched structures have for protein conjugation.

The bifunctional linker **53** has been used for the attachment of mPEG of up to 40 kDa to proteins by means of urethane bond formation. The fluorenyl moiety releases the native unmodified protein at a slow controlled rate via hydrolysis (equation 47) [200]. This approach has been used, for example, for the reversible PEGylation of exendine-4 [200], human growth hormone, interferon α2 [201], enkephalin [202], atrial natriuretic peptide [203] and insulin [204].



53

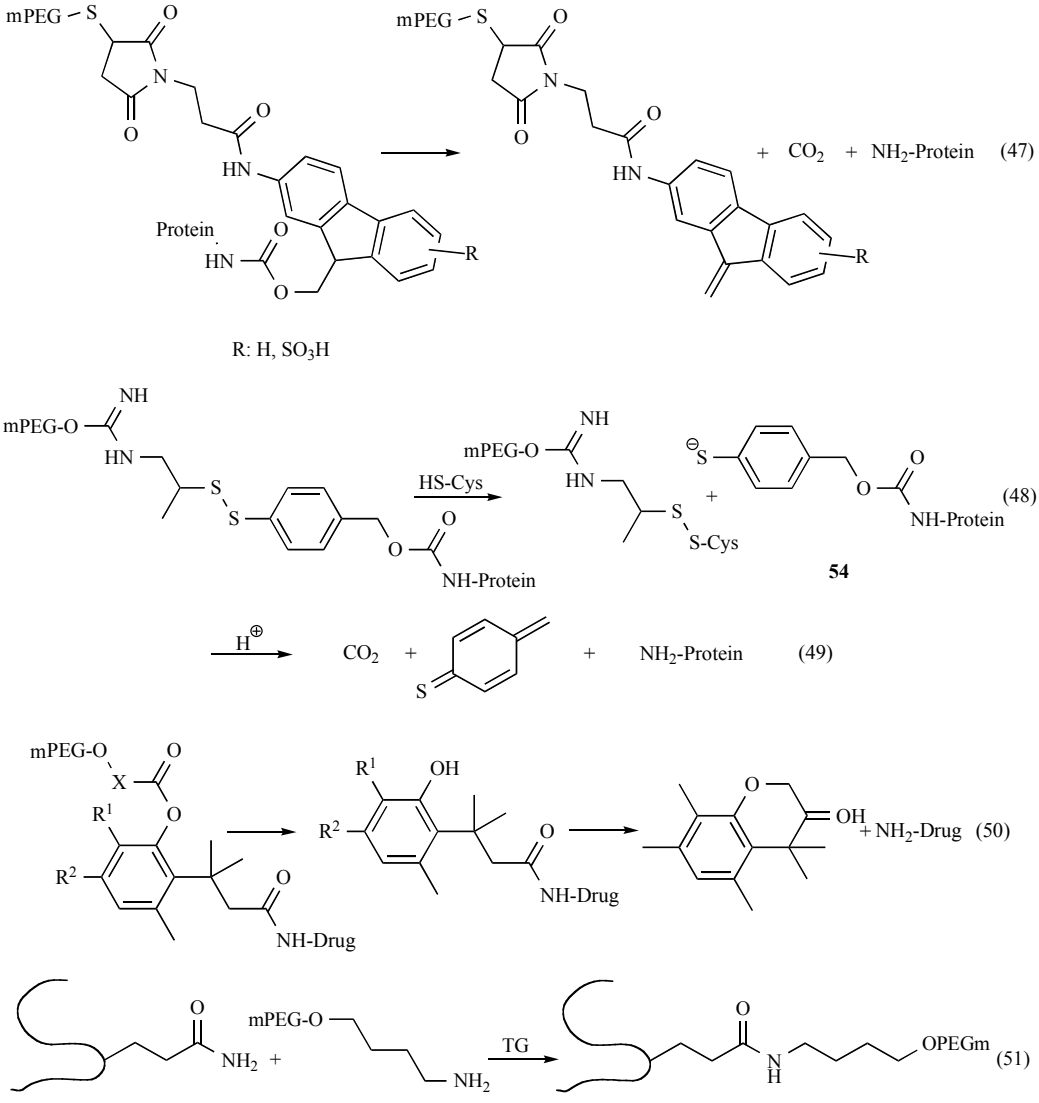
R: H, SO_3H

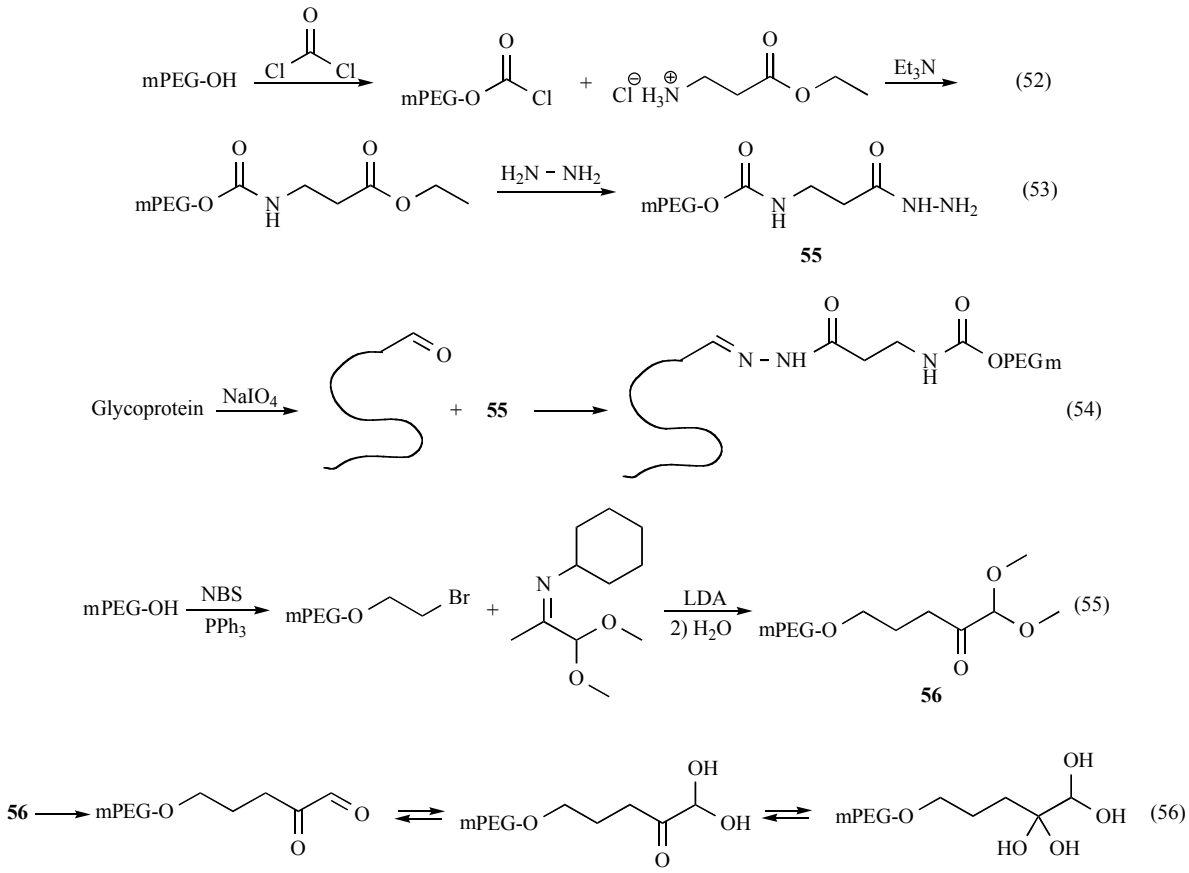
Cleavable disulfide bridges have also been exploited for the reversible PEGylation of cysteine residues of proteins. The underlying chemistry of the process using a crowded carbamate is presented in equations 48 and 49. Reduction of the disulfide bridge by a cysteine residue of the protein, or any other entity containing a thiol group, such as glutatione, releases the polymer and delivers sulfide **54.** Finally, 1,6-elimination delivers the unmodified native protein (equation 49) [205,206].

Reversible PEGylation based on trimethyl lock lactonization is well established strategy that has mainly found applications for the preparation of small compound prodrugs [207]. The process begins by the controlled hydrolysis of the ester linkage by endogenous enzymes (equation 50, where X= spacer), followed by nucleophilic attack of a free hydroxyl group to form the lactone and delivering the drug.

7. ENZYMATIC PEGYLATION

Transglutaminase has been used for site-selective PEGylation. The concept of these conjugations is based in the enzymatic catalyzed acyl transfer reaction between the carboxamide group of glutamine and the amino group of PEG-amine to afford a new and stable amide linkage (equation 51). The approach can be used for the PEGylation of native or chimeric proteins, yielding homogeneous conjugates.





Human granulocyte colony-stimulating factor [208], α-lactalbumin, interleukin-2 [209], salmon calcitonin and IgGH [210], among other proteins, have all been PEGylated using transglutaminase.

8. PEGYLATION AT CARBOHYDRATES RESIDUES

Chemical or enzymatic oxidation of carbohydrate residues of glycoproteins generates aldehyde groups that can react with hydrazides to yield hydrazones, which may be reduced to more stable alkyl hydrazides [211]. For example, hydrazide **55**, which can be prepared from mPEG and the ethyl ester of β-alanine (equations 52 and 53), was reacted with oxidized ovoalbumin and immunoglobulin G to yield diacyl-hydrazone linkages (equation 54) [212].

Although PEGylation of the reactive aldehyde groups with PEG-amines has been proposed, it has been suggested that this process is difficult, given that the amino groups of proteins show a similar reactivity to those of PEG-amine, leading to cross-linking of the protein. In this regard, the *p*Ka of hydrazides is around 3, allowing for selective conjugation at acidic *p*Hs, at which most of protein amino acids are protonated (*p*Ka around 10).

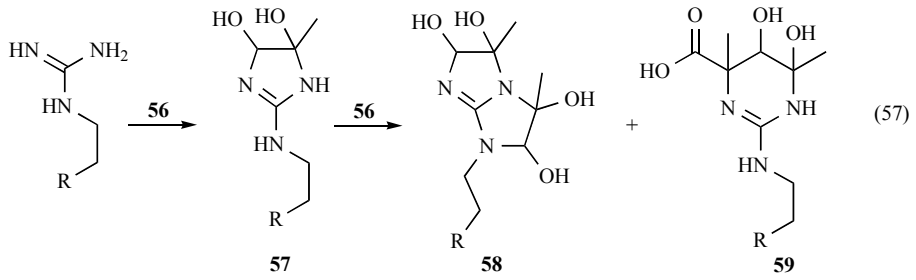
In a related method, serine or threonine terminated proteins were oxidized to the corresponding glyoxylyl groups, which were then reacted with aminooxy mPEG derivatives to achieve site-selective PEGylation. Furthermore, the reactive terminal carbonyl groups can be introduced by metal catalyzed transamination [213].

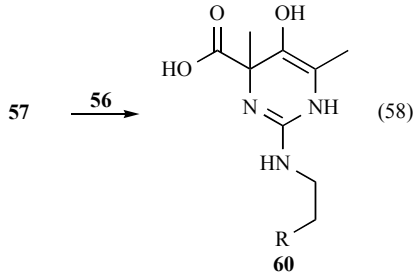
9. MISCELANEOUS MPEG REAGENTS AND REACTIONS

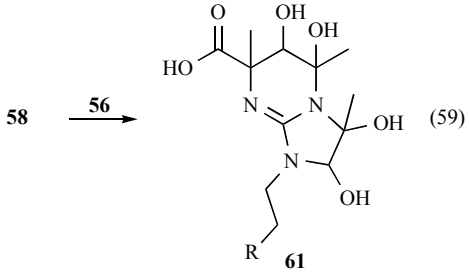
The PEGylation of arginine residues with mPEG phenylglyoxal has been reported. The usefulness of this methodology is limited due to the long reaction times that are required to achieve good yields of conjugation. In addition, the PEGylation with mPEG phenylglyoxal is not selective and conjugation at histidine and lysine residues is also usually observed [214,215].

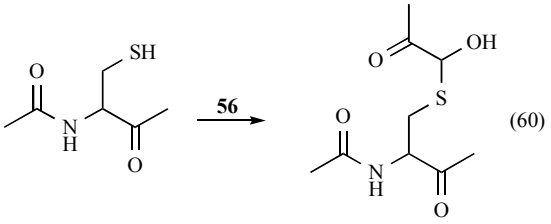
The site-selective and permanent arginine PEGylation has recently been disclosed [216,217]. The process relies on the thermodynamic selectivity obtained upon reaction of the protein with methyl glyoxal analog **56**. The dimethyl acetal derivative **56** which can easily be obtained as shown in equation 55, is in equilibrium with unprotected forms in aqueous media, and this mixture was used as the actual PEGylating reagent (equation 56).

A detailed study of the reaction of methyl glyoxal with *N*-acetyl arginine and *N*-acetyl cysteine at *p*H 9.0 (borate buffer) and 7.4 (phosphate buffer) (equations 57-60) suggest that although cysteine reacts quickly with the dicarbonyl compound (equation 60), the formed adduct can be hydrolyzed at *p*H 3. Moreover, this adduct is unstable under dilution or by re-suspension of the purified product at *p*H 7.0. On the other hand, at *p*H 9.0 adducts **57** and **58**, which arise from arginine modification with one and two molecules of **56**, were the only observed products. At *p*H 7.4 compounds **60** and **61** (equations 58 and 59) were also formed, indicating that complexation of diols **57** and **58** prevented the reaction with another molecule of **56**. In addition, the formation **61** indicates that, at some degree, **59** was also obtained. Hydrolysis studies suggested that the adducts obtained upon reaction with **56** are more stable than those obtained with *N*-acetyl cysteine, and also that the adducts obtained in the reaction with *N*-acetyl arginine are more stable than the adducts obtained with the same compound and phenyl glyoxal. Importantly, it has been demonstrated that with reagent **56,** PEGylation at lysine residues does not occur.





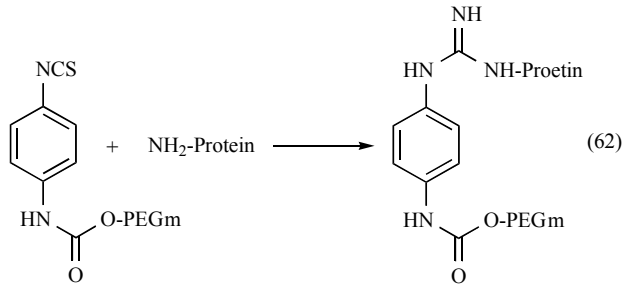




The site-selective conjugation at tyrosine residues of salmon calcitonin using diazonium salt **62** has recently been accomplished (equation 61) [218]. This PEGylation approach is reversible, and the starting materials can be obtained by reaction with suitable reducing reagents.

PEGylation of amino groups of human serum albumin and human keratinocyte growth factor 2 using mPEG isothiocyanate has been reported [219,220]. The conjugation with this reagent affords hydrolytically stable thiourea linkages by mean of a simple and easily obtained PEG derivative (equation 62) [221].

Finally, the selective PEGylation of terminal carboxyl groups has been reported by Zalipsky. In this method mPEG-hydrazide reacts selectively with the carboxyl group by means of carbodiimide activation at low *p*H. At the *p*H values at which the conjugations are performed (4.5-5.0) all amino groups of the protein are protonated and cross-linking of the biomolecule does not occurs [222].

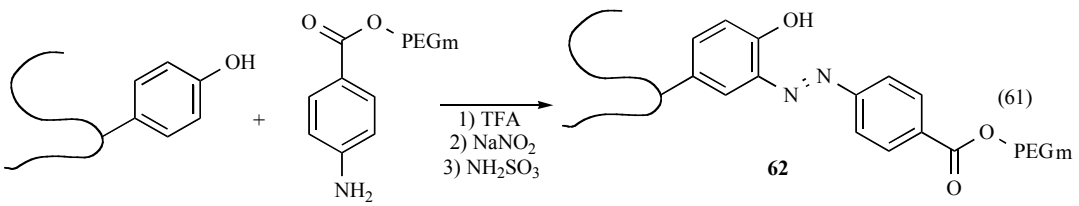


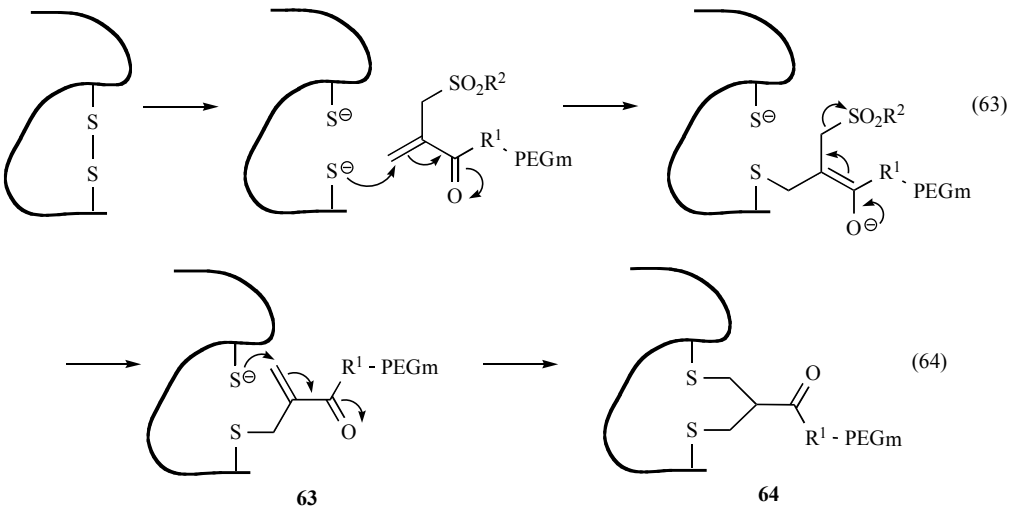
10. ACTUAL TRENDS

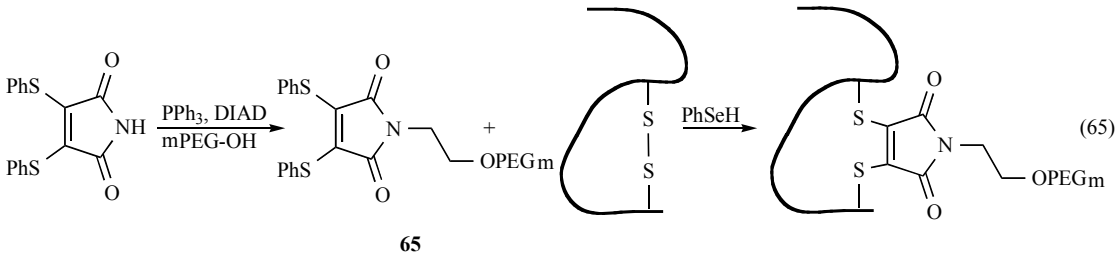
The number of peptide and protein drugs amenable for bioconjugation, as well as of new PEG tailor-made reagents and PEGylation strategies, are still nowadays increasing. Actual developments in PEGylation technology tend to tackle the two major difficulties found in bioconjugation, namely: i) to guarantee product homogeneity by improving site-specific approaches, and ii) to diminish the cost of production processes by improving the efficiency of the conjugation reaction, while maintaining batch-to-batch reproducibility.

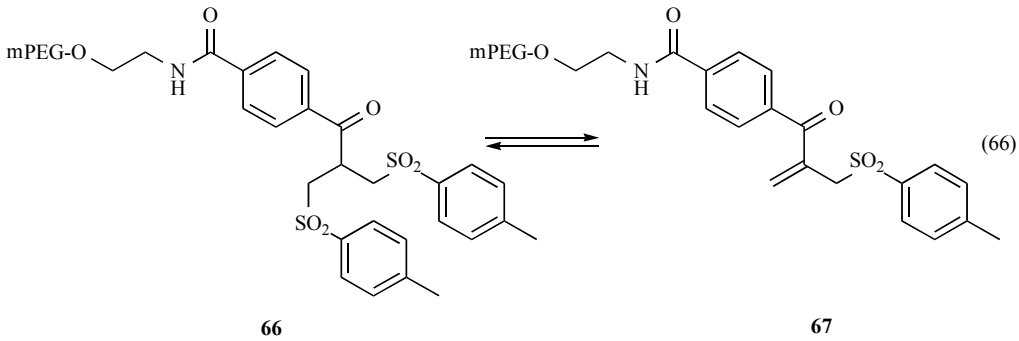
As was shown earlier, one of the most effective strategies to achieve site-selective conjugation involves PEGylation at scarce cysteine residues located at the surface of the protein. If these residues are not available at suitable location they may be introduced by genetic engineering methods. In connection, PEGylation of cysteine involved in accessible disulfide bridges is nowadays well established for site-specific conjugation. Based on these concepts, Brocchini has developed the TheraPEG^{TM} technology for site-selective PEGylation (equations 63-64) [149]. The disulfide bridge of the protein is reduced under mild reaction conditions to afford nucleophilic thiol group. The sulfide group reacts with the double bond of the acceptor in a Michael-type 1,4-addition reaction (equation 63), which releases a sulfonyl anion to give intermediate **63**. A second Michael 1,4-addition reaction with the sulfide anion finally yields hydrolytically stable cyclic sulfide **64** (equation 64).

It has been demonstrated that the conjugation reaction does not affect the tertiary structure of the protein and, importantly, that, due









to steric hindrance at the conjugation site, only one polymer chain is attached at each disulfide bridge.

A related approach using dithiophenolmaleimides **65** has been published. The conjugation reaction involves the generation of the free thiol group, which is quickly trapped *in situ* by added **65** (equation 65). It has been shown that this simple and fast method restrict the unfolding, aggregation and disulfide scrambling of the protein [223]. A related method for disulfide site-specific PEGylation that employs dibromomaleimide derivatives has recently been devised [224].

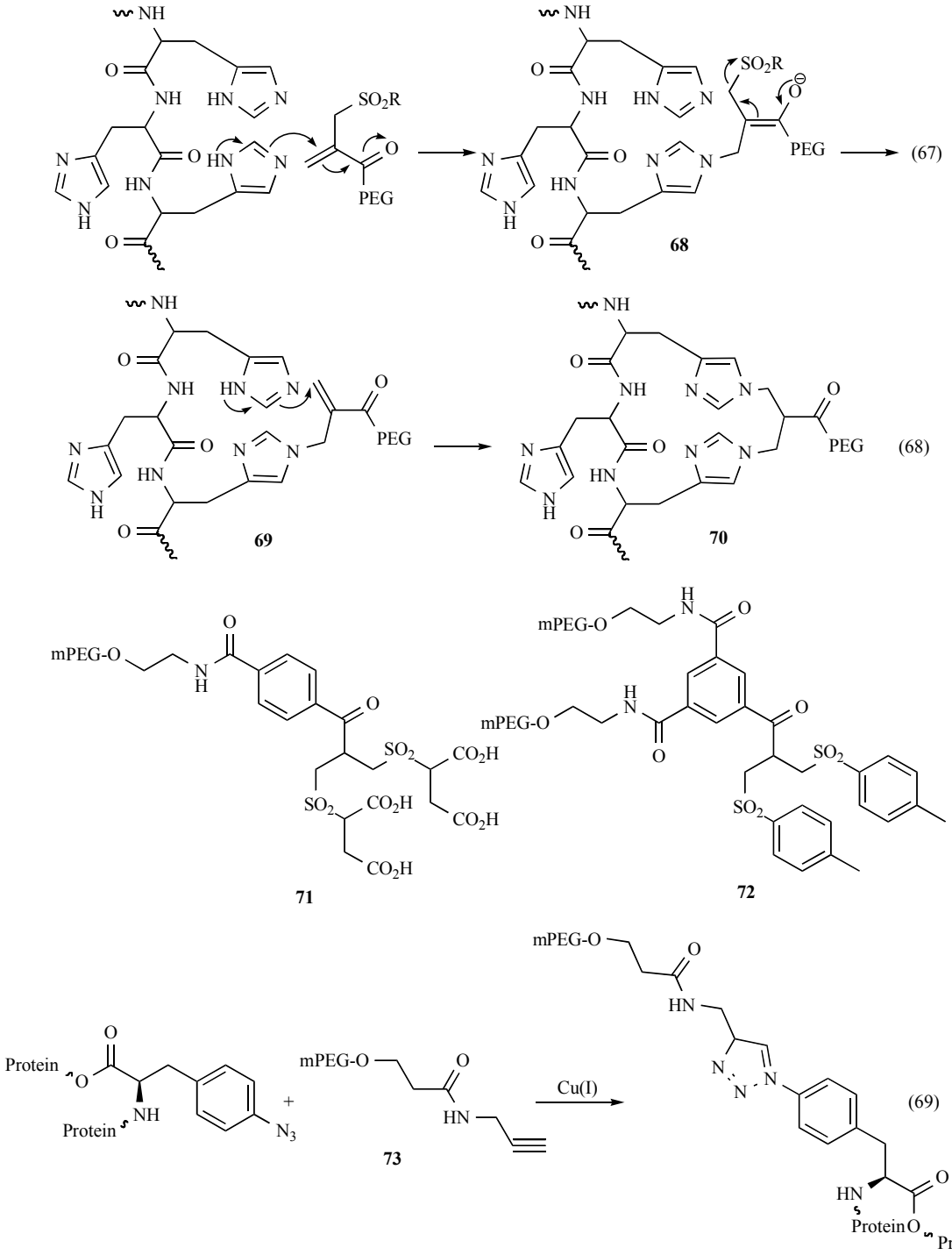
Recombinant proteins expressed in several prokaryotic expression systems are often produced with histidine-tags (HisTag), which allows the purification of proteins using affinity chromatography with matrices that contains nickel or other metal ions. Tailor made mPEG reagents have been created to selectively react with these tags and, importantly, as the tags are expressed at the N or C terminal of proteins, the biological activity is not compromised [225]. The concept of the HisTag PEGylation is closely related to that of the TheraPeg® technology, namely, a *bis*-alkylation through a double 1,4-Michael-type addition. Monosulfone **67** is formed by elimination of sulfinic acid from mPEG bis-sulfone **66**. Intermediate **67** is the actual PEGylating reagent (equation 66).

The proposed mechanism of conjugation reaction involves the nucleophilic addition of the heterocyclic nitrogen of the aromatic ring to the double bond to deliver intermediate **68** (equation 67), that upon elimination of sulfinic acid forms an α,β-unsaturated ester **69** (equation 68). A second Michael addition finally gives cyclic *bis*-alkylated product **70** (equation 68).

Bis-sulfone **71** and branched *bis*-sulfone **72** have also been prepared and carefully evaluated in HisTag PEGylation [225].

Proteins that have been PEGylated by HisTag technology include cytokines (IFN α-2a), antibody fragments (anti-TNF, scFV), endostatin and pro-brain natriuretic peptide.

The PEGylation of non-natural amino acids incorporated to the protein by genetic engineering methods is an increasingly important approach for site-specific conjugation. For example, the genetically introduced *para*-azidophenylalanine residue was used to achieve the site-specific conjugation of SOD by mean of a [3+2] cycloaddition reaction with mPEG-alkyne **73** (equation 69) [226].



Other related methods for the conjugation of non-natural amino acids includes the PEGylation of non-natural *p*-acetylphenylalanine with mPEG-oxyamine [227,228], pyrrolysinine and pyrroline-carboxy-lysine with mPEG carbonyl compounds [229], among several other methods [230-232].

A new two-step process, known as glycoPEGylation, has become increasingly important in the last years for the site-specific bioconjugation of proteins [233]. The sequence consists of a first step of enzymatic glycosylation with *N*-acetylgalatosamine and *N*-acetylgalatosamine transferase at specific threonine and serine residues. In the second step, the protein is PEGylated at the *N*-acetylgalatosamine residue with a sialic acid mPEG derivative using sialyltransferase. Examples of proteins that has been conjugated by this strategy includes factor VIIa [234,235], G-CSF, IFN α-2b and GM-CSF [233]. In a related approach, a transpeptidase (sortase A), has recently been employed for the site-selective attachment of mPEG to citokines [236].

The solid-phase PEGylation methodology involves the conjugation if protein to a tethered PEG derivative that is attached to a solid matrix. The process has been aimed at overcoming some of the drawbacks of the commonly used solution-phase methods. Several approaches have been developed, including site-specific PEGylation of keratinocyte growth factor 1 [237] and the use of heparin-sepharose columns to conjugate recombinant human fibroblast

growth factor 2 to PEG-butyraldehyde through reductive alkylation [238]. Other biomolecules that have been PEGylated using solid-phase procedures include albumin and staphylokinase [239], lipopetides [240], interferon α-2a [241] and hemoglobin among other [242].

Further innovative PEGylation strategies include: i) C-terminal PEGylation of recombinant proteins expressed as intein-fusion proteins, using hydrazone-forming ligation reactions [243]; ii) PEGylation of latex surfaces beads for immunocamouflage [244], iii) PEGylation of immunonanoparticles [245], and iv) monoPEGylation using an adapted Dock and Lock (DNL) method [246].

11. CONCLUSION

PEGylation is undoubtedly a diverse and hot field, which is embedded within emerging technologies for the development of improved and new protein therapeutics among other applications. PEGylation takes place at the interface of chemistry, biotechnology, polymer, pharmaceutical and biomedical sciences. Thus, it is synonym of inter- and multi-disciplinary research, a key factor for success that is not new but scarce in a situation where finding a common language is sometimes difficult. The combination of these and other factors, such as the ever growing demand and increasing complexity emerging from the need for more effective therapeutic targets, still today stimulates the development of research projects aimed towards the design, synthesis and evaluation of tailor-made mPEG reagents and technologies. We believe that these efforts will provide, in the near future, further interesting and technologically relevant results.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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REFERENCES

[1] González, M.G.; Vaillard, S.E.; Grau, R.J.A. Pegylation: An overview and recent advances reported in the patent literature. *Recent Pat. Chem. Eng.*, **2011**, *4(3)*, 241-264.

[2] Working, P.K.; Newman, M.S.; Johnson J.B. Cornacoff, C.J. Safety of Poly(ethylene glycol) and Poly(ethylene glycol) Derivatives. *ACS Symp. Ser.*, **1997**, *680*, 45-47.

[3] Dreborg, S.; Akerblom, E.B. Immunotherapy with monomethoxypolyethylene glycol modified allergens. *Crit. Rev. Ther. Drug Carrier Syst.*, **1990**, *6(4)*, 315-365.

[4] Leger, R.M.; Arndt, P.; Garratty, G.; Armstrong, J.K.; Meiselman, H.J.; Fisher, T.C. Normal donor sera can contain antibodies to polyethylene glycol (PEG). *Transfusion*, **2001**, *41*, 29S.

[5] Armstrong, J.K.; Leger, R.; Wenby, R.B.; Meiselman, H.J.; Garratty, G.; Fisher, T.C. Occurrence of an antibody to poly(ethylene glycol) in normal donors. *Blood*, **2003**, *102*, 556A.

[6] Caliceti, P.; Schiavon, O.; Veronese, F.M.; Chaiken, I.M. Effects of monomethoxypoly(ethylene glycol) modification of ribonuclease on antibody recognition, substrate accessibility and conformational stability. *J. Mol. Recognit.*, **1990**, *3(2)*, 89-93.

[7] Pomroy, N.C.; Deber, C.M. Solubilization of hydrophobic peptides by reversible cysteine PEGylation. *Biochem. Biophys. Res. Commun.*, **1998**, *245(2)*, 618-621.

[8] Kwon, G.S. Polymeric micelles for delivery of poorly water-soluble compounds. *Crit. Rev. Ther. Drug*, **2003**, *20(5)*, 357-403.

[9] Greish, K.; Fang, J.; Inutsuka, T.; Nagamitsu, A.; Maeda, H. Macromolecular Therapeutics: Advantages and prospects with special emphasis on solid tumour targeting. *Clin. Pharmacokinet.*, **2003**, *42(13)*, 1089-1105.

[10] Iyer, A.K.; Khaled, G.; Fang, J.; Maeda, H. Exploiting the enhanced permeability and retention effect for tumor targeting. *Drug Discov. Today*, **2006**, *11(17-18)*, 812-818.

[11] Maeda, H.; Wu, J.; Sawa, T.; Matsumura, Y.; Hori, K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Control. Release*, **2000**, *65(1-2)*, 271-284.

[12] Caliceti, P.; Veronese, F.M. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. *Adv. Drug Deliver. Rev.*, **2003**, *55(10)*, 1261-1277.

[13] Itoh, A.; Honda, S.; Matsui, T.; Hayashi, Y. Polyoxyalkylene monoalkyl ether, process for producing the same, polymerizable polyoxyalkylene monoalkyl ether derivative, polymer of said derivative and dispersant comprising said polymer. U. S. Patent 6,780,928, August 24, **2004**.

[14] Zhang, J.; Zhao, Y.-J.; Su, Z.-G.; Ma, G.-H. Synthesis of monomethoxy poly(ethylene glycol) without diol poly(eyhylene glycol). *J. Appl. Pol. Sci.*, **2007**, *105(6)*, 3780-3786.

[15] Jadur-Grodzinski, J. Preparation of polymers using living and controlled polymerizations. *J. React. Funct. Polym.*, **2001**, *49(1)*, 1-54.

[16] Veronese, F. Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials*, **2001**, *22(5)*, 405-417.

[17] Roberts, M.J.; Bentley, M.D.; Harris, J.M. Chemistry for peptide and protein PEGylation. *Adv. Drug Deliver. Rev.*, **2002**, *54(4)*, 459-476.

[18] Bentley, M.D.; Harris, M.J.; Kozlowski, A. Heterobifunctional poly(ethylene glycol) derivatives and methods for their preparation. U. S. Patent 7,642,323, January 5, **2010**.

[19] Harris, J.M. Laboratory Synthesis of polyethylene glycol derivatives. *Rev. Macromol. Chem. Phys.*, **1985**, *C25(3)*, 325-373.

[20] Herman, S.; Hooftman, G.; Schacht, E. Poly(ethylene glycol) with reactive endgroups: I. Modification of proteins. *J. Bioact. Compat. Pol.*, **1995**, *10(2)*, 145-187.

[21] Hooftman, G.; Herman, S.; Schacht, E. Review: poly(ethylene glycol) with reactive endgroups: II. Practical consideration for the preparation of protein-PEG conjugates. *J. Bioact. Compat. Pol.*, **1996**, *11(2)*, 135-159.

[22] Pesek, J.J.; Matyska, M.T.; Krishnamoorthi, V. Separation of polyethylene glycol-modified proteins by open tubular capillary electrochromatography. *J. Chromatogr. A*, **2004**, *1044(1-2)*, 317-322.

[23] Abuchowski, A.; Van Es, T.; Palczuk, N.C.; Davis, F.F. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem.*, **1977**, *252(11)*, 3578-3581.

[24] Abuchowski, A.; McCoy, J.R.; Palczuk, N.C. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.*, **1977**, *252(11)*, 3582-3586.

[25] Pasut, G.; Veronese, F.M. PEGylation of proteins as tailored chemistry for optimized bioconjugates. *Adv. Polym. Sci.*, **2006**, *192(1)*, 95-134.

[26] Alconcel, S.N.S.; Baas, A.S.; Maynard, H.D. FDA-approved poly(ethylene glycol)-protein conjugate drugs. *Polym. Chem.*, **2011**, *2(7)*, 1442-1448.

[27] Lee, W.Y.; Sehon, A.H. Abrogation of reaginic antibodies with modified allergens. *Nature*, **1977**, *267(5612)*, 618-619.

[28] Jackson, C.-J.C.; Charlton, J.L.; Kuzminski, K.; Lang, G.M.; Sehon, A.H. Synthesis, isolation, and characterization of conjugates of ovalbumin with monomethoxypolyethylene glycol using cyanuric chloride as the coupling agent. *Anal. Biochem.*, **1987**, *165(1)*, 114-127.

[29] Pyatak, P.S.; Abuchowski, A.; Davis, F.F. Preparation of a polyethylene glycol: superoxide dismutase adduct, and an examination of its blood circulating life and anti-inflammatory activity. *Res. Commun. Chem. Path.*, **1980**, *29(1)*, 113-127.

[30] Koide, A.; Kobayashi, S. Modification of amino groups in porcine pancreatic elastase with polyethylene glycol in relation to binding ability towards anti-serum and to enzymic activity. *Biochem. Bioph. Res. Co.*, **1983**, *111(2)*, 659-667.

[31] Tomiya, N.; Watanabe, K.; Awaya, J.; Kurono, M.; Fujii, S. Modification of acyl-plasmin-streptokinase complex with polyethylene glycol: reduction of sensitivity to neutralizing antibody. *FEBS Lett.*, **1985**, *193(1)*, 44-48.

[32] Gaertner, H.F.; Puigserver, A.J. Increased activity and stability of poly(ethylene glycol)-modified trypsin. *Enzyme Microb. Tech.*, **1992**, *14(2)*, 150-155.

[33] Gotoh, Y.; Tsukada, M.; Minoura, N. Chemical modification of silk fibroin with cyanuric chloride-activated poly(ethylene glycol): analyses of reaction site by proton NMR spectroscopy and conformation of the conjugates. *Bioconjugate Chem.*, **1993**, *4(6)*, 554-559.

[34] Brumeanu, T.D.; Zaghouani, H.; Bona, C. Purification of antigenized immunoglobulins derivatized with monomethoxypolyethylene glycol. *J. Chromatogr. A*, **1995**, *696(2)*, 219-225.

[35] Matsushima, A.; Sasaki, H.; Kodera, Y.; Inada, Y. Reduction of immunoreactivity of bovine serum albumin conjugated with polyethylene glycol (PEG) in relation to its esterase activity. *Biochem. Int.*, **1992**, *26(3)*, 485-490.

[36] Matsushima, A.; Nishimura, H.; Ashihara, Y. 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**, *9(7)*, 773-776.

[37] Ono, K.; Kai, Y.; Maeda, H.; Samizo, F.; Sakurai, K.; Nishimura, H.; Inada, Y. Selective synthesis of 2,4-bis(O-methoxypolyethylene glycol)-6-chloro-s-triazine as protein modifier. *J. Biomater. Sci. Polym. Ed.*, **1991**, *2(1)*, 61-65.

[38] Saito, T.; Nishimura, H.; Sekine, T.; Urushibara, T.; Kodera, Y.; Hiroto, M.; Matsushima, A.; Inada, Y. Tolerogenic capacity of poly(ethylene glycol) (PEG)-modified ovalbumins in relation to their immunoreactivity towards anti-ovalbumin antibody. *J. Biomat. Sci. Polym. Ed.*, **1996**, *8 (4)*, 311-332.

[39] Ikeda, K.; Schiltz, E.; Fujii, T.; Takahashi, M.; Mitsui, K.; Kodera, Y.; Matsushima, A.; Inada, Y.; Schulz, G.E.; Nishimura, H. Phenylalanine ammonia-lyase modified with polyethylene glycol: potential therapeutic agent for phenylketonuria. *Amino Acids*, **2005**, *29(3)*, 283-287.

[40] Delgado, C.; Patel, J.N.; Francis, G.E.; Fisher, D. Coupling of poly(ethylene glycol) to albumin under very mild conditions by activation with tresyl chloride: characterization of the conjugate by partitioning in aqueous two-phase systems. *Biotech. Appl. Bioc.*, **1990**, *12(2)*, 119-128.

[41] Nilsson, K.; Mosbach, K. Immobilization of ligands with organic sulfonyl chlorides. *Method. Enzymol.*, **1983**, *104*, 56-69.

[42] Sperinde J.J.; Martens, B.D.; Griffith, L.G. Tresyl-mediated synthesis: kinetics of competing coupling and hydrolysis reactions as a function of pH, temperature, and steric factors. *Bioconjugate Chem.*, **1999**, *10(2)*, 213-220.

[43] Gais, H.-J.; Ruppert, S. 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.*, **1995**, *36(22)*, 3837-3838.

[44] Koops, B.C.; Verheij, H.M.; Slotboom, A.J.; Egmond, M.R. Effect of chemical modification on the activity of lipases in organic solvents. *Enzyme Microb. Tech.*, **1999**, *25(7)*, 622-631.

[45] Yoshinga, K.; Harris, J.M. Effects of Coupling Chemistry on the Activity of poly(ethylene glycol)-modified alkaline phosphatase. *J. Bioact. Compat. Polym.*, **1989**, *4(1)*, 17-24.

[46] Knusli, C.; Delgado, C.; Malik, F.; Domine, M.; Tejedor, M.C.; Irvine, A.E.; Fisher, D.; Francis, G.E. Polyethylene glycol (PEG) modification of granulocyte-macrophage colony stimulating factor (GM-CSF) enhances neutrophil priming activity but not colony stimulating activity. *Brit. J. Haematol.*, **1992**, *82(4)*, 654-663.

[47] Chen, G.; Ito, I.; Imanishi Y. Mitogenic activities of water-soluble and -insoluble insulin conjugates. *Bioconjugate Chem.*, **1997**, *8(2)*, 106-110.

[48] Röstin, J.; Smeds, A.-L.; Åkerblom, E. B. Domain deleted recombinant coagulation factor VIII modified with monomethoxy polyethylene glycol. *Bioconjugate Chem.*, **2000**, *11(3)*, 387-396.

[49] Treetharnmathurot, B.; Ovartlarnporn, C.; Wungsintaweekul, J.; Duncand, R.; Wiwattanapatapee, R. Effect of PEG molecular weight and linking chemistry on the biological activity and thermal stability of PEGylated trypsin. *Int. J. Pharm.*, **2008**, *357(1-2)*, 252-259.

[50] Magnusson, J.P.; Saeed, A.O; Fernández-Trillo, F.; Alexander, C. Synthetic polymers for biopharmaceutical delivery. *Polym. Chem.*, **2011**, *2(1)*, 48-59.

[51] Zalipsky, S.; Gilon, C.; Zilkha, A. Attachment of drugs to polyethylene glycols. *Eur. Polym. J.*, **1983**, *19(12)*, 1177-1783.

[52] Philippova, O.E.; Topchieva I.N.; Kuchanov, S.I. On the possibility of chemical modification of polyethylene glycol in melt. *Pol. Bull.*, **1986**, *15(4)*, 297-302.

[53] M.C. Carter, M.C; Meyerhoff, M.E. Inestability of succinyl ester linkages in O2'-monosuccinyl cyclic AMP-protein conjugates at neutral pH. *J. Immunol. Methods*, **1985**, *81(2)*, 245-257.

[54] Sartore, L.; Caliceti, P.; Schiavon, O.; Monfardini, C.; Veronese, F.M. Accurate evaluation method of the polymer content in monomethoxy(polyethylene glycol) modified proteins based on amino acid analysis. *Appl. Biochem. Biotechnol.*, **1991**, *31(3)*, 213-222.

[55] Veronese, F.M.; Saccà, B.; Polverino de Laureto, P.; Sergi, M.; Caliceti, P.; Schiavon, O.; Orsolini, P. New PEGs for peptide and protein modification, suitable for identification of the PEGylation site. *Bioconjugate Chem.*, **2001**, *12(1)*, 62-70.

[56] Zalipsky, S.; Active carbonates of polyalkylene oxides for modification of polypeptides. U. S. Patent 5,324,844, June 28, **1994**.

[57] Abuchowski, A.; Kazo, G.M.; Verhoest, Jr. C.R. Cancer therapy with chemically modified enzymes. I. Antitumor properties of polyethylene glycol-asparaginase conjugates. *Cancer Biochem. Bioph.*, **1984**, *7(2)*, 175-186.

[58] Joppich, M.; Luisi, P.L. Peptides flanked by two polymer chains, 1. Synthesis of glycyl-L-tryptophylglycine substituted by poly(ethylene oxide) at both the carboxy and the amino end groups. *Makromolekul. Chem.*, **1979**, *180(5)*, 1381-1384.

[59] Satake-Ishikawa, R.; Ishikawa, M.; Okada, Y.; Kakitani, M.; Kawagishi, M.; Matsuki, S.; Asano, K. Chemical modification of recombinant human granulocyte colony-stimulating factor by polyethylene glycol increases its biological activity in vivo. *Cell Struct. Funct.*, **1992**, *17(3)*, 157-160.

[60] Holtsberg, F.W.; Ensor, C.M.; Steiner, M.R.; Bomalaski, J.S.; Clark, M.A. Poly(ethylene glycol) (PEG) conjugated arginine deiminase: effects of PEG formulations on its pharmacological properties. *J. Control. Release*, **2002**, *80(1-3)*, 259-271.

[61] Rubinstein, M.; Simon, S.; Bloch, R. Process for the cross-linking of proteins. U. S. Patent 4,101,380, July 18, **1978**.

[62] Harris, J.M.; Kozlowski, A.; Guo, L. Method of preparing carboxylic acid functionalized polymers. U. S. Patent 8,067,505, November 29, **2011**.

[63] Harris, J.M.; Kozlowski, A. Poly(ethylene glycol) and related polymers monosubstituted with propionic or butanoic acids and functional derivatives thereof for biotechnical applications. U. S. Patent 5,672,662, September 30, **1997**.

[64] Sedaghat-Herati, M.R.; Miller, P.; Kozlowski, A.; Harris, J.M. Synthesis of methoxypoly(oxyethylene)propionic acid. *Polym. Bull.*, **1999**, *43(1)*, 35-41.

[65] Koslowski, A.; McManus, S.P. Method of preparing propionic acid-terminated polymers. U. S. Patent 6,608,663, October 27, **2009**.

[66] Bentley, M.D.; Zhao, X.; Shen, X.; Guo, L. Sterically hindered poly(ethylene glycol) alkanoic acids and derivatives thereof. U. S. Patent 7,674,879, March 9, **2010**.

[67] Bentley, M.D.; Zhao, X.; Shen, X.; Guo, L. Sterically hindered poly(ethylene glycol) alkanoic acids and derivatives thereof. U. S. Patent 7,405,266, July 29, **2008**.

[68] Kozlowski, A. Method for preparing water-soluble polymer derivatives bearing a terminal carboxylic acid. U. S. Patent 5,569,214, August 4, **2009**.

[69] Bailon, P.S.; Won, C.-Y. Polyalkylene glycol acid additives. U. S. Patent 7,193,031, March 20, **2007**.

[70] Tan, Y.; Sun, X.; Xu, M.; An, Z.; Tan, X.; Tan, X.; Han, Q.; Milijkovic, D. A.; Yang, M.; Hoffman, R.M. Polyethylene glycol conjugation of recombinant methioninase for cancer therapy. *Protein Expres. Purif.*, **1998**, *12(1)*, 45-52.

[71] Hinds, K.D.; Kim, S.W. Effects of PEG conjugation on insulin properties. *Adv. Drug Deliver. Rev.*, **2002**, *54(4)*, 505-530.

[72] Sainathan, S.K.; Tu, L.; Bishnupuri, K.S.; Han, M.; Li, A.; Newberry, R.D.; McDonald, K.G.; Crimmins, D.L.; Houchen, C.; Anant, S.; Dieckgraefe, B.K.; PEGylated murine granulocyte-macrophage colony-stimulating factor: production, purification, and characterization. *Protein Expres. Purif.*, **2005**, *44(2)*, 94-103.

[73] Lee, S.-H.; Lee, S.; Youn, Y.S.; Na, D.H.; Chae, S.Y.; Byun, Y.; Lee, K.C. Synthesis, characterization, and pharmacokinetic studies of PEGylated glucagon-like peptide-1. *Bioconjugate Chem.*, **2005**, *16(2)*, 377-382.

[74] Chang L.-C.; Lee H.-F.; Chung M.-J.; Yang, V.C. PEG-modified protamine with improved pharmacological/pharmaceutical properties as a potential protamine substitute: synthesis and in vitro Evaluation. *Bioconjugate Chem.*, **2005**, *16(1)*, 147-155.

[75] Youn, Y.S.; Jung, J.Y.; Oh, S.H.; Yoo, S.D.; Lee, K.C. Improved intestinal delivery of salmon calcitonin by Lys18-amine specific PEGylation: stability, permeability, pharmacokinetic behavior and in vivo hypocalcemic efficacy. *J. Control. Release*, **2006**, *114(3)*, 334-342.

[76] Molek, J.R.; Zydney, A.L. Separation of PEGylated α-lactalbumin from unreacted precursors and byproducts using ultrafiltration. *Biotechnol. Prog.*, **2007**, *23(6)*, 1417-1424.

[77] Tsui, S.-M.; Lam, W.-M.; Lam, T.-L.; Chong, H.-C.; So, P.-K.; Kwok, S.-Y.; Arnold, S.; Cheng, P.; Wheatly, D.N.; Lo, W.-H.; Leung, Y.-C. Pegylated derivatives of recombinant human arginase (rhArg1) for sustained in vivo activity in cancer therapy: preparation, characterization and analysis of their pharmacodynamics in vivo and in vitro and action upon hepatocellular carcinoma cell (HCC). *Cancer Cell Int.*, **2009**, *9(1)*, 9.

[78] Wen, Z.; Niemeyer, B. Preparation and characterization of PEGyated concanavalin A for affinity chromatography with improved stability. *J. Chromatogr. B*, **2011**, *879(20)*, 1732-1740.

[79] Huang, Y.; Feng, S.; Tang, R.; Du, B.; Xu, K.; Pan, X. Efficacy of pretreatment of allografts with methoxypolyethylene glycol-succinimidyl-propionic acid ester in combination with an anti-OX40L monoclonal antibody in relieving graft-versus-host disease in mice. *Int. J. Hematol.*, **2010**, *92(4)*, 609-616.

[80] Crowther, C.; Ely, A.; Hornby, J.; Mufamadi, S.; Salazar, F.; Marion, P.; Arbuthnot, P. Efficient inhibition of hepatitis B virus replication in vivo, using polyethylene glycol-modified adenovirus vectors. *Hum. Gene Ther.*, **2008**, *19(11)*, 1325-1331.

[81] Eto, Y.; Gao, J.Q.; Sekiguchi, F.; Kurachi, S.; Katayama, K.; Mizuguchi, H.; Hayakawa, T.; Tsutsumi, Y.; Mayumi, T.; Nakagawa, S. Neutralizing antibody evasion ability of adenovirus vector induced by the bioconjugation of methoxypolyethylene glycol succinimidyl propionate (MPEG-SPA). *Biol. Pharm. Bull.*, **2004**, *27(6)*, 936-938.

[82] Zhang, Y.Z.; Xiong, W.; Li, Z.; Shao, C.P.; Li, T.J.; Zhao, F.; Yang, B.C. Effect of platelet CD42a modification by mPEG-SPA with different molecular masses. *Nan Fang Yi Ke Da Xue Xue Bao*, **2007**, *27(3)*, 392-393.

[83] Qiu, Y.; Zha, Y.; Tan, Y.X.; Zhang, Y.P. Rh antigen stability of mPEG modified red blood cells. *Exp. Hematol.*, **2006**, *14(5)*, 1020-1023.

[84] Muthuvel, A.; Rajamani, R.; Senthilvelan, M.; Manikandan, S.; Sheeladevi, R. Modification of allergenicity and immunogenicity of formate dehydrogenase by conjugation with linear mono methoxy poly ethylene glycol: improvement in detoxification of formate in methanol poisoning. *Clin. Chim. Acta*, **2006**, *374(1-2)*, 122-128.

[85] Larson, R.S.; Menard, V.; Jacobs, H.; Kim, S.W. Physicochemical characterization of poly(ethylene glycol)-modified anti-GAD antibodies. *Bioconjugate Chem.*, **2001**, *12(6)*, 861-869.

[86] Boccu, E.; Largajolli, R.; Veronese, F.M. Coupling of monomethoxypolyethyleneglycols to proteins via active esters. *Z. Naturforsch. C*, **1983**, *38(1-2)*, 94-99.

[87] Zalipsky, S.; Barany, G. Facile Synthesis of α-Hydroxy-ω-Carboxymethylpolyethylene Oxide. *J. Bioact. Compat. Polym.*, **1990**, *5(2)*, 227-231.

[88] Bailon, P.S.; Erythropoietin conjugates. U. S. Patent 6,583,272, June 24, **2003**.

[89] Veronese, F.; Largajolli, R.; Boccú, E.; Benassi, C.; Schiavon, O. Surface modification of proteins activation of monomethoxy-polyethylene glycols by phenylchloroformates and modification of ribonuclease and superoxide dismutase. *App. Biochem. Biotech.*, **1985**, *11(2)*, 141-152.

[90] Laliberte, M.; Gayet, J.C.; Fortier, G. Surface modification of horseradish peroxidase with poly(ethylene glycol)s of different molecular masses (Mr): relationship between the Mr of the poly(ethylene glycol) and the stability of horseradish peroxidase-poly(ethylene glycol) adducts under various denaturing conditions. *Biotechnol. Appl. Bioc.*, **1994**, *20(3)*, 397-413.

[91] Veronese, F.; Sartore, L.; Orsolini, P.; Deghenghi, R. Biologically active drug polymer derivatives and method for preparing the same. U. S. Patent 5,286,637, February 15, **1994**.

[92] Verral, D.E. Method for making polyethylene glycol carbonates. U. S. Patent 7,816,484, October 19, **2010**.

[93] Kito, M.; Miron, T.; Wilchek, M.; Kojima, N.; Ohishi, N.; Yai, K. A simple and efficient method for preparation monomethoxypolyethylene glycol activated with p-nitrophenylchloroformate and its applications to modification of L-asparaginase. *J. Clin. Biochem. Nutr.*, **1996**, *21(2)*, 101-111.

[94] Caliceti, P.; Monfardini, C.; Sartore, L.; Schiavon, O.; Baccichetti, F.; Carlassare, F.; Veronses, F.M. Preparation and properties of monomethoxy poly(ethylene glycol) doxorubicin conjugates linked by an amino acid or a peptide as spacer. *Farmaco*, **1993**, *48(7)*, 919-932.

[95] Kelly, S.J.; Delnomdedieu, M.; Oliverio, M.I.; Williams, L.D.; Saifer, M.G.P.; Sherman, M.R.; Coffman, T.M.; Johnson, G.A.; Hershfield, M.S. Diabetes insipidus in uricase-deficient mice: a model for evaluating therapy with poly(ethylene glycol)-modified uricase. *J. Am. Soc. Nephrol.*, **2001**, *12(5)*, 1001-1009.

[96] da Silva Freitas, D.; Spencer, P.J.; Vassão, R.C.; Abrahão-Neto, J. Biochemical and biopharmaceutical properties of PEGylated uricase. *Int. J. Pharm.*, **2009**, *387(1-2*), 215-22.

[97] da Silva Freitas, D.; Abrahão-Neto, J. Biochemical and biophysical characterization of lysozyme modified by PEGylation. *Int. J. Pharm.*, **2010**, *392(1-2)*, 111-117.

[98] Zalipsky, S.; Seltzer, R.; Nho, K. In: *Polymeric Drugs and Drug Delivery Systems*. ACS Symposium Series; Dunn, R.L; Ottenbrite, R.M. (Eds). American Chemical Society, Washington, **1991**.

[99] Zalipsky, S.; Seltzer, R.; Menon-Rudolph, S. Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins. *Biotech. Appl. Bioc.*, **1992**, *15(1)*, 100-114.

[100] Chiu, H.C.; Zalipsky, S.; Kopeckova, P.; Kopecek, J. Enzymatic activity of chymotrypsin and its poly(ethylene glycol) conjugates toward low and high molecular weight substrates. *Bioconjugate Chem.*, **1993**, *4(4)*, 290-295.

[101] Sirokman, G.; Fasman, G.D. Refolding and proton pumping activity of a polyethylene glycol-bacteriorhodopsin water-soluble conjugate. *Protein Sci.*, **1993**, *2(7)*, 1161-1170.

[102] Lee, K.C.; Moon, S.C.; Park, M.O.; Lee J.T.; Na, D.H.; Yoo, S.D.; Lee, H.S.; DeLuca, P.P. Isolation, characterization, and stability of positional isomers of mono-PEGylated salmon calcitonins. *Pharmaceut. Res.*, **1999**, *16(6)*, 813-818.

[103] Wang, Y.-S.; Youngster, S.; Grace, M.; Bausch, J.; Bordens, R.; Wyss, D.F. Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications. *Adv. Drug Deliver. Rev.*, **2002**, *54(4)*, 547-570.

[104] Wang, M.; Basu, A.; Palm, T.; Hua, J.; Youngster, S.; Hwang, L.; Liu, H.-C.; Li, X.; Peng, P.; Zhang, Y.; Zhao, H.; Zhang, Z.; Longley, C.; Mehlig, M.; Borowski, V.; Sai, P.; Viswanathan, M.; Jang, E.; Petti, G.; Liu, S.; Yang, K.; Filpula, D. Engineering an arginine catabolizing bioconjugate: biochemical and pharmacological characterization of PEGylated derivatives of arginine deiminase from Mycoplasma arthritidis. *Bioconjugate Chem.*, **2006**, *17(6)*, 1447-1459.

[105] Miron, T.; Wilchek, M. A simplified method for the preparation of succinimidyl carbonate polyethylene glycol for coupling to proteins. *Bioconjugate Chem.*, **1993**, *4(6)*, 568-569.

[106] Grace, M.; Youngster, S.; Gitlin, G.; Sydor, W.; Xie, L.; Westreich, L.; Jacobs, S.; Brassard, D.; Bausch, J. Structural and biologic characterization of pegylated recombinant IFN-α2b. *J. Interferon Cytokine Res.*, **2001**, *21(12)*,1103-1115.

[107] Zalipsky, S.; Funtionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconjugate Chem.*, **1995**, *6(2)*, 150-165.

[108] Wu, D.; Zhao, H.; Greewald, B. Process for the preparation of polymer conjugates. U. S. Patent 7,365,127, April 29, **2008**.

[109] Nitecki, D.E.; Preparation of an activated polymer ester for protein conjugation. U. S. Patent 5,281,698, January 25, **1994**.

[110] Dolence, E.K.; Hu, C.-Z.; Tsang, R.; Sanders, C.G.; Osaki, S. Electrophilic polyethylene oxides for the modification of polysaccharides, polypeptides (proteins) and surfaces. U. S. Patent, 5,650,234, July 22, **1997**.

[111] Zhang, G.; Hu, Q.; Braunlin, E.A.; Suggs, L.J.; Zhang, J. Enhancing efficacy of stem cell transplantation to the heart with a PEGylated fibrin biomatrix. *Tissue Eng. Part A*, **2008**, *14(6)*, 1025-1036.

[112] Kozlowsky, A. Method for the preparation of 1-benzotriazolyl carbonate esters of poly(ethylene glycol). U. S. Patent 6,624,246, September 23, **2003**.

[113] Guiotto, A.; Pozzobon, M; Sanavio, C.; Schiavon, O.; Orsolini, P.; Veronese, F.M. An improved procedure for the synthesis of branched polyethylene glycols (PEGs) with the reporter dipeptide Met-βAla for protein conjugation. *Bioorg. Med. Chem. Lett.*, **2002**, *12(2)*, 177-180.

[114] Beauchamp, C.O.; Gonias, S.L.; Menapace, D.P.; Pizzo, S.V. A new procedure for the synthesis of polyethylene glycol-protein adducts; effects on function, receptor recognition, and clearance of superoxide dismutase, lactoferrin, and α2-macroglobulin. *Anal. Biochem.*, **1983**, *131(1)*, 25-33.

[115] Brygier, J.; Gelbcke, M.; Guermant, C.; Nijs, M.; Baeyens-Volant, D.; Looze, Y. Covalent attachment of poly(ethyleneglycol) to peptides and proteins. *App. Biochem. Biotech.*, **1993**, *42(2)*, 127-135.

[116] González, M.; Grau, R.J.A.; Vaillard, S.E. New method for the synthesis and purification of branched mPEG2Lys. *React. Funct. Polym.*, **2012**, *72(1)*, 107-113.

[117] Rajagopalan, S.; Gonias, S.L.; Pizzo, S.V. A nonantigenic covalent streptokinase-polyethylene glycol complex with plasminogen activator function. *J. Clin. Invest.*, **1985**, *75(2)*, 413-419.

[118] Cunningham-Rundles, C.; Zhuo, Z.; Griffith, B.; Keenan, J. Biological activities of polyethylene-glycol immunoglobulin conjugates resistance to enzymatic degradation. *J. Immunol. Methods*, **1992**, *152(2)*, 177-190.

[119] Sawai, K.; Kurono, M.; Awaya, J.; Iwata, T. Modified urokinase composition. E. Patent 0,154,432, September 11, **1985**.

[120] Nakagawa, Y.; Ito, T. Chemically modified proteins with polyethyleneglycol. U. S. Patent 4,791,192, December 13, **1988**.

[121] Chamow, S.M.; Kogan, T.P.; Venuti, M.; Gadek, T.; Harris R.J.; Peers, D.H.; Mordenti, J.; Shak, S.; Ashkenasi, A. Modification of CD4 immunoadhesin with monomethoxypoly(ethylene glycol) aldehyde via reductive alkylation. *Bioconjugate Chem.*, **1994**, *5(2)*, 133-140.

[122] Capon, D.J.; Shak, S.; Ward, R.H.R. CD4 polypeptides derivatives. E. Patent 0,352,752, June 13, **1990**.

[123] Harris, J.M.; Struck, E.C.; Case, M.G.; Paley, M.S. Synthesis and characterization of poly(ethylene glycol) derivatives. *J. Polymer Sci.: Polymer Chem. Ed.*, **1984**, *22(2)*, 341-352.

[124] Harris, J.M.; Sedaghat-Herati, M.R. Preparation and use of polyethylene glycol propionaldehyde. U. S. Patent 5,252,714, October 12, **1993**.

[125] Fox, M.E.; Appell, R.B. Novel mPEG propionaldehyde precursor. U. S. Patent 0,249,119, December 9, **2004**.

[126] Bentley, M.D.; Harris, J.M. Poly(ethylene glycol) aldehyde hydrates and related polymers and application in modifying amines. U. S. Patent 5,990,237, November 23, **1999**.

[127] Wirth, P.; Souppe, J.; Tritsch, D.; Biellmann, J.-F. Chemical modification of horseradish peroxidase with ethanal-methoxypolyethylene glycol: solubility in organic solvents, activity, and properties. *Bioorg. Chem.*, **1991**, *19(2)*, 133-142.

[128] Pendri, A.; Gilbert, C.W.; Soundararajan, S.; Bolikal, D.; Shorr, R.G.L.; Greenwald, R.B. PEG modified anticancer drugs: synthesis and biological activity. *J. Bioact. Compat. Polym.*, **1996**, *11(2)*, 122-134.

[129] Kinstler, O.B.; Gabriel, N.E.; Farrar, C.E.; Deprince, R.B. N-terminally chemically modified protein compositions and methods. U. S. Patent 5,824,784, October 20, **1998**.

[130] Molineux, G. The design and development of pegfilgrastim (PEG-rmetHuG-CSF, Neulasta). *Curr. Pharm. Design*, **2004**, *10(11)*, 1235-1244.

[131] Kinstler, O.; Molineaux, G.; Treuheit, M.; Ladd, D.; Gegg, C. Mono-N-terminal poly(ethylene glicol)-protein conjugates. *Adv. Drug Deliver. Rev.*, **2002**, *54(4)*, 477-485.

[132] Bentley, M.D.; Roberts, M.J.; Harris, J.M. Reductive amination using poly(ethylene glycol) acetaldehyde hydrate generated in situ: applications to chitosan and lysozyme. *J. Pharm. Sci.* **1998**, *87(11)*, 1446-1469.

[133] Edwards III, C.K. PEGylated recombinant human soluble tumour necrosis factor receptor type I (r-Hu-sTNF-RI): novel high affinity TNF receptor designed for chronic inflammatory diseases. *Ann. Rheum. Dis.*, **1999**, *58(s1)*, I73-I81.

[134] Arduini, R.M.; Li, Z.; Rapoza, A.; Gronke, R.; Hess, D.M.; Wen, D.; Miatkowski, K.; Coots, C.; Kaffashan, A.; Viseux, N.; Delaney, J.; Domon, B.; Young, C.N.; Boyton, R.; Chen, L.L.; Betzenhauser, M.; Miller, S.; Gill, A.; Pepinsky, R.B.; Hochman, P.S.; Baker, D.P. Expression, purification, and characterization of rat interferon-β, and preparation of an N-terminally PEGylated form with improved pharmacokinetic parameters. *Protein Expres. Purif.*, **2004**, *34(2)*, 229-242.

[135] Na, D.H.; Lee, K.C.; DeLuca, P.P. PEGylation of octreotide: II. Effect of N-terminal mono-PEGylation on biological activity and pharmacokinetics. *Pharm. Res.*, **2005**, *22(5)*, 743-749.

[136] Wang, Y.-J.; Hao, S.-J.; Liu, Y.-D.; Hu, T.; Zhang, G.-F.; Zhang, X.; Qi, Q.-S.; Ma, G.-H.; Su, Z.-G. PEGylation markedly enhances the in vivo potency of recombinant human non-glycosylated erythropoietin: a comparison with glycosylated erythropoietin. *J. Control. Release*, **2010**, *145(3)*, 306-313.

[137] Uchiyama, Y.; Hosoe, M.; Sato, T.; Takahashi, T. Biological and immunological characteristics of porcine follicle-stimulating hormone chemically modified with a polyethylene glycol derivative. *Vet. J.*, **2010**, *184(2)*, 208-211.

[138] Emoto K.; Van Alstine, J.M.; Harris, J.M. Stability of poly(ethylene glycol) graft coatings. *Langmuir*, **1998**, *14(10)*, 2722-2729.

[139] Fukai, R.; Dakwa, P.H.R.; Chen, W. Strategies toward biocompatible artificial implants: grafting of functionalized poly(ethylene glycol) to poly(ethylene terephthalate) surfaces. *J. Polym. Sci. Pol. Chem.*, **2004**, *42(21)*, 5389-5400.

[140] Ikeda, I.; Horie, H.; Suzuki, K. Synthesis of graft poly(ester ether) by ring-opening copolymerization of epoxy-terminated poly(ethylene glycol) with lactones. *J. Appl. Polym. Sci.*, **1994**, *51(11)*, 1931-1935.

[141] Emoto K.; Harris, J.M.; Van Alstine, J.M. Grafting poly(ethylene glycol) epoxide to amino-derivatized quartz: effect of temperature and pH on grafting density. *Anal. Chem.*, **1996**, *68(21)*, 3751-3757.

[142] Bergström, K.; Holmberg, K.; Safranj, A.; Hoffman, A.S.; Edgell, M.J.; Kozlowski, A.; Hovanes, B.A.; Harris, J.M. Reduction of fibrinogen adsorption on PEG-coated polystyrene surfaces. *J. Biomed. Mater. Res.*, **1992**, *26(6)*, 779-790.

[143] Head, D.M; Andrews, B.A.; Asenjo, J.A. Epoxy-oxirane activation of PEG for protein ligand coupling. *Biotechnol. Tech.*, **1989**, *3(1)*, 27-32.

[144] Elling, L.; Kula, M.R. Immunoaffinity partitioning: synthesis and use of polyethylene glycol-oxirane for coupling to bovine serum albumin and monoclonal antibodies. *Biotechnol. Appl. Bioc.*, **1991**, *13(3)*, 354-362.

[145] Pitha, J.; Kociolek, K.; Caron, M.G. Detergents Linked to Polysaccharides: preparation and effects on membranes and cells. *Eur. J. Biochem.*, **1979**, *94(1)*, 11-18.

[146] Ba, H.; Peng, X.; Qi, Y. Study on epoxy networks solid electrolytes. *Makromol. Chem.*, **1991**, *191(11)*, 2529-2536

[147] Wang, H.; Lizhong, H.; Lensch, M.; Gabius, H.-J.; Fee, C.J.; Middelberg, A.P.J. Single-site Cys-substituting mutation of human lectin galectin-2: modulating solubility in recombinant production, reducing long-term aggregation, and enabling site-specific monoPEGylation. *Biomacromolecules*, **2008**, *9(11)*, 3223-3230.

[148] Rosendahl, M.S.; Doherty, D.H.; Smith, D.J.; Carlson, S.J. A long-acting, highly potent interferon α-2 conjugate created using site-specific PEGylation. *Bioconjugate Chem.*, **2005**, *16(1)*, 200-207.

[149] Brocchini, S.; Godwin, A.; Balan, S.; Choi, J.-W.; Zloh, M.: Shaunak, S. Disulfide bridge based PEGylation of proteins. *Adv. Drug Deliver. Rev.*, **2008**, *60(1)*, 3-12.

[150] Morpurgo, M.; Veronese F.M.; Kachensky, D.; Harris, J.M. Preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjugate Chem.*, **1996**, *7(3)*, 363-368.

[151] Yu, Y.; Chau, Y. One-step "Click" method for generating vinyl sulfone groups on hydroxyl-containing water-soluble polymers. *Biomacromolecules*, **2012**, *13(3)*, 937-942.

[152] Nho, K.; Hyun, C.; Lee, J.; Pak, Y. Preparation method of PEG-maleimide derivatives. U. S. Patent 6,828,401, December 7, **2004**.

[153] Goodson, R.J.; Katre, N.V. Site-directed PEGylation of recombinant interleukin-2 at its glycosylation site. *Bio/Technology*, **1990**, *8(4)*, 343-346.

[154] Kogan, T.P. The synthesis of substituted methoxy-poly(ethylene glycol) derivatives suitable for selective protein modification. *Synth. Commun.*, **1992**, *22(16)*, 2417-2424.

[155] Sakanoue, K.; Kubo, K.; Ohashi, S. Polyoxyalkylene derivative and process of producing the same. E. Patent 1,283,233, July 18, **2007**.

[156] Shen, X. N-Maleimidyl polymer derivatives. U. S. Patent 6,602,498, August 5, **2003**.

[157] Chapman, A.P.; Antoniw, P.; Spitali, M.; West, S.; Stephens, S.; King, D.J. Therapeutic antibody fragments with prolonged in vivo half-lives. *Nat. Biotech.*, **1999**, *17(8)*, 780-783.

[158] Vanwetswinkel, S.; Plaisance, S.; Zhi-yong, Z.; Vanlinthout, I.; Brepoels, K.; Lasters, I.; Collen, D.; Jespers, L. Pharmacokinetic and thrombolytic properties of cysteine-linked polyethylene glycol derivatives of staphylokinase. *Blood*, **2000**, *95(3)*, 936-942.

[159] Long, D.L.; Doherty, D.H.; Eisenberg, S.P.; Smith, D.J.; Rosendahl, M.S.; Christensen, K.R.; Edwards, D.P.; Chlipala, E. A.; Cox, G.N. Design of homogeneous, monopegylated erythropoietin analogs with preserved in vitro bioactivity. *Exp. Hematol.*, **2006**, *34(6)*, 697-704.

[160] Zappe, H.; Snell, M.E.; Bossard, M.J. PEGylation of cyanovirin-N, an entry inhibitor of HIV. *Adv. Drug Deliver. Rev.*, **2008**, *60(1)*, 79-87.

[161] Colonna, C.; Conti, B.; Perugini, P.; Pavanetto, F.; Modena, T.; Dorati, R.; Iadarola, P.; Genta, I. Site-directed PEGylation as successful approach to improve the enzyme replacement in the case of prolidase. *Int. J. Pharm.*, **2008**, *358(1-2)*, 230-237.

[162] Jung, S.; Lee, S.H.; Mok, H.; Chung, H.J.; Park, T.G. Gene silencing efficiency of siRNA-PEG conjugates: effect of PEGylation site and PEG molecular weight. *J. Control. Release*, **2010**, *144(3)*, 306-313.

[163] Hu, J.; Duppatla, V.; Harth, S.; Schmitz, W.; Sebald, W. Site-specific PEGylation of bone morphogenetic protein-2 cysteine analogues. *Bioconjugate Chem.*, **2010**, *21(10)*, 1762-1772.

[164] Gong, N.; Ma, A.N.; Zhang, L.J.; Luo, X.S.; Zhang, Y.H.; Xu, M.; Wang, Y.-X. Site-specific PEGylation of exenatide analogues markedly improved their glucoregulatory activity. *Br. J. Pharmacol.*, **2011**, *163(2)*, 399-412.

[165] Da Pieve, C.; Williams, P.; Haddleton, D.M.; Palmer, R.M.; Missailidis, S. Modification of thiol functionalized aptamers by conjugation of synthetic polymers. *Bioconjugate Chem.*, **2010**, *21(1)*, 169-174.

[166] Shaw, G.; Veldman, G.; Wooters, J. Cysteine added variants of polypeptides and chemical modifications thereof. W. O. Patent 9,012,874, November 1, **1990**.

[167] Doherty, D.H.; Rosendahl, M.S.; Smith, D.J.; Hughes, J.M.; Chlipala, E.A.; Cox, G.N. Site-Specific PEGylation of engineered cysteine analogues of recombinant human granulocyte-macrophage colony-stimulating factor. *Bioconjugate Chem.*, **2005**, *16(5)*, 1291-1298.

[168] Danial, M.; van Dulmen, T.H.H; Alejsandrowicz, J.; Pötgens, A.J.G.; Klok, H.-A. Site-specific PEGylation of HR2 peptides: effects of PEG conjugation position and chain lenght on HIV-1 membrane fusion inhibition and proteolytic degradation. *Bioconjugate Chem.*, **2012**, *23(8)*, 1648-1660.

[169] Guo, Z.Y.; Chang, C.C.Y.; Lu, X.; Chen, J.; Li, B.L.; Chang, T.Y. The disulfide linkage and the free sulfhydryl accessibility of acyl-coenzyme A:cholesterol acyltransferase 1 as studied by using mPEG5000-maleimide. *Biochemistry-Us.*, **2005**, *44(17)*, 6537-6546.

[170] Kozlowski, A.; McManus, S.P. Stabilized polymeric thiol reagents. U. S. patent, 8,217,123, July 10, **2012**.

[171] Woghiren, C.; Sharma, B.; Stein, S. Protected thiol-polyethylene glycol: new activated polymer for reversible protein modification. *Bioconjugate Chem.*, **1993**, *4(5)*, 314-318.

[172] El Tayar, N.; Roberts, M.J.; Harris, M.J.; Sawlivich, W. Polyol-IFN-beta conjugates. W. O. Patent 9,955,377, November 4, **1999**.

[173] Monfardini, C.; Schiavon, O.; Caliceti, P.; Morpurgo, M.; Harris, J.M.; Veronese, F.M. A branched monomethoxypoly(ethylene glycol) for protein modification. *Bioconjugate Chem.*, **1995**, *6(1)*, 62-69.

[174] Bailon, P.S.; Palleroni, A.V. Interferon conjugates. U. S. Patent 7,201,897, April 10, **2007**.

[175] Dhalluin, C.; Ross, A.; Huber, W.; Gerber, P.; Brugger, D.; Gsell, B.; Senn, H. Structural, kinetic, and thermodynamic analysis of the binding of the 40 kDa PEG-interferon-α2a and its individual positional isomers to the extracellular domain of the receptor IFNAR2. *Bioconjugate Chem.*, **2005**, *16(3)*, 518-527.

[176] Foser, S.; Schacher, A.; Weyer, K.A.; Brugger, D.; Dietel, E.; Marti, S.; Schreitmüller, T. Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon α-2a (PEGASYS). *Protein Expres. Purif.*, **2003**, *30(1)*, 78-87.

[177] Ramon, J.; Saez, V.; Baez, R.; Aldana, R.; Hardy, E. PEGylated Interferon-α2b: a branched 40K polyethylene glycol derivative. *Pharm. Res.*, **2005**, *22(8)*, 1375-1387.

[178] Basu, A.; Yang, K.; Wang, M.;, Liu, S.; Chintala, R.; Palm, T.; Zhao, H.; Peng, P.; Wu, D.; Zhang, Z.; Hua, J.; Hsieh, M.-C.; Zhou, J.; Petti, G.; Li, X.; Janjua, A.; Mendez, M.; Liu, J.; Longley, C.; Mehlig, M.; Borowski, V.; Viswanathan, M.; Filpula, D. Structure-function engineering of interferon-β-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. *Bioconjugate Chem.*, **2006**, *17(3)*, 618-630.

[179] Noberini, R.; Mitra, S.; Salvucci, O.; Valencia, F.; Duggineni, S.; Prigozhina, N.; Wei, K.; Tosato, G.; Huang, Z.; Pasquale, E.B.. PEGylation potentiates the effectiveness of an antagonistic peptide that targets the EphB4 receptor with nanomolar affinity. *PloS one*, **2011**, *6(12)*, e28611.

[180] Mezo, A.R.; Low, S.C.; Hoehn, T.; Palmieri, H. PEGylation enhances the therapeutic potential of peptide antagonists of the neonatal Fc receptor, FcRn. *Bioorg. Med. Chem. Lett.*, **2011**, *21(21)*, 6332-6335.

[181] Zheng, J.C.; Lei, N.; He, Q.C.; Hu, W.; Jin, J.G.; Meng, Y.; Deng, N.-h.; Meng, Y.-F.; Zhang, C.-J.; Shen, F.-b.et al. PEGylation is effective in reducing immunogenicity, immunotoxicity, and hepatotoxicity of alpha-momorcharin in vivo. *Immunopharm. Immunot.*, **2012**, *34(5)*, 866-873.

[182] Bian, X.; Shen, F.; Chen, Y.; Wang, B.; Deng, M.; Meng, Y. PEGylation of alpha-momorcharin: synthesis and characterization of novel anti-tumor conjugates with therapeutic potential. *Biotechnol. Lett.*, **2010**, *32(7)*, 1-8.

[183] Novikov, B.N.; Grimsley, J.K.; Kern, R.J.; Wild, J.R.; Wales, M.E. Improved pharmacokinetics and immunogenicity profile of organophosphorus hydrolase by chemical modification with polyethylene glycol. *J. Control. Release*, **2010**, *146(3)*, 318-325.

[184] Nojima, Y.; Iguchi, K.; Suzuki, Y.; Sato, A. The pH-dependent formation of PEGylated bovine lactoferrin by branched polyethylene glycol (PEG)-N-hydroxysuccinimide (NHS) active esters. *Biol. Pharm. Bull.*, **2009**, *32(3)*, 523-526.

[185] Harris, J.M.; Veronese, F.M. Caliceti, P.; Schiavon, O. Multiarmed, monofunctional, polymer for coupling to molecules and surfaces. U. S. Patent 5,932,462, August 3, **1999**.

[186] Greewald, R.B.; Martinez, A.J. Water-soluble non-antigenic polymer linkable to biologically active material. U. S. Patent 6,313,906, September 5, **2000**.

[187] Greenwald, R.B.; Choe, Y.H.; McGuire, J.; Conocer, C.D. Effective drug delivery by PEGylated drug conjugates. *Adv. Drug. Deliver. Rev.*, **2003**, *55(2)*, 217-250.

[188] Filpula, D.; Zhao, H. Releasable PEGylation of proteins with customized linkers. *Adv. Drug. Deliver. Rev.*, **2008**, *60(1)*, 29-49

[189] Greenwald, R.B.; Choe, Y.H.; McGuire, J.; Conover, C.D. Effective drug delivery by PEGylated drug conjugates. *Adv. Drug Deliver. Rev.*, **2003**, *55(2)*, 217-250.

[190] Greenwald, R.B. PEG drugs: an overview. *J. Control. Release*, **2001**, *74(1-3)*, 159-171.

[191] Greenwald, R.B.; Pendri, A.; Bolikal, D.; Gilbert, C.W. Highly water soluble taxol derivatives: 2'-Polyethyleneglycol esters as potential prodrugs. *Bioorg. Med. Chem. Lett.*, **1994**, *4(20)*, 2465-2470.

[192] Greenwald, R.B.; Pendri, A.; Conover, C.D.; Zhao, H.; Choe, Y.H.; Martinez, A.; Shum, K.; Guan, S.; Drug delivery systems employing 1,4- or 1,6- elimination: poly(ethylene glycol) prodrugs of amine-containing compounds. *J. Med. Chem.*, **1999**, *42(18)*, 3657-3667.

[193] Greenwald, R.B.; Zhao, H.; Yang, K.; Reddy, P.; Martinez, A. A New aliphatic amino prodrug system for the delivery of small molecules and proteins utilizing novel PEG derivatives. *J. Med. Chem.*, **2004**, *47(3)*, 726-734.

[194] Conover, C.D.; Zhao, H.; Logley, C.B.; Shum, K.L.; Greewald, R.B. Utility of poly(ethylene glycol) conjugation to create prodrugs of amphotericin B. *Bioconjugate Chem.*, **2003**, *14(3)*, 661-666.

[195] Greewald, R.B.; Zhao, H.; Xia, J.; Martinez, A. Poly(ethylene glycol) transports forms of vancomycin: a long-lived continous release delivery system. *J. Med. Chem.*, **2003**, *46(23)*, 5021-5030.

[196] Greenwald, R.B.; Yang, K.; Zhao, H.; Conover, C.D.; Lee, S.; Filpula, D. Controlled release of proteins from their poly(ethylene glycol) conjugates: drug delivery systems employing 1,6- eliminations. *Bioconjugate Chem.*, **2003**, *14(2)*, 395-43.

[197] Lee, S.; Greenwald, R.B.; McGuire, J.; Yang, K.; Shi, C. Drug delivery systems employing 1,6-elimination : releasable poly(ethylene glycol) conjugates of proteins. *Bioconjugate Chem.*, **2001**, *12(12)*, 163-169.

[198] Greenwald, R.B.; Zhao, H.; Yang, K.; Reddy, P.; Martinez, A. A new aliphatic amino prodrug system for the delivery of small molecules and proteins utilizing novel PEG derivatives. *J. Med. Chem.*, **2004**, *47(3)*, 726-734.

[199] Zhao, H.; Yang, K.; Martinez, A.; Basu, A.; Chintala, R.; Liu, H.C.; Janjua, A.; Wang, M.; Filpula, D. Linear and branched bicin linkers for releasable PEGylation of macromolecules: controlled release in vivo and in vitro from mono- and multi-PEGylated proteins. *Bioconjugate Chem.*, **2006**, *17(2)*, 341-351.

[200] Tsubery, H.; Mironchik, M.; Fridkin, M.; Shechter, Y. Prolonging the action of protein and peptide drugs by a novel approach of reversible polyethylene glycol modification. *J. Biol. Chem.*, **2004**, *279(37)*, 38118-38124.

[201] Peleg-Shulman, P.; Tsubery, H.; Mironchik, M.; Fridkin, M.; Schreiber, G.; Shechter, Y.; Reversible PEGylation: a novel technology to release native interferon α2 over a prolonged time period. *J. Med. Chem.* **2004**, *47(20)*, 4897-4904.

[202] Shechter, Y.; Heldman, E.; Sasson, K.; Bachar, T.; Popov, M.; Fridkin, M.; Delivery of neuropeptides to the brain: studies with enkephalin. *ACS Chem. Neurosci.*, **2010**, *1(5)*, 399-406.

[203] Nesher, M.; Vachutinsky, Y.; Fridkin, G.; Schwarz, Y.; Fridkin, M.; Shechter, Y.; Lichtstein, D. Reversible PEGylation prolongs the hypotensive effect of atrial natriuretic peptide. *Bioconjugate Chem.*, **2008**, *19(1)*, 342-348.

[204] Shechter, Y.; Mironchik, M.; Rubinraut, S.; Tsubery, H.; Sasson, K.; Marcus, Y.; Fridkin, M. Reversible PEGylation of insulin facilitates its prolonged action in vivo. *Eur. J. Pharm. Biopharm.*, **2008**, *70(1)*, 19-28.

[205] Zalipsky, S.; Qazen, M.; Walker J.A.; Mullah, N.; Quinn, Y.P.; Huang, S.Q. New detachable poly(ethylene glycol) conjugates: cysteine-cleavable lipopolymers regenerating natural phospholipid, diacylphosphatidylethanolamine. *Bioconjugate Chem.*, **1999**, *10(5)*, 7037-707.

[206] Zalipsky, S.; Mullah, N.; Engbers, C.; Hutchins, M. U.; Kiwan, R. Thiolytically cleavable dithiobenzyl urethane-linked polymer-protein conjugates as macromolecular prodrugs: reversible PEGylation of proteins. *Bioconjugate Chem.*, **2007**, *18(6)*, 1869-1878.

[207] Greenwald, R.B.; Choe, Y. H.; Conover, C.D.; Shum, K.; Wu, D.; Royzen, M. Drug delivery systems based on trimethyl lock lactonization: poly(ethylene glycol) prodrugs. *J. Med. Chem.*, **2000**, *43(3)*, 475-487.

[208] Maullu, C.; Raimondo, D.; Caboi, F.; Giorgetti, A.; Sergi, M.; Valentini, M.; Tonon, G.; Tramontano, A. Site-directed enzymatic PEGylation of the human granulocyte colony-stimulating factor. *FEBS J.*, **2009**, *276(22)*, 6741-6750.

[209] Fontana, A.; Spolaore, B.; Mero, A.; Veronese, F.M. Site-specific modification and PEGylation of pharmaceutical proteins mediated by transglutaminase. *Adv. Drug Deliver. Rev.*, **2008**, *60(1)*, 13-28.

[210] Mero, A.; Schiavon, M.; Veronese, F.M.; Pasut, G. A new method to increase selectivity of transglutaminase mediated PEGylation of salmon calcitonin and human growth hormone. *J. Control. Release*, **2011**, *154(1)*, 27-34.

[211] Zalipsky, S.; Menon-Rudolph, S. Hydrazide derivatives of poly(ethylene glycol) and their bioconjugates, in *Poly(ethylene glycol) Chemistry and Biological Applications*. Harris, J.M.; Zalipsky, S. (Eds.), ACS Books, Washington, D.C., **1997**.

[212] Zalipsky, S. Hydrazine containing conjugates of polypeptides and glycopolypeptides with polymers. W. O. Patent 92/16555, October 1, **1992**.

[213] Gaertner, H.F.; Offord, R.E. Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins. *Bioconjugate Chem.*, **1996**, *6(1)*, 38-44.

[214] Sano, A.; Maeda, H.; Kai, Y.; Ono, K. Polyethylene glycol derivatives, modified peptides and production thereof. E. Patent, 0,340,741, November 11, **1989**.

[215] Yankeelov, Jr., J.A. Modification of arginine with diketones. *Methods Enzymol.*, **1972**, *25C*, 566-584.

[216] Gauthier, M.A.; Ayer, M.; Kowal, J.; Wurm, F.R.; Klok, H.-R. Arginine-specific protein modification using α-oxo-aldehyde functional polymers prepared by atom transfer radical polymerization. *Polym. Chem.*, **2011**, *2(7)*, 1490-1498.

[217] Gauthier, M.A.; Klok, H.-A. Arginine-specific modification of proteins with polyethylene glycol. *Biomacromolecules*, **2011**, *12(2)*, 482-493.

[218] Jones, M.W.; Mantovani, G.; Blindauer, C.A.; Ryan, S.M.; Wang, X.; Brayden, D.J.; Haddleton, D.M. Direct peptide bioconjugation/PEGylation at tyrosine with linear and branched polymeric diazonium salts. *J. Am. Chem. Soc.*, **2012**, *134(17)*, 1406-1413.

[219] Meng, F.; Manjula, B.N.; Smith, P.K.; Acharya, S.A. PEGylation of human serum albumin: reaction of PEG-phenylisothiocyanate with proteins. *Bioconjugate Chem.*, **2008**, *19(7)*, 1352-1360.

[220] Huang, Z.; Ni, C.; Chu, Y.; Wang, S.; Yang, S.; Wu, X.; Wang, X.; Li, X.; Feng, W.; Lin. S. Chemical modification of recombinant human keratinocyte growth factor 2 with polyethylene glycol improves biostability and reduces animal immunogenicity. *J. Biotechnol.*, **2009**, *142(3-4)*, 242-249.

[221] Smith, P.K. Activated polyethylene glycol compounds. U. S. Patent 6,737,524, May 18, **2004**.

[222] Zalipsky, S.; Menon-Rudolph, S. Hydrazide derivatives of poly(ethylene glycol) and their bioconjugates. Poly(ethylene glycol) chemitry and biological applications. *ACS Symp. Ser.*, **1997**, 680, 318-341.

[223] Schumacher, F.F.; Nobles, M.; Ryan, C.P.; Smith, M.E.; Tinker, A.; Caddick S.; Baker, J.R. In situ maleimide bridging of disulfides and a new approach to protein PEGylation. *Bioconjugate Chem.*, **2011**, *22(2)*, 132-136.

[224] Jones, M.W.; Strickland, R.A.; Schumacher, F.F.; Caddick, S.; Baker, J.R.; Gibson, M.I.; Haddleton, D.M.; Polymeric dibromomaleimide as extremely efficient disulfide bridging bioconjugation and PEGylation agents. *J. Am. Chem. Soc.*, **2012**, *134(3)*, 1847-1852.

[225] Cong, Y.; Pawlisz, E.; Bryant, P.; Balan, S.; Laurine, E.; Tommasi, R.; Singh, R.; Dubey, S.; Peciak, K.; Bird, M.; Sivasankar, A.; wierkosz, J.; Muroni, M.; Heidelberg, S.; Farys, M.; Khayrzad, F.; Edwards, J.; Badescu, G.; Hodgson, I.; Heise, C.; Somavarapu, S.; Liddell, J.; Powell, K.; Zloh, M.; Godwin, A.; Brocchini, S. Site-specific PEGylation at histidine tags. *Bioconjugate Chem.*, **2012**, *23(2)*, 248-263.

[226] Deiters, A.; Cropp, T.A.; Summerer, D.; Mukherji, M.; Schultz, P.G. Site-specific PEGylation of proteins containing unnatural amino acids. *Bioorg. Med. Chem. Lett.*, **2004**, *14(23)*, 5743-5745.

[227] Cho, H.; Daniel, T; Buechler, Y.J.; Litzinger, D.C.; Maio, Z.; Putnam, A.-M.H; Kraynov, V.S.; Sim, B.-C.; Bussell, S.; Javahishvili, T.; Kaphle, S.; Viramontes, G.; Ong, M.; Chu, S.; Gc, B.; Lieu, R.; Knidsen, N.; Castiglioni, P.; Norman, T.C.; Axelrod, D.W.; Hoffman, A.R.; Schultz, P.G.; DiMarchi, R.D.; Kimmel, B.E. Optimized clinical performance of growth hormone with an expanded genetic code. *P. Natl. Acad. Sci. USA*, **2011**, *108(22)*, 9060-9065.

[228] Chen, H.; Lu, Y.; Fang, Z.; Liu, J.; Tian, H.; Gao, X.; Yao, W. High-level production of uricase containing keto functional groups for site-specific PEGylation. *Biochem. Eng. J.*, **2011**, *58-59(1)*, 25-32.

[229] Ou, W.; Uno, T.; Chiu, H.P.; Grünewald, J.; Cellitti, S.E.; Crossgrove, T.; Hao, X.; Fan, Q.; Quinn, L.L.; Patterson, P.; Okach, L.; Jones, D.H.; Lesley, S.A.; Brock, A.; Geierstanger, B.H.. Site-specific protein modifications through pyrroline-carboxy-lysine residues. *P. Natl. Acad. Sci. USA*, **2011**, *108(26)*, 10437-10442.

[230] Grabstein, K.H.; Wang, A.; Nairn, N.; McCraith, S.; Datta, D. Modified human interferon-ß polypeptides. U. S. 7,632,492, December 15, **2009**.

[231] Dirks, A.J.; Cornelissen, J.L.M.; Nolte, R.J.M.; Monitoring protein-polymer conjugation by a fluorogenic Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition. *Bioconjugate Chem.*, **2009**, *20(6)*, 1129-1138.

[232] Serwa, R.; Majkut, P.; Horstmann, B.; Swiecicki, J.-M.; Gerrits, M.; Krause, E.; Hackenberger, C.P.R. Site-specific PEGylation of proteins by a Staudinger-phosphite reaction. *Chem. Sci.*, **2010**, *1(5)*, 596-602.

[233] DeFrees, S.; Wang, Z.-G.; Xing, R.; Scott, A.E.; Wang, J.; Zopf, D.; Gouty, D.L.; Sjoberg, E.R.; Panneerselvam, K.; Brinkman-Van der Linden, E.C.M; Bayer, R.J.; Tarp, M.A.; Clausen, H. GlycoPEGylation of recombinant therapeutic proteins produced in Escherichia coli. *Glycobiology*, **2006**, *16(9)*, 833-843.

[234] Stennicke, H.R.; Ostergaard, H.; Bayer, R.J.; Kalo, M.S.; Kinealy, K.; Holm, P.K.; Sørensen, B.B.; Zopf, D.; Bjørn, S.E.; Generation and biochemical characterization of glycoPEGylated factor VIIa derivatives. *Thromb. Haemost.*, **2008**, *100(5)*, 920-928.

[235] Sen, P.; Ghosh, S.; Ezban, M.; Pendurthi, U.R.; Vijaya Mohan Rao, L. Effect of glycoPEGylation on factor VIIa binding and internalization. *Haemophilia*, **2010**, *16(2)*, 339-348.

[236] Popp, M.W.; Dougan, S.K.; Chuang, T.Y.; Spooner, E.; Ploegh, H.L. Sortase-catalyzed transformations that improve the properties of cytokines. *P. Natl. Acad. Sci. USA*, **2011**, *108(8)*, 3169-3174.

[237] Huang, Z.; Zhu, G.; Sun, C.; Zhang, J.; Zhang, Y.; Ye, C.; Wang, X.; Ilghari, D.; Li, X. A novel solid-phase site-specific PEGylation enhances the in vitro and in vivo biostabilty of recombinant human keratinocyte growth factor 1. *PLoS One*, **2012**, *7(5)*, e36423.

[238] Huang, Z.; Ye, C.; Liu, Z.; Wang, X.; Chen, H.; Liu, Y.; Tang, L.; Zhao, H.; Wang, J.; Feng, W.; Li, X. Solid-phase N-terminus PEGylation of recombinant human fibroblast growth factor 2 on heparin-sepharose column. *Bioconjugate Chem.*, **2012**, *23(4)*, 740-750.

[239] Suo, X.; Lu, X.; Hu, T.; Ma, G.; Su, Z. A solid-phase adsorption method for PEGylation of human serum albumin and staphylokinase: preparation, purification and biochemical characterization. *Biotechnol. Lett.*, **2009**, *31(8)*, 1191-1196.

[240] Jølck, R.I.; Berg, R.H.; Andresen, T.L. Solid-phase synthesis of PEGylated lipopeptides using click chemistry. *Bioconjugate Chem.*, **2010**, *21(5)*, 807-810.

[241] Lee, B.K.; Kwon, J.S.; Kim, H.J.; Yamamoto, S.; Lee, E.K. Solid-phase PEGylation of recombinant interferon α-2a for site-specific modification: process performance, characterization, and in vitro bioactivity. *Bioconjugate Chem.*, **2007**, *18(6)*, 1728-1734.

[242] Suo, X.; Zheng, C.; Yu, P.; Lu, X.; Ma, G.; Su, Z. Solid phase pegylation of hemoglobin. *Artif. Cell. Blood Sub.*, **2009**, *37(4)*, 147-155.

[243] Thom, J.; Anderson, D.; McGregor, J.; Cotton, G. Recombinant protein hydrazides: application to site-specific protein PEGylation. *Bioconjugate Chem.*, **2011**, *22(6)*, 1017-1020.

[244] Le, Y.; Li, L.; Wang, D.; Scott, M.D. Immunocamouflage of latex surfaces by grafted methoxypoly(ethylene glycol) (mPEG): proteomic analysis of plasma protein adsorption. *Sci. China Ser. C*, **2012**, *55(3)*, 191-201.

[245] Olivier, J.-C.; Huertas, R.; Lee, H.J.; Calon, F.; Pardridge, W.M. Synthesis of PEGylated immunonanoparticles. *Pharm. Res.*, **2002**, *19(8)*, 1137-1143.

[246] Chang, C.-H.; Rossi, E.A.; Cardillo, T.M.; Nordstrom, D.L.; McBride, W.J.; Goldenberg, D.M. A new method to produce produce monoPEGylated dimeric cytokines shown with human interferon-α2b. *Bioconjugate Chem.*, **2009**, *20*, 10, 1899-1907.