

Review

Peptide and protein PEGylation: a review of problems and solutions

Francesco M. Veronese*

Department of Pharmaceutical Sciences, CNR, Center for Chemical Investigation of Drugs, University of Padova, Via F. Marzolo 5, 35131 Padova, Italy

Received 8 March 2000; accepted 30 May 2000

Abstract

The paper discusses general problems in using PEG for conjugation to high or low molecular weight molecules. Methods of binding PEG to different functional groups in macromolecules is reported together with their eventual limitations. Problems encountered in conjugation, such as the evaluation of the number of PEG chains bound, the localisation of the site of conjugation in polypeptides and the procedure to direct PEGylation to the desired site in the molecule are discussed. Finally, the paper reports on more specific methods regarding reversible PEGylation, cross-linking reagents with PEG arms, PEG for enzyme solubilization in organic solvent and new polymers as alternative to PEG. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: PEG; PEGylation; PEGylation chemistry; Problems in PEGylation; Site-directed PEGylation

Contents

1. Introduction	405
2. Problems in PEGylation related to the starting PEG	406
3. Activated PEGs for amino group conjugation	406
3.1. Alkylating PEGs	407
3.2. Acylating PEGs	407
4. PEG with amino acid arm	408
5. Characterization of PEG conjugates	408
6. Unexpected reaction in PEGylation chemistry	409
7. PEGylation at cysteine residue	410
8. Arginine modification	410
9. Carboxyl group modification	410
10. Hydroxyl group conjugation	411
11. Active site protection from PEGylation	411
12. PEG as flexible, hydrophilic moiety in bifunctional reagents	412
13. Enzyme PEGylation for solubilization in organic solvents	412
14. Reversible grafting of PEG to proteins or fatty acid	413
15. Identification of PEGylation site in peptide and protein	413
16. New monofunctional polymers for protein conjugation	414
17. Sources of PEG	415
18. Conclusions	415
Acknowledgements	415
References	415

1. Introduction

PEGylation is a procedure of growing interest for enhancing the therapeutic and biotechnological potential of peptides and proteins. When poly(ethylene glycol) (PEG) is properly linked to a polypeptide, it modifies

* Corresponding author. Tel. +39-049-827-5694. Fax +39-049-827-5366.

E-mail address: veronese@pdfar3.dsfarm.unipd.it (F.M. Veronese).

many of its features while the main biological functions, such as enzymatic activity or receptor recognition, may be maintained. PEG conjugation masks the protein's surface and increases the molecular size of the polypeptide, thus reducing its renal ultrafiltration, preventing the approach of antibodies or antigen processing cells and reducing the degradation by proteolytic enzymes. Finally, PEG conveys to molecules its physico-chemical properties and therefore modifies also biodistribution and solubility of peptide and non-peptide drugs. PEGylation is therefore of interest in applied biotechnology because, upon modification, enzymes may become soluble and active in organic solvent. This property opens new techniques in biocatalysis and in pharmaceutical technology where many insoluble drugs are solubilized by PEG conjugation and thus more easily administered [1].

Several authors reviewed different aspects of PEGylation with emphasis on the polypeptide and protein conjugation and also on the modification of liposomes, particles and non-peptide drugs [2–9]. This article provides a general survey of the PEGylation technology, with special emphasis being given to contributions for the solution of specific problems encountered during PEG conjugation.

2. Problems in PEGylation related to the starting PEG

The unusual properties that PEG conveys to proteins such as solubility, biocompatibility, protein rejection, and modification of pharmacokinetics, to name only a few, are the “reason d'être” of this technology. However, PEG, being a synthetic polymer, is polydispersed and, even in the best of cases, a polydispersity value (M_w/M_n) ranging approximately from 1.01 for low molecular weight oligomer (3–5 kDa), to 1.2 for high molecular weight (20 kDa) may be expected. This polydispersity is a negative property since it is reflected in polydispersity of the conjugates. At present, the best way for testing the M_w/M_n ratio is MALDI mass spectroscopy (Fig. 1), but GPC with a refractive index detector is also useful.

The monomethoxylated form of PEG is generally used in protein conjugation, since its monofunctionality yields cleaner chemistry. However, a certain amount of PEG diol is always present, in the range of 1–10%, depending upon the molecular weight (lower for low-mass PEG). The level of polydispersity may vary greatly among the different suppliers and, as a general rule, we would recommend to evaluate product homogeneity before its application by using one of the techniques mentioned above. In the case of GPC a peak or shoulder in front of the main elution peak is observed, corresponding to the diol by-product, which is characterised by a molecular weight approximately double that of the mono-functional methoxy-PEG. High diol concentration will yield unwanted cross-linked conjugates.

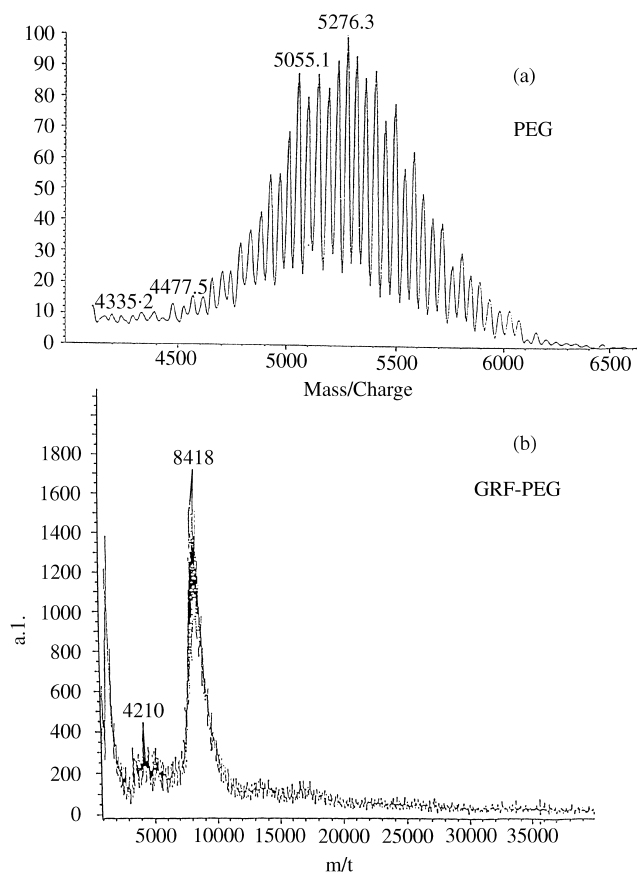


Fig. 1. PEG polydispersity: (a) polydispersity of PEG of 5 kDa as assessed by MALDI mass spectrometry and (b) mass spectrum of the conjugate of this PEG species with the growth hormone releasing factor, a peptide of 3358 kDa. A broad peak centred at 8418, corresponding to the sum of PEG and peptide masses, is obtained. The polydispersity of PEG is reflected into the polydispersity of the conjugate.

PEG molecular weight has a great influence on the biological properties of conjugates. The data reported in Table. 1 on the residence time of SOD and SOD conjugates of different mass demonstrate this point.

3. Activated PEGs for amino group conjugation

Since most applications of PEG conjugation involve labile molecules, the coupling reaction generally requires mild chemical conditions. In case of polypeptides, the most common reactive groups involved in coupling are nucleophiles with the following decreasing rank order of reactivity: thiol, alpha amino group, epsilon amino group, carboxylate, hydroxylate. However, this order is not absolute, since it depends also on the reaction pH, furthermore other residues may react in special conditions, as the imidazole group of histidine.

The thiol group is rarely present in proteins, furthermore it is often involved in active sites. The carboxylic groups cannot be easily activated without having

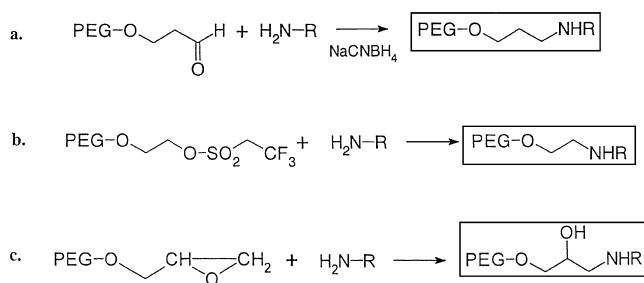


Fig. 2. *Alkylating PEGs*: (a) PEG aldehyde: Best reaction takes place with α - and ϵ -amino groups in protein at neutral or mild alkaline pHs, while good selectivity for the α -amino terminal aminoacid at pH, 5–6, was found. (b) Tresylated-PEG: Best reaction takes place with amines around pH 8 and low temperature. The positive amino charge is maintained. The reaction rate is depending upon the properties of the peptide, but doubts remain on the structure of the reaction products. (c) PEG epoxide: Reaction at high pH, 8.5–9.5, is preferred for amino conjugation, although binding to hydroxyl, imidazole and thiol groups also may take place.

reaction with the protein amino groups, to yield intra or inter molecular cross linking. Therefore, amino groups, namely the alpha amino or the epsilon amino of lysine, are the usual sites of PEG linking. As a consequence, the problem is to transform the hydroxyl terminal group of PEG to an activated one. In the literature more than a dozen of such procedures are described and for a detailed discussion, the reader is referred to excellent reviews and, among these, the ones by Schact or Zalipsky [4,5,7].

For a general survey, these activated PEGs may be divided into two important classes.

3.1. Alkylating PEGs

These products do not modify the charge of amino residues. Among these stands *PEG-aldehyde* that gives a permanent linkage after Schiff base formation followed by cyanoborohydride reduction (Fig. 2a) [10]. This is a convenient way for conjugation when the amino positive charge is critical for the retention of biological activity. However, the reaction rate for the Schiff base formation is relatively low, sometimes up to a day is necessary to reach the completion, with consequent inactivation of labile molecules. The reaction pH is critical for selectivity, α -amino terminal modification can be achieved around pH 5 [11]. *PEG-tresyl chloride* activation is an alternative method to maintain positive charge [12] (Fig. 2b). However, the chemistry of conjugation and the conjugation products are not unique and well defined [12–14]. The use of *epoxy PEG* has been used, but again, as with PEG-aldehyde, the reactivity is low and furthermore the specificity is not certain since hydroxy groups may also react (Fig. 2c).

3.2. Acylating PEGs

Most of these are *hydroxysuccinimidyl esters* (–OSu) of carboxylated PEGs (Fig. 3a). It is important to know

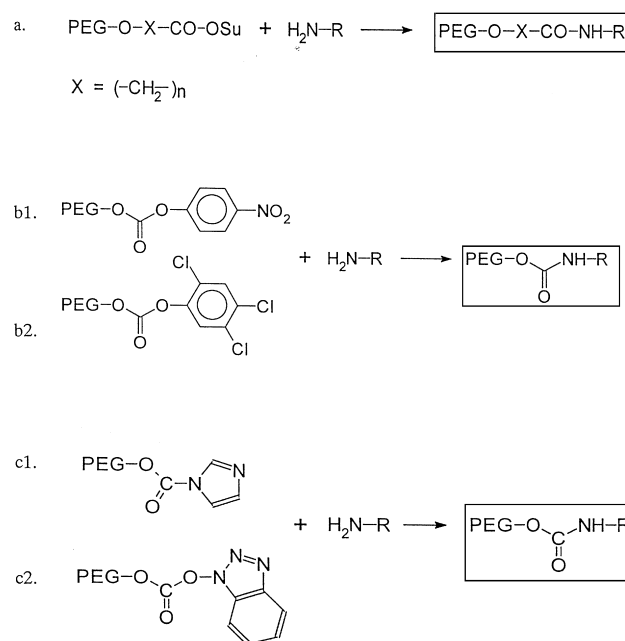


Fig. 3. *Acylating PEGs*: (a) Activated alkyl acids: These compounds are highly reactive towards amino groups, but the reaction rate may change significantly from the X structure. (b) PEG-*p*-nitrophenylcarbonate (b1) and PEG-trichlorophenylcarbonate (b2): The reaction rate is much slower of the OSu activated carboxylate-PEG. Carbamate derivatives are obtained. (c) PEG-oxycarbonylimidazole (c1) and PEG-benzotriazole carbonate (c2): Both reagents yield carbamate linkages with amino groups, but with different reaction rates.

that the distance between the active ester (–COOSu) and the last PEG ether can vary in different available products, by up to four methylene units, and this has profound influence on the reaction towards amino groups as well towards water. As an example, the $t^{1/2}$ hydrolysis rate of PEG–O–CH₂–CH₂–CH₂–COOSu is 23 h, while that of PEG–O–CH₂–COOSu is 0.75 h [15].

In another acylating chemistry the terminal PEG hydroxy group is activated by chloroformates or carbonylimidazole. We have special experience with the first ones, namely with *PEG-p-nitrophenylcarbonate* or *PEG-trichlorophenylcarbonate* [16] (Fig. 3b). These reagents exhibit lower reaction rate than the –OSu activated PEGs and this property allows exploitation of different reactivity among the amino groups of the macromolecule by stopping the reaction at the desired time of modification. In this way some selectivity in the conjugation based on amino group availability can be achieved. Furthermore, reactions in aqueous-organic solvents are feasible. There is an interesting difference between these two carbonate-activated PEGs: the first one has a pale yellow colour, whose intensity increases with the conjugation reaction for the formation of phenol as a leaving group, while the second is a colorless product. The extent of amino groups modification can be followed in the case of the second product by colorimetric analysis to titrate the residual amino group directly on aliquots of the reaction

mixture without the need for removing excess reagent or reaction by-products [17]. If the fluorimetric method, based on the reaction of fluorescamine, is used, the degree of amine modification can be evaluated using a much lower amount of product [18]. A urethane linkage with amino groups is also obtained with other electrophilically activated PEGs. Among these are *PEG-oxycarbonylimidazole* [19] and *PEG-benzotriazole carbonate* [20] (Fig. 3c). The first one is characterised by a lower reaction rate as compared to chloroformates, while the second one is very reactive, although less so than the succinimidyl activated carboxyl PEGs.

4. PEG with amino acid arm

The presence of an amino acid or peptide arm between PEG and the attached macromolecule gives several

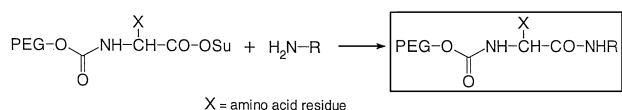


Fig. 4. *PEG amino acids*: A variety of properties may be conveyed to the PEG-conjugate using different amino acids or peptides. Examples may be: Nle for analytical purposes, ^{14}C or tritium labelled Gly for pharmacokinetic studies, Trp for fluorescence labelling, Lys for branching, Met-Nle or Met- β Ala for PEG removal by BrCN treatment.

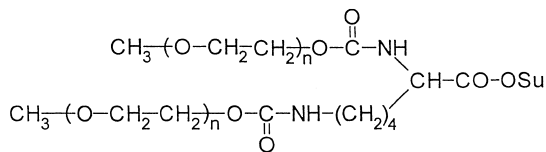


Fig. 5. *Branched PEG (PEG2)*: Lysine is the amino acid spacer between two PEG molecules and the activated carboxylate. This PEG offers the advantages of higher protection of protein surface for single point of conjugation, as compared to the linear form. Generally, it conveys to its products higher retention in blood, lower immunogenicity and decreased enzyme inactivation during the conjugation.

advantages due to the variability of properties that may be introduced using a suitable amino acid or peptide. For the convenience of synthesis the amino acid or peptide can be linked through its amino group by urethane linkage to PEG, while the carboxyl group, properly activated, is bound to the amine of the macromolecule [21] (Fig. 4). Among these arms, norleucine is of interest for the advantage in analysis of PEGylated proteins [22]. A second is the so-called branched PEG or PEG2 [23]. This PEG derivative is characterized by two linear PEG chains linked together through two functions of a trifunctional spacer, while the third function is used to bind the protein. Lysine is the tri-functional amino acid spacer and the two PEG chains are linked to its alpha and epsilon amino groups while the carboxylic group is activated as $-\text{OSu}$ for protein binding (Fig. 5). This PEG has the advantage of a lower inactivation of the enzymes during conjugation (see below). Furthermore, and more important, for its “umbrella-like” structure it is more effective also in protecting proteins from proteolysis, in the approach of antibodies, and in reducing immunogenicity [24,25] (Fig. 6).

5. Characterization of PEG conjugates

The observations reported above on the degree of modification obtained by the colorimetric titration of the non-conjugated amino groups, opens the general problem of the precise evaluation of the number of polymer chains bound to peptide or proteins. PEG is transparent and non-fluorescent and therefore non-detectable by itself, furthermore it does not release products easy to quantify upon hydrolysis. The colorimetric reaction of PEG with iodine can be used for its direct evaluation [26] but it possess low-sensitivity relative to the high values of the blanks. The different retention time in GPC elution, shown by differently modified conjugates, was also suggested as a tool for the evaluation of the degree of

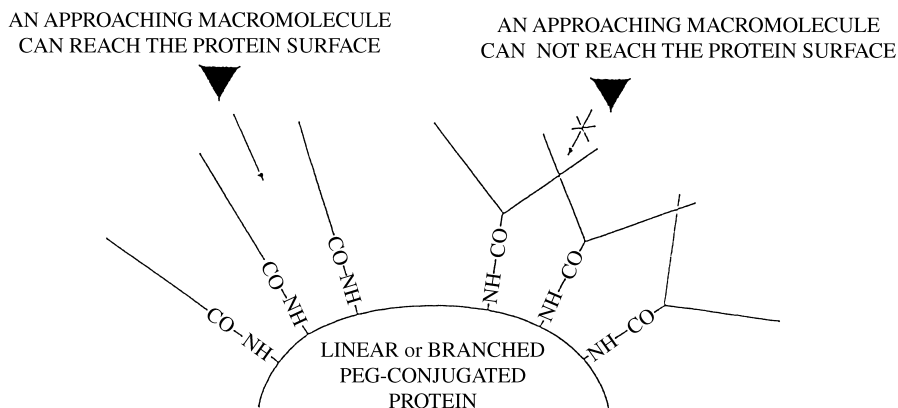


Fig. 6. The “umbrella-like” structure of branched PEG explains the higher capacity in rejecting approaching molecules or cells as compared to linear PEG of the same size.

polymer substitution. However, the elution time in GPC is not directly related to the increase in molecular weight due to the linked PEG, since the hydrodynamic volumes of PEG and the protein are very different, the first being higher by about two fold of that of a protein with the same molecular weight.

Usually, the number of PEG-bound chains is calculated indirectly from the decrease of unreacted amino groups, with respect to the total present before the reaction, by using the colorimetric or fluorimetric methods as reported above. However, these methods may not always give reliable results, due to the intrinsic variability of any colorimetric or fluorimetric determination and to the contemporaneous need for a separate evaluation of protein concentration.

As an alternative, MALDI mass spectrometry is now frequently employed because it yields a complete picture of the mixture of components of the conjugation reaction based on the mass (Fig. 7). However, in this case, the method is not quantitative, due to the different extraction

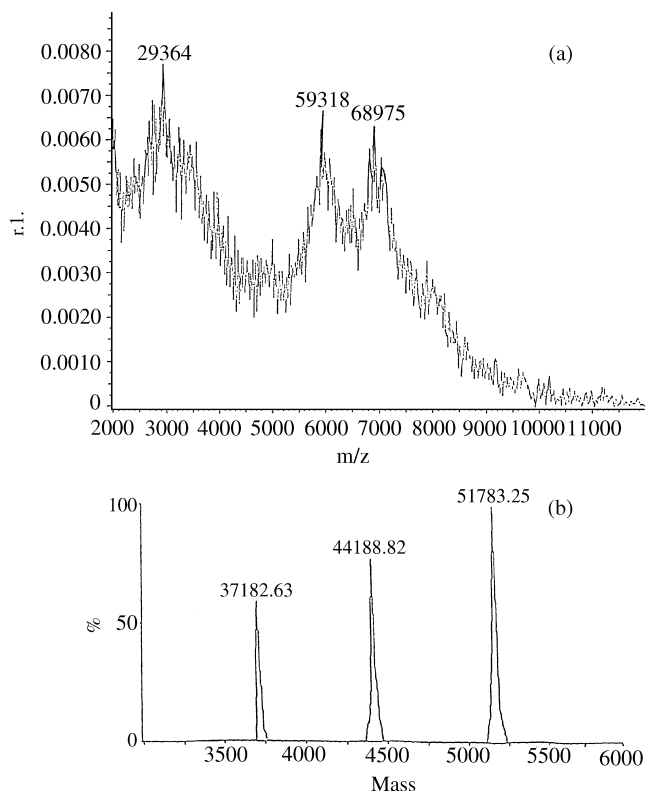


Fig. 7. (a) Maldi mass spectrometry of a subtilisin (MW 29 kDa) PEGylated with branched PEG 10 kDa. Two major peaks of equal area are present corresponding to subtilisine conjugated with 3 and 4 branched PEG molecules and a minor peak with 5 PEG molecules. The first large peak (centred at 29.3 kDa) refers to the conjugate with multiple charges. To note that the amount of the conjugates with 3 and 4 PEG chains, that appears equal in this spectrum, when evaluated by GPC chromatography was found to be largely different, a ratio of approximately 1 : 2. (b) Electrospray mass spectrometry of PEG-subtilisine after deconvolution. The presence of conjugates with 2, 3 and 5 PEG chains is shown (from Giacchini AM et al. [84]).

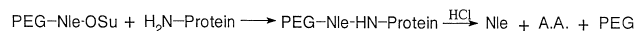


Fig. 8. *PEG-norleucine*: This PEG is useful for absolute evaluation of the number of PEG chains bound to a protein. This number is obtained from Nle by amino acid analysis after acid hydrolysis.

yield from the matrix of compounds of different weight. Electrospray mass spectrometry was also proposed for the evaluation of poly(ethylene glycol)–protein conjugates. Subtilisine and lipase and PEG 5000 Da were the first models successfully investigated by this technique [29]. The advantage of both these mass spectrometric methods resides in the fact that they can be used with any of the PEG coupling methods employed: acylating or alkylating, involving amino, thiol, carboxylic or arginine groups.

In order to overcome the problems connected with the evaluation of the degree of polypeptide modification, a procedure was devised that does not yield the pattern of modified products as mass spectrometry does (for this a preliminary chromatographic step would be necessary), but it gives the overall modification extent of the conjugation product [22]. The method exploits the use, as a spacer arm between PEG and protein, of the amino acid norleucine (which is absent in proteins) activated as –OSu at its carboxylic group. The PEG-Nle conjugated protein is hydrolysed by HCl and amino acid analysis is carried out according to a standard procedure: the number of bound PEG chains corresponds to the number of Nle. A stable amino acid is used as internal reference standard to calculate the ratio between Nle and protein (Fig. 8).

Capillary electrophoresis has also being reported for protein PEG-conjugate characterization. This method allows a wonderful separation of PEG-protein isomers that cannot be easily reached by HPLC or by ion-exchange chromatography [27,28]. Finally, SDS electrophoresis may also be of use but its success is limited to PEGylated peptides or low molecular weight proteins since the great hindrance of the conjugates interferes with the penetration inside the gel.

6. Unexpected reaction in PEGylation chemistry

To get a precise characterization of PEG conjugates more than a single analytical method must be employed. A typical example of this need comes from a recent study carried out in our laboratory for the modification of a genetic variant of epidermal growth factor (EGF) [30]. In this case, using the colorimetric procedure of Habeeb [17] to evaluate the number of bound polymer chains, we could not realise that PEG was bound to the peptide at the level of a tyrosine residue in addition to two amino groups.

This tyrosine conjugation, unexpected for a PEG activated as hydroxysuccinimidylester, could in fact be revealed only by MALDI mass spectrometry or by the

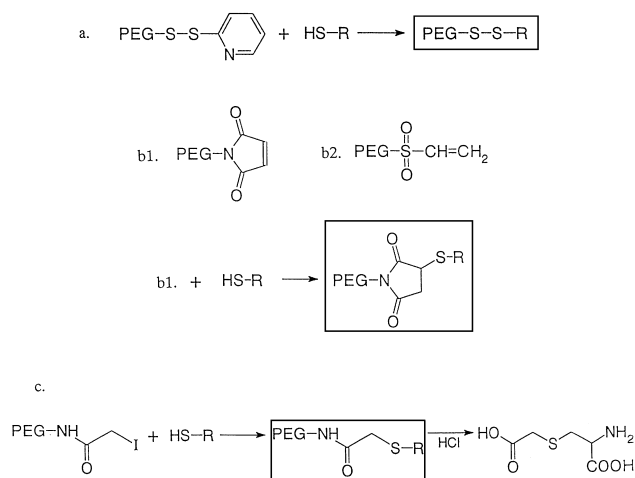


Fig. 9. Thiol reactive PEGs: (a) PEG-ortho-pyridyl-disulphide: The reaction conjugate with this PEG may be quantitatively reversed by thiol treatment. (b) PEG-maleimide (b1) and PEG-vinylsulphone(b2): The thiols give stable addition to the double bond of these PEGs. The reaction rate is pH dependent, best conditions being around 8. To note that the amine addition is not excluded specially increasing the pH or when organic cosolvents are present. (c) PEG-iodoacetamide: A stable thioether is formed in mild basic media. By strong acid hydrolysis carboxymethylcysteine (CMC) is quantitatively obtained from the cysteine derivative. CMC may be evaluated by standard amino acid analysis thus offering a method to assess the cysteine conjugation.

norleucine procedure of analysis described above, which showed an additional PEG chain. This extra chain could be removed from the conjugate by mild hydrolysis, as expected for an ester linkage. Tyrosine modification in the EGF molecule was probably related to the special amino acid arrangement in the sequence of this polypeptide, since it did not take place in the homologous human protein.

7. PEGylation at cysteine residue

Few proteins possess thiols suitable for PEG binding. However, this rare residue may be introduced at the desired position of the sequence by genetic engineering, a strategy that offers the conditions for site-directed PEGylation. This sequence-specific modification may take advantage of the few thiol reactive PEGs. Among these there is an activated disulphide, namely *PEG-ortho-pyridyl-disulphide* [31], that reacts with thiols yielding the more stable symmetric disulphide (Fig. 9a). A second reagent, *PEG-maleimide*, takes advantage of thiol addition to the activated double bond, known from many years in protein chemistry as the Michael reaction [32]; a new reagent, based on thiol addition to *PEG-vinylsulphone* double bond was devised in a joint collaboration between our laboratory and Prof. M. Harris in Huntsville [33] (Fig. 9b). Both of these last two reagents react with the sulphhydryl group only

at neutral or mild alkaline pH (7.5–8.5) while, when the pH is increased, the amino group addition may also take place, although with significantly lower kinetics. It may be interesting to note that this unwanted amino reaction is enhanced by the presence of an organic co-solvent in the reaction mixture [34]. *PEG-iodoacetamide* was also employed (Fig. 9c) as a well-known reaction in protein chemistry. This modification procedure presents the advantage that, by strong acid hydrolysis, the PEGylated cysteine gives rise to carboxymethylcysteine, a stable cysteine derivative that can be identified and quantified by standard amino acid analysis [35].

8. Arginine modification

Only few examples of polypeptide PEGylation at the level of arginine were reported so far, mainly in the patent literature, all based on the use of *PEG-1-3-dioxo compounds* (Fig. 10). The disadvantage of this method is related to the long reaction time needed for complete coupling that may be harmful to the protein stability, and to the non-specificity of its chemistry since other amino acids, histidine and lysine in particular, may also react [36,37].

9. Carboxyl group modification

Amino PEG may be used to PEGylate carboxyl groups, by the use of *N,N'*-dicyclohexylcarbodiimide or a water-soluble coupling agent as *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride. However, the procedure is successful only when amines are not present in the compound, as for instance in the case of non-peptide drugs. In peptides and proteins the risk of cross-linking is difficult to avoid.

An original strategy was devised by Zalipsky to specifically PEGylate carboxylic groups in proteins without cross-linking formation with amino groups: it takes advantage of the linkage of PEG to carboxyl groups only, by the use of *PEG-hydrazide* [38] (Fig. 11). The very low pK of the hydrazide group allows, in the presence of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, its binding to carboxyl groups in acid media (pH 4.5–5), where all the protein amino groups are protonated and not reactive. This condition assures that intra- or inter-molecular protein cross linking between carboxylates and a PEG amine takes place.

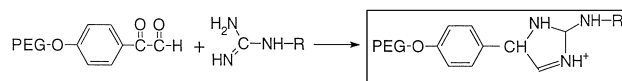


Fig. 10. PEG-phenylglyoxale: The reaction rate of this PEG with arginine is low and other amino acids are modified as well.

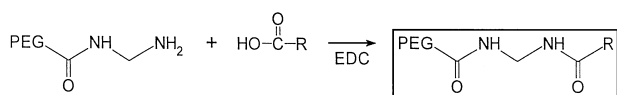


Fig. 11. *PEG-hydrazide*: At pH 4–5.5 only the conjugation between PEG-hydrazide and the carboxylic groups takes place, because under such pHs the protein or peptide amino groups are protonated and not available for binding.

10. Hydroxyl group conjugation

PEG-isocyanate is useful for hydroxyl group conjugation yielding a stable urethane linkage [39]. However, its reactivity may be best exploited for non-peptide moieties such as drugs or hydroxyl-containing matrices for chromatography and also to yield biocompatible surfaces. PEG-isocyanate is in fact highly reactive with amines also (Fig. 12).

11. Active site protection from PEGylation

The same mechanism that prevents the approach of proteolytic enzymes or antibodies to PEGylated protein can also reject a substrate from the protein active site. This mainly occurs with enzymes possessing high molecular weight substrates such as peptides, proteins, polysaccharides, and also in the receptor-binding of PEGylated molecules. In both cases this effect prevents or largely reduces the advantages of PEG conjugation technology. Methods that can be exploited under different circumstances were devised to overcome this problem. These are summarised as follows:

1. In a few cases, the problem was reduced by carrying out the conjugation in the presence of an active-site protecting agent, such as a substrate, an inhibitor, or other agent with specific affinity for the macromolecule. Protection of the binding site from PEG conjugation was observed to some extent [40]. However, the results, using this approach, are generally unsatisfactory because PEGylation may still occur in the surroundings of the protected site.

With this in mind, a procedure was devised, based on the use of an inhibitor (as a benzamidine derivative in case of serine protease) covalently linked to an insoluble resin (agarose) that, at proper pH and ionic strength binds reversibly, by affinity, to the enzyme and protects both the active site and its close surroundings from reacting with PEG. After the removal of the insoluble inhibitor by changing pH, the enzyme was found to maintain activity also towards high molecular weight substrates such as albumin in case of trypsin, or blood clots in the case of urokinase [41,42] (Fig. 13). In another case the activity of proteolytic enzymes was instead maintained by the use of very large polypeptide inhibi-

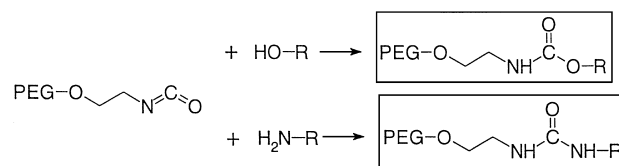


Fig. 12. *PEG-isocyanate*: This PEG is mostly used for PEGylation of polysaccharide gels or for surface modification. Attention must be paid to the presence of dimers.

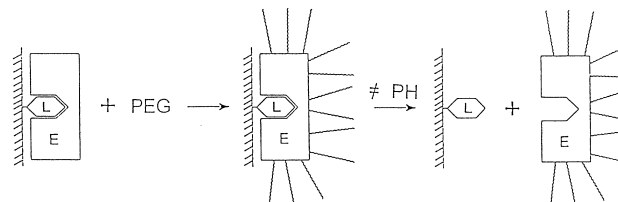


Fig. 13. A ligand, such as a substrate or inhibitor (L), linked to an insoluble matrix gives reversible binding with the enzyme and prevents the PEGylation around the binding site. The PEGylated macromolecule is released from the ligand by any condition that reverses the binding such as pH, salts, solvents.

tors (as kunitz inhibitor) added in solution to the conjugation mixture [43]. Also in this case the protecting agent was removed, at the end of conjugation reaction, by changing the pH to a value that minimizes non-covalent binding forces.

2. The problem of loss of biological activity during conjugation may be circumvented by exploiting a different structure in the polymer used for modification. In this regard, observations are of interest with some enzymes, uricase or asparaginase as typical examples. It was found that the activity loss of these enzymes was largely prevented by using a special form of PEG, called branched PEG or PEG2 (see above). Using this PEG, enzyme activity is preserved to a larger extent as compared to linear PEG, most probably because polymer approaching to the active site cleft is hindered by its bulky structure (Fig. 14). As in the case of uricase conjugation, the residual PEGylated enzyme activity is 32% of the starting one, if a branched PEG is employed for the conjugation, but 2.5% only if a linear PEG of the same mass is used [25].

The same mechanism limits also the sites of PEGylation in the polypeptide allowing a conjugation, in the case of the branched form, to the more accessible amino acid residues only. This is well demonstrated in α -interferon conjugation that, once modified with the linear polymer, gave rise to eleven positional isomers, whereas, with the branched polymer, only one or two lysine residues were sites of PEGylation [45–47].

3. In case of smaller macromolecules, such as peptide hormones or cytokines, activity loss may be prevented by using specific chemical strategies aiming to tailor PEGylation at the level of non-essential residues. This may be reached taking advantage of different residues reactivity due to solvent exposure or nucleophilicity.

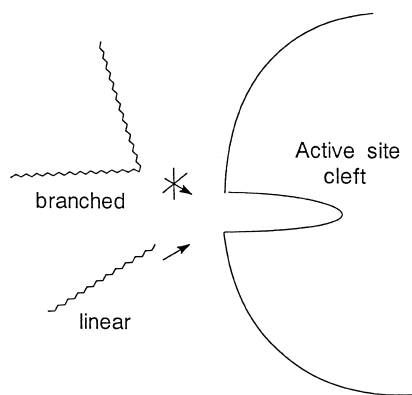


Fig. 14. The high steric hindrance of branched PEG may be advocated to explain the lower inactivation of enzymes as compared to linear PEG of the same size.

4. If nature does not offer the lucky situation reported above, another possibility is to carry out the modification in a stoichiometric deficiency of PEG to obtain isomers that can be separated later by chromatography. Each isolated isomer is assayed for biological activity so that the most promising one can be selected for further development. This strategy was followed in our laboratory with the 1–29 sequence of the growth hormone releasing factor that, having three reactive amino groups, could lead to seven potential conjugation products: three mono-PEGylated isomers, three di-PEGylated and one tri-PEGylated form. In this case, however, this number was drastically reduced because of a low reactivity of the alpha amino residue and, when the reaction was carried out in stoichiometric deficiency of PEG, two mono-PEGylated isomers only (in positions 12 and 21, respectively) were obtained as the main products. One PEG isomer proved to be the most biologically active, and a procedure for its convenient isolation was optimised [44]. A similar strategy has been used for the modification of alpha-interferon where eight mono-substituted isomers were obtained and, after purification to homogeneity, assayed for a structure activity relationship [45]. A further successful example of separation between mono and di-PEGylated products was reported in salmon calcitonin conjugation by PEG 12 kDa. In this case it could be demonstrated that the conjugated species were equally bioactive as the native peptide [48].

5. A more sophisticated strategy to reach specific PEGylation is based on a preliminary chemical reversible site-directed protection of the peptide to leave some groups free for PEG conjugation. This was the case of insulin modification since in alkaline conditions glycine A1 and lysine B29 were preferentially protected by BOC to PEGylate Phe B1 only [49–51]. Further example is the reversible protection of the alpha or the epsilon amino groups of a somatostatin analogue achieved carrying out the reaction at different pHs with a suitable reagent [52].

6. A quite different approach was instead followed by Campbell [53], who obtained GRF-PEGylated peptides during the synthetic step by using PEGylated amino acids.

12. PEG as flexible, hydrophilic moiety in bifunctional reagents

Bifunctional reagents are a major interest in biology or applied sciences, and a list of reagents with different chemical properties were described and reviewed [54]. The placing of a PEG chain between two reactive moieties extends the application of cross-linking reagents where hydrophilicity, flexibility and biocompatibility are needed. Furthermore, the variety of PEG length available in the market allows tailoring of the distance between the two reactive entities and cross-linking macromolecules when the reaction would be hampered by steric hindrance. New applications are in the preparation of supports for solid or liquid phase peptide synthesis [55,56], targetable polymeric drugs [57], linking mobile cofactors to apoenzymes [58], grafting on surfaces and conjugating fluorescence probes to macromolecules. For bifunctional PEG preparation a diol PEG of desired molecular length is the starting polymer, while the two terminal hydroxyls are activated with the same reactive group or with groups of different reactivity. In the last case, since amino and thiols are the residues most reactive in macromolecules, α -OSu-activated carboxylic groups on one side and vinyl sulphone, maleimide or the pyridyl disulfide on the other, are the bifunctional PEG preferred end groups. This choice allows carrying out the cross-linking in a sequential order thanks to the different reaction conditions of the two groups (Fig. 15).

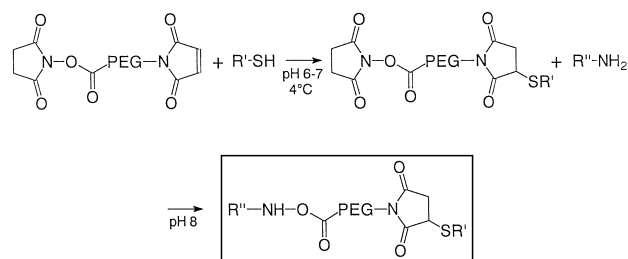


Fig. 15. α -OSu- ω -maleimide-PEG: This is a typical cross-linking reagent that may react in a sequential order with different groups in protein, taking advantage of different pH and temperatures. With this strategy two different macromolecules may be stoichiometrically linked together.

13. Enzyme PEGylation for solubilization in organic solvents

Enzymes are becoming new tools for specific bioconversions in organic synthesis. However, they suffer from

insolubility in most solvents where many compounds of pharmaceutical and cosmetic interest may be dissolved. Poly(ethylene glycol) chains bound to the enzymes overcome such limitation because they convey organic solvent solubility without impairing, in many instances, enzymatic activity [59]. For this purpose, enzyme PEGylation may be carried out with any of the above reported methods. Most used, however, is the one that takes advantage of trichlorotriazine (*PEG-dichlorotriazine*), since it is easy to prepare, gives stable products with both amino and thiol groups and its chemistry is well known [60]. The risk of toxicity of the intermediate of this reaction, that is of concern in therapeutic enzymes, does not hold for this application as well as the possibility of cross-linking. A branched form of PEG-triazine was also prepared that exploits two chlorides of trichlorotriazine for PEG binding while the third is used for enzyme conjugation [61] (Fig. 16).

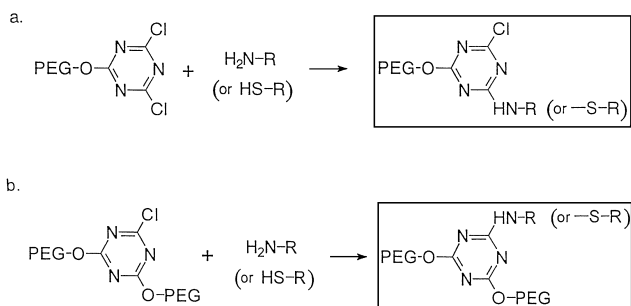


Fig. 16. *PEG-triazine*: Linear (a) or branched (b) PEG based on trichlorotriazine activating moiety. The linear form may yield cross-linked products for the presence of the remaining chloride in the triazine ring.

14. Reversible grafting of PEG to proteins or fatty acid

In several instances it may be useful to selectively remove PEG from its conjugates. Some strategies were reported that take advantage of the introduction of labile bonds between PEG and the conjugated moiety. One of these exploits the use of maleic acid between PEG and protein. A cleavage takes place in mild acid medium at room temperature thanks to the anchimeric assistance of the carboxylate, enhanced by the presence of a double bond, to the nearby amide bond [62] (Fig. 17). In two further reports cleavage is achieved by thiols: in the first

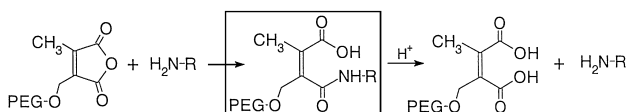


Fig. 17. *PEG-maleic-anhydride*: The PEGylation may be reversed by anchimeric assistance of the neighbouring free carboxyl function which is facilitated by the presence of the close double bond.

the conjugating moiety is an aliphatic disulphide, namely 3,3'- dithiodipropionate that is cleaved by 1,4-dithiothreitol treatment [63]; in the second the conjugating moiety is a thiophenyl group that releases PEG from distearoylphosphotidyl ethanolamine by mild thiol treatment [64].

15. Identification of the PEGylation site in peptide and protein

While with small peptides the exact location of the PEG chain can be easily identified, because the Edman degradation may be carried out on the conjugate, this is very difficult or impossible with larger conjugates. Note also that in the stepwise degradation of a conjugate the PEGylated site is identified by a missing amino acid. On the other hand, in proteins, Edman degradation may be carried out only after cleavage to smaller peptides. In this case, a problem resides in the difficulty to isolate and purify the individual products of the digestion, as often the polymer chain makes them equivalent in properties. Furthermore, the hindrance of the bound polymer chains may prevent the specific cleavage by proteolytic enzymes needed in sequence studies. For these reasons few investigations of sequence characterization in PEGylated proteins have so far been reported.

A successful exception concerns the characterization of a PEGylated α -interferon mixture, obtained in stoichiometric deficiency of PEG as reported previously, in which the monoPEGylated isomers were separated by ion-exchange chromatography and each peptide identified by Edman degradation [45]. In a more complex case, poly-PEGylated growth hormone was hydrolysed by trypsin and the site of modification identified by the indirect (and dubious) evidence based on the absence of the expected tryptic peptides as compared to the digestion of the native growth hormone [65]. Incomplete proteolysis is in fact expected at the level of PEG.

These difficulties may be greatly reduced by a recent method that takes advantage of the properties of two special PEGs with methionine in the peptide arm, PEG-Met-Nle or PEG-Met- β Ala [66] (Fig. 18). The presence

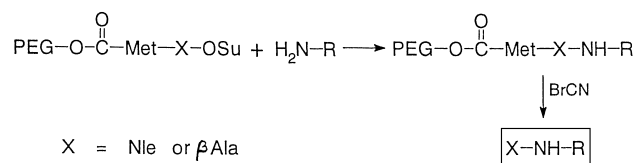


Fig. 18. *PEG-Met-Nle-OSu* and *PEG-Met- β Ala-OSu*: PEG protein conjugation, and PEG release by BrCN treatment, leaving Nle or β Ala as reporter groups bound to protein at the residues where the polymer was present.

of the methionine allows the removal of the polymer chains from the protein molecule by cyanogen bromide treatment following a reaction well known in protein chemistry. After cleavage at the level of methionine, nor-leucine in the first case, or β -alanine in the second, remains linked to the protein as a reporter group in the position of the PEG chains. The proteins, now devoid of the bulky polymer, may be more easily fractionated into the position isomers than the PEGylated form and the different components analyzed by any sequence procedure. The protein or the peptides obtained by digestion, with linked unnatural amino acid Nle or β -Ala, may also be analysed by MALDI or electrospray mass spectrometry to reveal the eventual increase in weight due to this new amino acid, or characterised by amino acid analysis after acid hydrolysis or sequenced by Edman degradation. The procedure described here was successfully tested in our laboratory using as models lysozyme, a decapeptide partial sequence of glucagone, and insulin with PEG linked in different positions [67,85].

16. New monofunctional polymers for protein conjugation

The basic reason for the success of methoxypoly(ethylene glycol) among other polymers resides in its monofunctionality. This prevents the formation of cross-linked conjugates that would take place with polyfunctional polymers as polysaccharides, most of polyacrylates or serum albumin, all reagents already proposed for conjugation [68–71].

To extend the potential of peptide and protein conjugation, new monofunctional oligomers were investigated that convey different properties to the conjugates. Among these are *poly(N-acryloylmorpholine)* (PACM) (a) and *poly(N-vinylpyrrolidone)* (PVP) (b) (Fig. 19). They are obtained by a special polymerization process that yields oligomers with the desired molecular weight (in the range of 3000–6000 Da) and with a single terminating functional group [72,73].

The studies carried out so far with these two polymers using superoxide dismutase, ribonuclease, uricase and

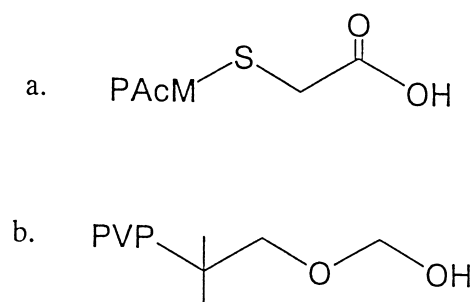


Fig. 19. Carboxyl (a) or hydroxyl (b) terminating *poly(N-acryloylmorpholine)* (PACM) and *poly(N-vinylpyrrolidone)* (PVP) as alternative polymers to PEG for protein or liposomes modification.

asparaginase as model proteins, demonstrated that the enzymes may be modified with retention of activity. Furthermore, it was found that the properties of the conjugates, organ targeting, blood residence time and immunogenicity are different from the ones obtained with PEG [74] (Table 1).

It is also worth noting that both the polymers were also successfully used to mask the liposome surface for obtaining new forms of “stealth liposomes” (see Table 2) [75]. This result is related to the great flexibility and hydrophilicity of PVP and PACM, requisites that are considered essential to convey stealth properties [76] (Table 3).

Table 1
Influence of PEG molecular mass on the clearance time in rats^a or mice^b. In each case three PEG chains were bound to SOD molecule

Protein ^c	Half-life
SOD	0.08 ^a
SOD-PEG 1900 Da	1.5 ^a
SOD-PEG 5000 Da	11.0 ^a
SOD-PEG 72000 Da	36.0 ^b

^cAn equal number of PEG chains were bound to SOD molecule.

Table 2

Comparison between degree of modification and residual enzymatic activity of uricase and superoxide dismutase after linear PEG, PACM and PVP modification^a

	PEG		PACM		PVP	
	Modification degree %	Activity %	Modification degree %	Activity %	Modification degree %	Activity %
Uricase ^b	44	40	18	24		
SOD	70 ^c	65 ^c			37 ^b	80 ^b

^aThe modification was carried out with a 5 KDa PEG and 6 KDa PACM or PVP.

^bUricase modification was carried out in the presence of uric acid as active site protection (38).

^cData from (65).

Table 3

Half life in mouse circulation of “stealth” liposomes obtained with linear PEG, branched PEG, PAcM and PVP^a

Liposomes ^b		Half life (min)
3 mol%	PEG/PE	80
”	PEG2/PE	140
”	PAcM/PE	40
”	PVP/PE	90
7 mol%	PEG/PE	230
”	PEG2/PE	230
”	PAcM/PE	130
”	PEV/PE	170
Without any polymer		10

^aThe polymer molecular masses were: PEG 5 KDa, PEG2 10 KDa, (2 × 5 KDa), PAcM and PVP 6 KDa.

^bThe polymer content (3 mol % or 7 mol %) is calculated as a fraction of PE (phosphatidylethanolamine) residue.

17. Sources of PEG

Fluka Chemie AG (Germany) is a source of PEGs of different molecular weights but the quality is not so sure. Polymer Laboratories Ltd (England) supplies ultra-pure PEG or methoxy PEG of low polydispersity to use as standards and also, on demand, small quantities of PEG or functionalized PEG for R & D purposes. Shearwater Polymers Inc. (Huntsville AL, USA) is a company specialized on PEG that supplies a great variety of products activated towards any amino acid residues as well as heterofunctional PEGs and PEG conjugates also.

18. Conclusions

The investigations reviewed here demonstrate how rich in research potential is this recent field of research. In fact, the search for new solutions to still unsolved problems, such as organ-specific targeting of conjugates, or the discovering linear or branched polymers with original structure, may still fascinate the chemist. Furthermore the coming of new PEGylation tools such as dendrimer PEG will further develop the biological and therapeutic applications that, began in the 1970s with the pioneering albumin and catalase PEGylation studies by Davis and Abouchowski [77,78], are now expanding to the field of peptide and non-peptide drugs [79], to immunology [80,81], diagnostics and biocatalysis [82,83].

Acknowledgements

The author would like to thank the CNR (Target Project on Biotechnology) project for financial support. Prof. Milton J. Harris is also acknowledged for reading the manuscript.

References

- [1] Herman S, Hooftman G, Schacht E. Poly(ethylene glycol) with reactive endgroups. I. Modification of proteins. *J Bioactive Compatible Polym* 1995;10:145–87.
- [2] Nucci ML, Shorr R, Abuchowski A. The therapeutic value of poly(ethylene glycol)-modified proteins. *Adv Drug Delivery Rev* 1991;6:133–51.
- [3] Dreborg S, Akerblom EB. Immunotherapy with monomethoxy polyethylene glycol modified allergens. *Critical Rev Ther Drug Carrier Syst* 1990;6:315–65.
- [4] Hooftman G, Herman S, Schacht E. Poly(ethylene glycol)s with reactive endgroups. II. Practical consideration for the preparation of protein-PEG conjugates. *J Bioactive Compatible Polym* 1996;11:135–59.
- [5] Delgado C, Francis GE, Derek FF. The uses and properties of PEG-linked proteins. *Critical Rev Ther Drug Carrier Syst* 1992;9:249–304.
- [6] Katre NV. The conjugation of proteins with polyethylene glycol and other polymers. Altering properties of proteins to enhance their therapeutic potential. *Adv Drug Delivery Rev* 1993; 10:91–114.
- [7] Zalipsky S. Chemistry of polyethylene glycol conjugates with biologically active molecules. *Adv Drug Delivery Rev* 1995;16:157–82.
- [8] Monfardini C, Veronese FM. Stabilization of substances in the circulation. *Bioconjugate Chem* 1998;9:418–50.
- [9] Veronese FM, Morpurgo M. Bioconjugation in pharmaceutical chemistry. *II Farmaco* 1999;54:497–516.
- [10] Harris, JM, Herati, RM. Preparation and use of polyethylene glycol propionaldehyde. US Patent 5,252,714.
- [11] Kinstler OB, Brems DN, Lauren SL, Paige AG, Hamburger JB, Treuheit MJ. Characterization and stability of N-terminally PEGylated rhG-CSF. *Pharmaceut Res* 1996;13:996–1002.
- [12] Delgado C, Patel JN, Francis GE, Fisher G. 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. *Biotechnol Appl Biochem* 1990;12:119–28.
- [13] Gais HJ, Ruppert S. Modification and immobilization with poly(ethylene glycol) tresylates and polysaccharides tresylates: evidence suggesting a revision of the coupling mechanism and the structure of the polymer linkage. *Tetrahedron Lett* 1995;36: 3837–8.
- [14] Sperinde JJ, Martens BD, Griffiter LG. Tresyl mediated synthesis: kinetics of competing coupling and hydrolysis reaction as a function of pH, temperature and steric factors. *Bioconjugate Chem* 1999;10:213–20.
- [15] Harris JM, Guo L, Fang ZH, Morpurgo M. PEG-protein tethering for pharmaceutical applications. Seventh International Symposium on Recent Advances in Drug Delivery, Salt Lake City, February 27, March 2 1995.
- [16] Veronese FM, Largajolli R, Boccù E, Benassi CA, Schiavon O. Activation of monomethoxy poly(ethylene glycol) by phenylchloroformate and modification of ribonuclease and superoxide dismutase. *Appl Biochem Biotechnol* 1985;11:141–52.
- [17] Habeeb AFSA. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal Biochem* 1966;14:328–36.
- [18] Stocks SJ, Jones AJ, Ramey CW, Brooks DE. A fluorometric assay of the degree of modification of protein primary amines with polyethylene glycol. *Anal Biochem* 1986;154:232.
- [19] Beauchamp CO, Gonias SL, Menapace DP, Pizzo SV. A new procedure for the synthesis of polyethylene glycol-protein adducts, effects on function, receptor recognition and clearance of superoxide dismutase, lactoferrin and α_2 -macroglobulin. *Anal Biochem* 1983;131:25–33.

- [20] Dolence EK, Hu C, Tsang R, Sanders CG, Osaki S. Electrophilic polyethylene oxides for the modification of polysaccharides, polypeptides (proteins) and surfaces. US Patent 5,650,234.
- [21] Sartore L, Caliceti P, Schiavon O, Veronese FM. Enzyme modification by MPEG with amino acid or peptide as spacer arm. *Appl Biochem Biotech* 1991;27:55–63.
- [22] Sartore L, Caliceti P, Schiavon O, Monfardini C, Veronese FM. Accurate evaluation method of the polymer content in monomethoxy poly(ethylene glycol) modified proteins based on amino acid analysis. *Appl Biochem Biotechnol* 1991;31:213–22.
- [23] Monfardini C, Schiavon O, Caliceti P, Morpurgo M, Harris JM, Veronese FM. A branched monomethoxypoly(ethylene glycol) for protein modification. *Bioconjugate Chem* 1995;6:62–9.
- [24] Veronese FM, Monfardini C, Caliceti P, Schiavon O, Scrawen MD, Beer D. Improvement of pharmacokinetic, immunological and stability properties of asparaginase by conjugation to linear and branched monomethoxypoly(ethylene glycol). *J Control Release* 1996;40:199–209.
- [25] Veronese FM, Caliceti P, Schiavon O. Branched and linear poly(ethylene glycol): influence of the polymer structure on enzymological, pharmacokinetic and immunological properties of protein conjugates. *J Bioactive Compatible Polym* 1997;12:196–207.
- [26] Sims GEC, Snape TJ. A method for the estimation of polyethylene glycol in plasma protein fraction. *Anal Biochem* 1980;107:60–3.
- [27] Bullock J, Chowdhury S, Johnston D. Characterization of poly(ethylene glycol) modified superoxide dismutase: comparison of capillary electrophoresis and matrix assisted laser desorption/ionization mass spectrometry. *Anal Chem* 1996;68:3258–64.
- [28] Roberts MJ, Harris JM. Attachment of degradable poly(ethylene glycol) to protein has the potential to increase therapeutic efficacy. *J Pharm Sci* 1998;87:1440–5.
- [29] Giacchini AM, Carrea G, Secundo F, Baraldini M, Roda A. Electrospray mass spectrometric analysis of poly(ethylene glycol) — protein conjugates. *Rapid Comm Mass Spectrometry* 1997;11:1219–22.
- [30] Orsatti L, Veronese FM. An unusual coupling of poly(ethylene glycol) to tyrosine residues in epidermal growth factor. *J Bioactive Compatible Polym* 1999;14:429–36.
- [31] Woghiren C, Sharma B, Stein S. Protected thiol-polyethylene glycol: a new activated polymer for reversible protein modification. *Bioconjugate Chem* 1993;4:314.
- [32] Ishii Y, Lehrer SS. Effects of the state of the succinimido-ring on the fluorescence and structural properties of pyrene maleimide-labeled $\alpha\alpha$ tropomyosin. *Biophys J* 1986;50:75–80.
- [33] Morpurgo M, Schiavon O, Caliceti P, Veronese FM. Covalent modification of mushroom tyrosinase with different amphiphilic polymers for pharmaceutical and biocatalysis applications. *Appl Biochem Biotechnol* 1996;56:59–72.
- [34] Veronese FM. Personal communication.
- [35] Gard FRN. Carboxymethylation. *Methods Enzymol* 1972;B25:424–49.
- [36] Yankeelov Jr AJA. Modification of arginines by diketones. *Methods Enzymol* 1972;B25:566–84.
- [37] Akihito S, Maeda H, Kai Y, Ono K. Polyethylene glycol derivatives, modified peptides and production thereof. E.P. 0.340.741.
- [38] Zalipki S, Menon-Rudolph S. Hydrazide derivatives of poly(ethylene glycol) and their bioconjugates. Poly(ethylene glycol) chemistry and biological applications. *ACS Symp Ser* 1997;680:318–41.
- [39] Greenwald RB, Pendri A, Bolikal D. Highly soluble taxal derivatives: 7-polyethylene glycol carbamates and carbonates. *J Org Chem* 1995;60:331–6.
- [40] Schiavon O, Caliceti P, Veronese FM. Therapeutic proteins: a comparison of chemical and biological properties of uricase conjugated to linear and branched poly(ethylene glycol) or poly(N-acryloylmorpholine). *II Farmaco* 2000;55:264–9.
- [41] Caliceti P, Schiavon O, Sartore L, Monfardini C, Veronese FM. Active site protection of proteolytic enzymes by poly(ethylene glycol) surface modification. *J Bioactive Compatible Polym* 1993;8:41–50.
- [42] Caliceti P, Morpurgo M, Schiavon O, Monfardini C, Veronese FM. Preservation of thrombolytic activity of urokinase modified with monomethoxypoly(ethylene glycol). *J Bioactive Compatible Polym* 1994;9:252–66.
- [43] Huber R, Kukle D, Rode D, Schwager P, Baltels K, Deisenhofer J, Steigemann L. Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. II. Crystallographic refinement at 1.9 Å resolution. *J Mol Biol* 1974;89:73–101.
- [44] Caliceti P, Schiavon O, Veronese FM. Design, and characterization of new polymer conjugates of GHRH. *Acta Technologica Legis Medicamenti* 1999;10:86. and E.P. 97.12.1264.
- [45] Monkars SP, Spence C, Porter JE, Palleroni A, Nalin C, Rosen P, Bailon P. Isolation of positional isomers of monopoly(ethylene glycol)ylated interferon-2 α and the determination of their biochemical and biological characteristics. In: Harris JM, Zalipsky S, Poly(ethylene glycol) Chemistry and Biological Applications, *ACS Symposium Series*, 1997;680: 207–16.
- [46] Harris JM. Pre-clinical and clinical development of PEG-alpha-interferon treatment of hepatitis-C. Sixth Europ Symp Controll Drug Del 12–14 April 2000.
- [47] Baillon PS, Pallerani AV. Interferon conjugates. E.P. 0809996A2.
- [48] Lee KC, Tak KK, Park MO, Lee JT, Woo BH, Yoo SD, Lee HS, Dlluca PP. Preparation and characterization of polyethylene glycol modified salmon calcitonins. *Pharm Develop Technol* 1999;4:269–75.
- [49] Caliceti P, Veronese FM. Improvement of the physicochemical and biopharmaceutical properties of insulin by poly(ethylene glycol) conjugation. *STP Pharma Sci* 1999;9:107–13.
- [50] Uchio T, Baudyš M, Liu F, Song SC, Kim SW. Site-specific insulin conjugates with enhanced stability and extended action profile. *Adv Drug Delivery Rev* 1999;35:289–306.
- [51] Hinds K, Koh JJ, Joss L, Liu F, Baudyš M, Kim SW. Synthesis and characterization of poly(ethylene glycol)-insulin conjugates. *Bioconjugate Chem* 2000;11:195–201.
- [52] Veronese FM. personal communication.
- [53] Campbell RM, Heimer EP, Ahmad M, Heisenbeis HG, Lambas TJ, Lee Y, Miller RW, Stricker PR, Felix AM. PEGylated peptides. *J Peptide Res* 1997;49:527–37.
- [54] Hermanson GT. editor. *Bioconjugate techniques*. San Diego, CA: Academic Press, 1996.
- [55] Bonora CM, Toniolo C. Linear oligopeptides 6^a. Synthesis by the liquid phase method and characterization by amino acid analysis of two complete monodispersed homologous series derived from L-alanine and L-valine. *Makromol Chem* 1979;18:425.
- [56] Zalipsky S et al. In: Hruby VJ, Kopple KH, editors. *Peptides: structure and function*. Pierce Chem Co., Rockford IL, 1985, p. 257.
- [57] Zalipsky S, Barang G. Facile synthesis of α -hydroxy- ϵ -carboxymethylpolyethylene oxide. *J Bioactive Compatible Polym* 1990;5:227–31.
- [58] Nakamura A, Urabe I, Okada H. Anchimeric assistance in the intramolecular reaction of glucose-dehydrogenase-polyethylene glycol NAD conjugate. *J Biol Chem* 1986;261:16792.
- [59] Mabrouk PA. The use of poly(ethylene glycol) enzymes in non-aqueous enzymology. In: Harris JM, Zalipsky S, editors. *Poly(ethylene glycol) Chemistry and Biological Applications*. Washington, DC: ACS, 1997. p. 118–33.
- [60] Mabrouk PA. The use of non aqueous media to probe biochemically significant enzyme intermediates: the generation and stabilization of horseradish peroxidase compound II in neat benzene solution at room temperature. *J Am Chem Soc* 1995;117:2141–6.
- [61] Matsushima A, Nishimura H, Ashihara Y, Yakata Y, Inara Y. Modification of *E. Coli* asparaginase with 2,4-bis(omethoxypolyethylene glycol)-6-chloro-s-triazine (activated PEG2); disappearance of binding ability towards anti-serum and retention enzymatic activity. *Chem Lett* 1980:773–6.

- [62] German AJ, Kalindjian SB. The preparation and properties of novel reversible polymer-protein conjugates: 2- ω -methoxy-polyethylene(5000)glycomethylene-3-methylmaleyl conjugates of plasminogen activators. *FEBS Lett* 1987;223:361–5.
- [63] Kirpotin D, Hong K, Mullah N, Papahodiopulos D, Zalipsky S. Liposomes with detachable polymer coating: destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface grafted poly(ethylene glycol). *FEBS Lett* 1996;388:115–8.
- [64] Zalipsky S, Qazen M, Walker JA, Mullah N, Quinn YP, Huang SK. New detachable poly(ethylene glycol) conjugates: cysteine cleavable lipopolymers regenerating natural phospholipid, diocyl phosphotidylethanolamine. *Bioconjugate Chem* 1999;10:703–7.
- [65] Clark R, Olson K, Fuh G, Marian M, Mortensen D, Teshima G, Chang S, Chu H, Mukku V, Canova-Davis E, Somers T, Cronin M, Winkler M, Wells JA. Long-acting growth hormones produced by conjugation with poly(ethylene glycol). *J Biol Chem* 1996;271:21969–77.
- [66] Veronese FM, Saccà B, Schiavon O, Caliceti P, Orsatti L, Orsolini P. PEG peptide and protein drug delivery: a procedure to identify the pegylation site. *Proc Int Symp Control Rel Bioact Mater CRS* 1999; 26: 106–7. and E.P. 98.12.3307.
- [67] Veronese FM, Saccà B, Polverino de Lauro P, Sergi M, Caliceti P, Schiavon O. New PEGs for peptide and protein modification, suitable for identification of the PEGylation site. *Bioconjugate Chem*, submitted for publication.
- [68] Marshall JJ, Humpreys JD, Abramson SL. Attachment of carbohydrate to enzymes increases their circulation lifetime. *FEBS Lett* 1977;83:249–52.
- [69] Wileman T, Bennet M, Lilleyan J. Potential use of an asparaginase-dextran conjugate in acute lymphoblastic leukemia. *J Pharm Pharmacol* 1983;35:762–5.
- [70] Von Spect BW, Seifeld H, Brendel W. Poly-N-vinylpyrrolidone as a soluble carrier of proteins. *Seyler's Z Physiol Chem* 1973;354:1659–60.
- [71] Wong K, Cleland LG, Poznansky MJ. Enhanced antiinflammatory effect and reduced immunogenicity of bovine liver superoxide dismutase by conjugation with homologous albumin. *Agent Action* 1980;10:231–9.
- [72] Ranucci E, Spagnoli G, Sartore L, Ferruti P, Caliceti P, Schiavon O, Veronese FM. Synthesis and molecular weight characterization of low molecular weight end-functionalized poly(4-(1-oxo-2-propenyl)morpholine). *Macromolecular Chem Phys* 1994;195:9–79.
- [73] Caliceti P, Schiavon O, Morpurgo M, Veronese FM, Sartore L, Ranucci E, Ferruti P. Physico chemical and biological properties of monofunctional hydroxy terminating poly-(N-vinylpyrrolidone) conjugated superoxide dismutase. *J Bioactive Compatible Polym* 1995;10:103–20.
- [74] Caliceti P, Schiavon O, Veronese FM. Biopharmaceutical properties of uricase conjugated to neutral and amphiphilic polymers. *Bioconjugate Chem* 1999;10:638–46.
- [75] Torchilin VP, Trubetskoy VS, Whiteman KR, Caliceti P, Ferruti P, Veronese FM. New synthetic amphiphilic polymers for steric protection of liposomes in vivo. *J Pharm Sci* 1995;84:1049–53.
- [76] Torchilin VP, Omelyanenko VG, Papisov IM, Bagdanov AA Jr, Herron JN, Gentry CA. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer coated liposome longevity. *Biochim Biophys Acta* 1994;1195:11–20.
- [77] Abuchowski A, van Es T, Palczuk NC, Davis FF. Alteration of immunological properties of bovine serum albumin by covalent attachment of poly(ethylene glycol). *J Biol Chem* 1977;252:3578–81.
- [78] Abuchowski A, McCoy JR, Palczuk NC, van Es T, Davis FF. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J Biol Chem* 1977;252:3582–6.
- [79] Greenwald RB. Drug delivery systems: anticancer prodrugs and their polymeric conjugates. *Exp Opin Ther Patents* 1997;7:601–9.
- [80] Sehon AH. Suppression of antibody responses by conjugates of antigens and monomethoxypoly(ethylene glycol). *Adv Drug Delivery Rev* 1991;6:203–17.
- [81] Dreborg S, Akerblom EB. Immuno-therapy with monomethoxypolyethylene glycol modified allergens. *Critical Rev Ther Drug Carrier Systems* 1990;6:315–65.
- [82] Okahata Y, Mori T. Lipid-coated enzymes as efficient catalysts in organic media. *Tibtech* 1997;15:50–4.
- [83] Inada Y, Furukawa M, Sasaki H, Kodera Y, Hiroto M, Nishimura H, Matsushima A. Biomedical and biotechnological applications of PEG- and EG-modified proteins. *Tibtech* 1995;13:86–91.
- [84] Gioacchini AM, Carrea G, Secundo F, Baraldini M, Roda A. Electrospray mass spectrometric analysis of poly(ethylene glycol)-protein conjugates. *Rapid Commun Mass Spectrom* 1997;11:1219–22.
- [85] Veronese FM, Saccà B, Schiavon O, Caliceti P, Orsatti L, Orschin P. PEG peptide protein pegylation: a procedure to identify the pegylation site. *Proc Int Symp Control Bioact Mat* 1999;26:106–7.