EXHIBIT B

PART 3 OF 5

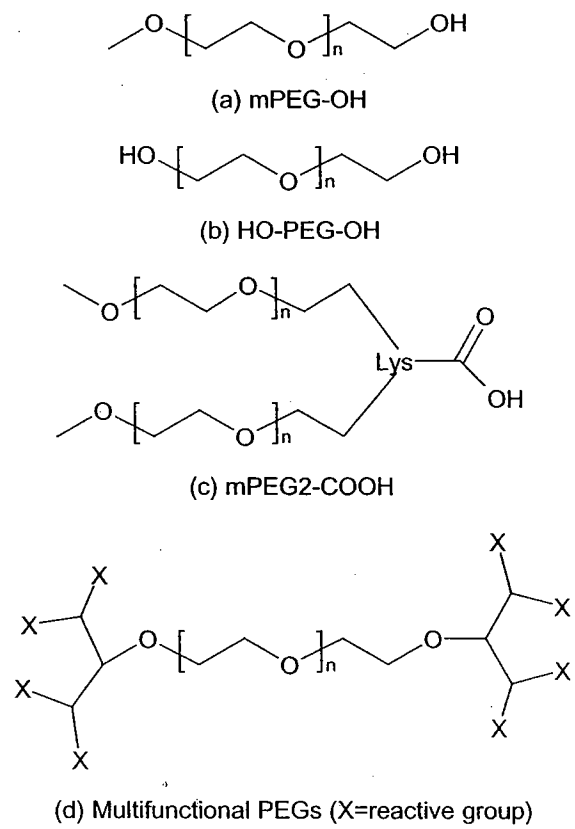


Fig. 1 PEG structures: (a) linear monomethoxy PEG, (b) linear diol PEG, (c) branched PEG, (d) multifunctional PEGs.

forming polymers with one or two end-chain hydroxyl groups, respectively (mPEG-OH or HO-PEG-OH; Fig. 1). Starting from these simple molecules, a wide series of activated PEGs were developed to address selectively different chemical groups in proteins. Moreover, PEGs with various shapes are now available: branched, multifunctional or heterobifunctional PEGs (Fig. 1). Monofunctional polymers (mPEG-OH), linear or branched, are suitable for protein modification, while those with multiple reactive groups are used to increase the drug/polymer ratio, a strategy that is useful in the case of therapeutics agents with low biological activity that otherwise would require the administration of a large amount of conjugates.

The optimization of both the polymerization procedure and purification process allowed developing PEGs with low polydispersivity, M_w/M_n, spanning from 1.01, for PEG below 5 kDa molecular weigh, to 1.1 for PEG with molecular weight as high as 50 kDa.

PEG has unique solvation properties that are due to the coordination of 2–3 water molecules per ethylene oxide unit[6] that, together with the great flexibility of the polymer backbone, are responsible of the PEG biocompatibility and rejecting properties towards protein, which are at the basis of the low immunogenicity and antigenicity of the conjugates.[14] Furthermore, these characteristics give to PEG molecules an apparent molecular weight 5–10 times higher than that of a globular protein of comparable mass, as it can be verified by gel permeation chromatography.[15] Due to this large hydrodynamic volume, a single PEG molecule covers an extended surface of the conjugated protein, preventing degradation by mammalian cells and enzymes.[16]

In vivo, PEG undergoes limited chemical degradation and the clearance depends upon its molecular weight: below 20 KDa, it is easily secreted into the urine; while at higher molecular weights, they are eliminated more slowly and the clearance through the liver becomes predominant. The threshold for kidney filtration is about 40–60 KDa (a hydrodynamic radius of approximately 45 Å[17]), that represents the albumin excretion limit. Over this limit, the polymer remains in circulation and it is mainly accumulated in the liver. Alcohol dehydrogenase can degrade low molecular weight PEGs, and chain cleavage can be catalyzed by P450 microsomial enzymes.[18] Some branched PEGs may undergo a molecular weight reduction when the hydrolysis and loss of one polymer chain is catalyzed by anchimeric assistance.[19] Finally, several years of PEG use as an excipient in foods, cosmetics and pharmaceuticals, without toxic effects, are a clear proof of its safety.[16]

The first generation of PEG conjugates was based on low molecular weight polymers (≤12 kDa), most commonly in their linear monomethoxylated form (mPEG). The batches of this polymer often contained a relevant percentage of PEG diol originated from the synthesis, an impurity that, after activation, could act as a potential cross-linking agent. Furthermore, the chemistry employed in mPEG synthesis often presented side reaction products or led to weak and reversible linkages. The drug PEG-adenosine deaminase (Adagen®)[20] and PEG-asparaginase (Oncaspar®)[21] belong to this generation.

The second generation of PEGs is characterized by improved polymer purity (reduction of both polydispersivity and diol content also for high molecular weights PEG) and in a wide selection of activated PEG

reagents that allow selectivity of reaction towards different protein sites. The availability of several new PEGs is now widening the opportunities of the PEGylation technology. Among all, we would like to report:

- PEG-propionaldehyde, also in the form of the more stable acetal: the reaction with amino group leads to a Shiff base that, once reduced by sodium ciano borohydride, yields a stable secondary amine that maintains the same neat charge of the parent drug.
- Several PEG-succinimidyl derivatives: highly reactive towards amino group. The reaction rate of these derivatives may significantly change, depending on the length and the composition of the alkyl chain between PEG and the succinimidyl moiety.[22]
- "Y" shaped branched PEG[23] (termed PEG2 or U-PEG) [see Fig. 1(c)]: provides a higher surface shielding effect and it is more effective in protecting the conjugated protein from degradative enzymes and antibodies (Fig. 2). Moreover, proteins modified with this PEG retain higher activity than the same enzyme modified by linear PEGs. This effect is probably due to the hindrance of the branched polymer that prevents the entrance

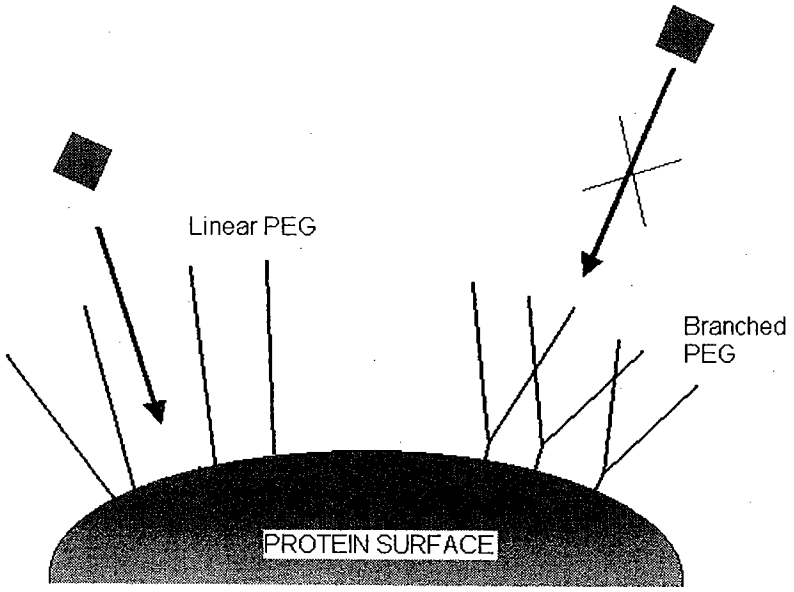


Fig. 2 Structure of linear and branched PEG on protein surface. The "umbrella like" structure of branched PEG explains the higher capacity in rejecting approaching molecules or cells as compared to linear PEG.

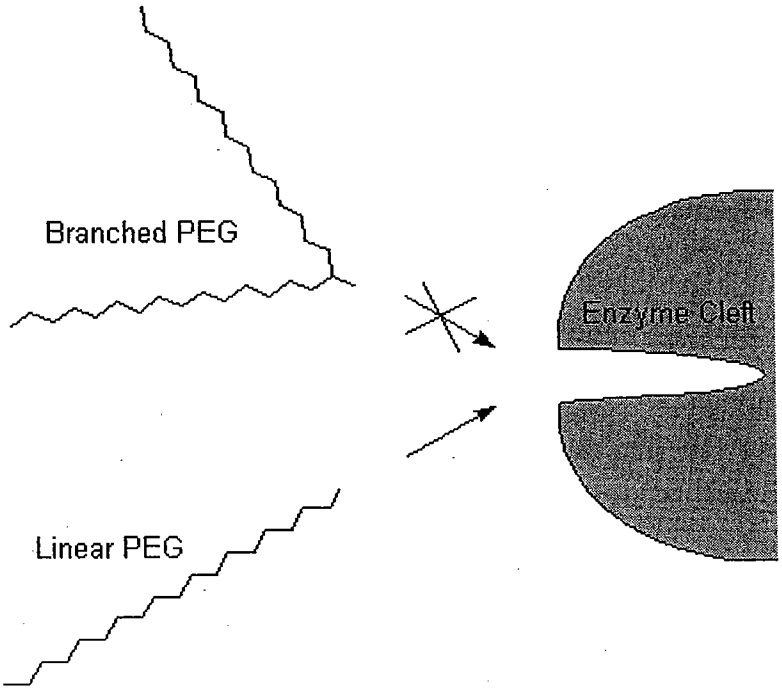


Fig. 3 Effect of PEG hindrance on the enzyme active site access. The high steric hindrance of branched PEG, that makes difficult the access to active site cleft, may be advocated to explain the lower inactivation of enzymes as compared to linear PEG of the same size.

of PEG inside the enzyme active site cleft or other sites involved in biological activity (Fig. 3).

- PEGs reactive toward thiol groups: PEG-maleimide (MAL-PEG), PEG-vinylsulfone (VS-PEG), PEG-iodoacetamide (IA-PEG) and PEG-orthopyridyl-disulfide (OPSS-PEG) have been specifically developed for this conjugation, but only the last strictly reacts with the thiol groups, avoiding any degree of amino modification that may occur instead in small amount using the other three (Fig. 4).
- Heterobifunctional PEGs[24,25]: these derivatives have different reactive groups at the two polymer ends that allow to link separately two different molecules to the same PEG chain. It is therefore possible to obtain conjugates that carry both a drug and a targeting molecule. Among the proposed and commercially available heterobifunctional PEGs, the ones mainly used are H_2N-PEG-COOH, HO-PEG-COOH, H_2N-PEG-OH and their reversibly protected and activated forms.

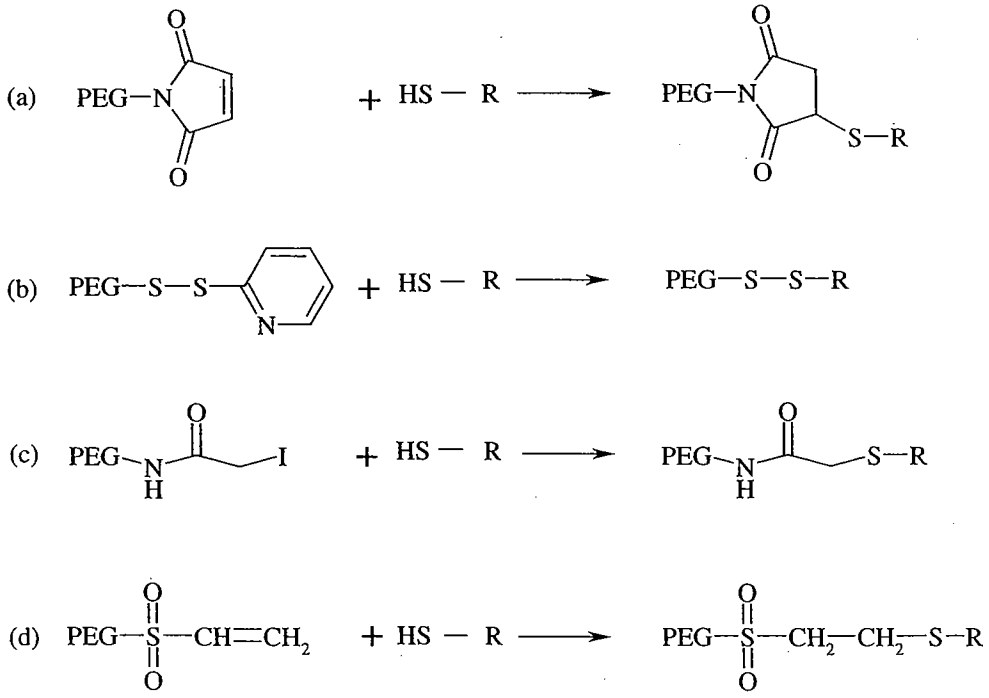


Fig. 4 Examples of activated PEG molecules reactive towards thiol groups: (a) PEG maleimide, (b) PEG orthopyridyl-disulfide, (c) PEG iodoacetamide, (d) PEG vinylsulfone.

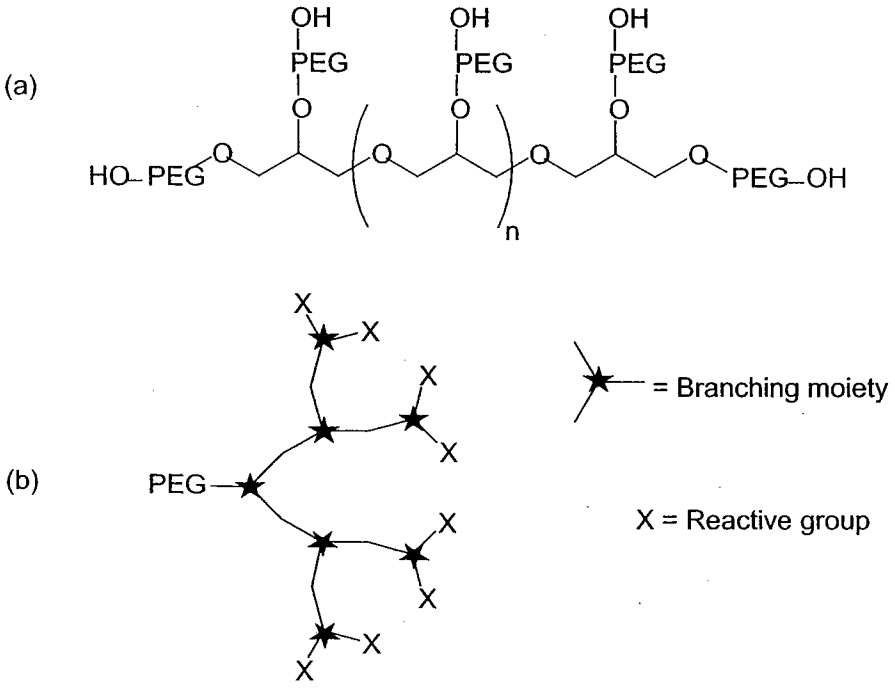


Fig. 5 Different strategies to achieve multifunctional high loading PEGs: (a) multiarm PEGs, (b) dendronized PEGs, the branching moiety may be a bicarboxylic amino acid, lysine or other bifunctional molecules.

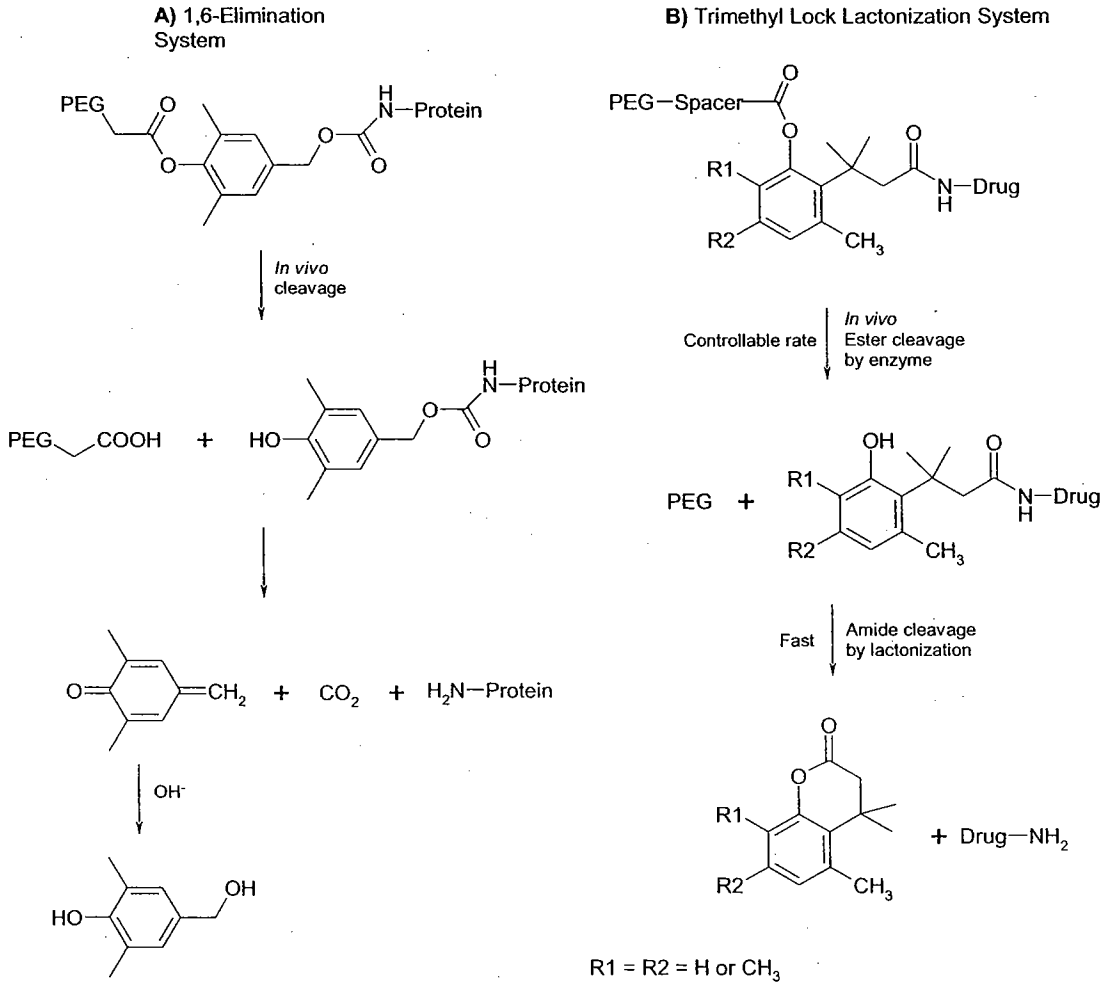
- Multiarm or "dendronized" PEGs (Fig. 5): the former are prepared by linking a linear PEG to a multimeric compound, whereas the latter are linear PEGs with a dentritic structure at one or both chain ends.[26,27] The aim of both derivatives is to increase the drug/polymer molar ratio, overcoming problems of high viscosity that may occur with solutions of mono-functional drug-polymer conjugates. This is particularly true for drugs that require high amount of product for the therapeutic treatments.
- Further advances have been achieved developing PEGs with designed linkers for a controlled release of the conjugated drugs; some of the most exploited linkers are peptides recognized and cleaved by lysosomal enzymes once the conjugates reach the intracellular compartment. Examples of such peptide linkers may be H-Gly-Phe-Leu-Gly-OH or H-Gly-Leu-Phe-Gly-OH.[28,29]
- Alternatively, linkers may respond to pH changes such as the cis-aconityl spacer.[30]
- The linker and the polymer together may be designed to form a double prodrug system, where the drug release is obtained after polymer hydrolysis (first prodrug) that triggers the linker (second prodrug), as reported for the drug delivery system based on 1,6 elimination reaction or trimethyl lock lactonization[31,32] (Scheme 1).

Most of these PEGs, also in the activated form, are now present in the market. Several protein bioconjugates derived from this second generation reached the market, like linear PEG-interferon α2b (PEG-Intron®),[33] branched PEG-interferon α2a (Pegasys®),[34,35] PEG-growth hormone receptor antagonist (Pegvisomant, Somavert®)[36] and PEG-G-CSF (pegfilgrastim, Neulasta®).[37] A PEG conjugate of a 24-mer oligonucleotide, a branched PEG-anti-VEGF aptamer (Pegaptanib sodium injection, Macugen™)[38] also reached the market, while many other products are under clinical trials and hopefully will be available in the near future.

3. PEGylation Chemistry

3.1. *Amino group PEGylation*

PEGylation at the level of protein amino groups may be carried out with PEGs having different reactive groups, but although the coupling reaction is based on the same chemistry (for instance acylation), the obtained



Scheme 1 Controlled release of active molecules from PEG based on (A) 1,6-elimination system and (B) trimethyl lock lactonization system.

products may be different. The difference can reside in the number of PEG chains linked per protein molecule, in the amino acids involved and in the chemical bond between PEG and protein. This paragraph will report the most common methods for random PEGylation, while procedures for site-direct modification are discussed later.

So far the products present on the market are mainly coming from random PEGylation (see the above mentioned Adagen®, Oncaspar®, PEG-Intron® and Pegasys®) since the FDA Authorities still approve these conjugate mixtures upon demonstration of their reproducibility.

The choice of an activated PEG for amino linking can rely on the wide range of polymers commercially available, the most known are: (a) PEG succinimidyl succinate (SS-PEG), (b) PEG succinimidyl carbonate (SC-PEG), (c) PEG *p*-nitrophenyl carbonate (pNPC-PEG), (d) PEG benzotriazolyl carbonate (BTC-PEG), (e) PEG trichlorophenyl carbonate

(TCP-PEG), (f) PEG carbonylimidazole (CDI-PEG), (g) PEG tresylate, (h) PEG dichlorotriazine and (i) PEG aldehyde (AL-PEG) and a branched form of PEG (PEG_2-COOH) (see Fig. 6).

The difference among the above reported PEGs lies in the kinetic rate of amino coupling and in the resulting link between polymer and drug. The derivatives with slower reactivity, such as the carbonate PEGs (pNPC-PEG, CDI-PEG and TCP-PEG) or the aldehyde ones, allow a certain degree of selective conjugation within the amino groups present in a protein, according to their nucleophilicity or accessibility.[7] A difference in reactivity is usually observed between the ε and the α amino group in proteins due to their p*K*a, of 9.3–9.5 for the ε-amino residue of lysine and 7.6–8 for the α-amino group. This difference in reactivity is therefore exploited in the α-amino modification performing the conjugation reaction at pH 5.5–6.0, as reported for G-CSF PEGylation with PEG-aldehyde.[37] The ε-amino groups of lysine, possessing high nucleophilicity at high pH, are preferred site of conjugation at pH 8.5–9.

It is noteworthy that the conjugation performed using PEG dichlorotriazine, PEG tresylate and PEG aldehyde (the latter after sodium ciano borohydride reduction) maintains the same total charge on the native protein surface, since these derivatives react through an alkylation reaction, yielding a secondary amine. In contrast, PEGylation conducted with acylating PEGs (i.e. SS-PEG, SC-PEG, pNPC-PEG, CDI-PEG, TCP-PEG and PEG_2-COOH) gives weak acidic amide or carbamate linkages with partial loss of positive charge.

The above reported PEG derivatives may sometime give side reactions involving the hydroxyl groups of serine, threonine and tyrosine and the secondary amino group of histidine. These linkages however are generally hydrolytically unstable. The reaction conditions or particular conformational disposition may enhance the percentage of these unconventional PEGylation reactions, for example α-interferon was conjugated by SC-PEG or BTC-PEG to His-34 under slightly acidic conditions[39] (the p*K*a value of histidine is between those of the α and ε amino groups). PEG was found linked to the hydroxyl groups of serine in the decapeptide antide or of tyrosine in epidermal growth factor (EGF) also.[40,41]

As examples of random amino PEGylation two case studies are here reported. *Interferon α-2a* (INF) was modified with linear succinimidyl carbonate PEG (SC-PEG; 5 KDa), in pH 10 buffer, through the formation of a urea linkage between protein and polymer. The coupling, performed at

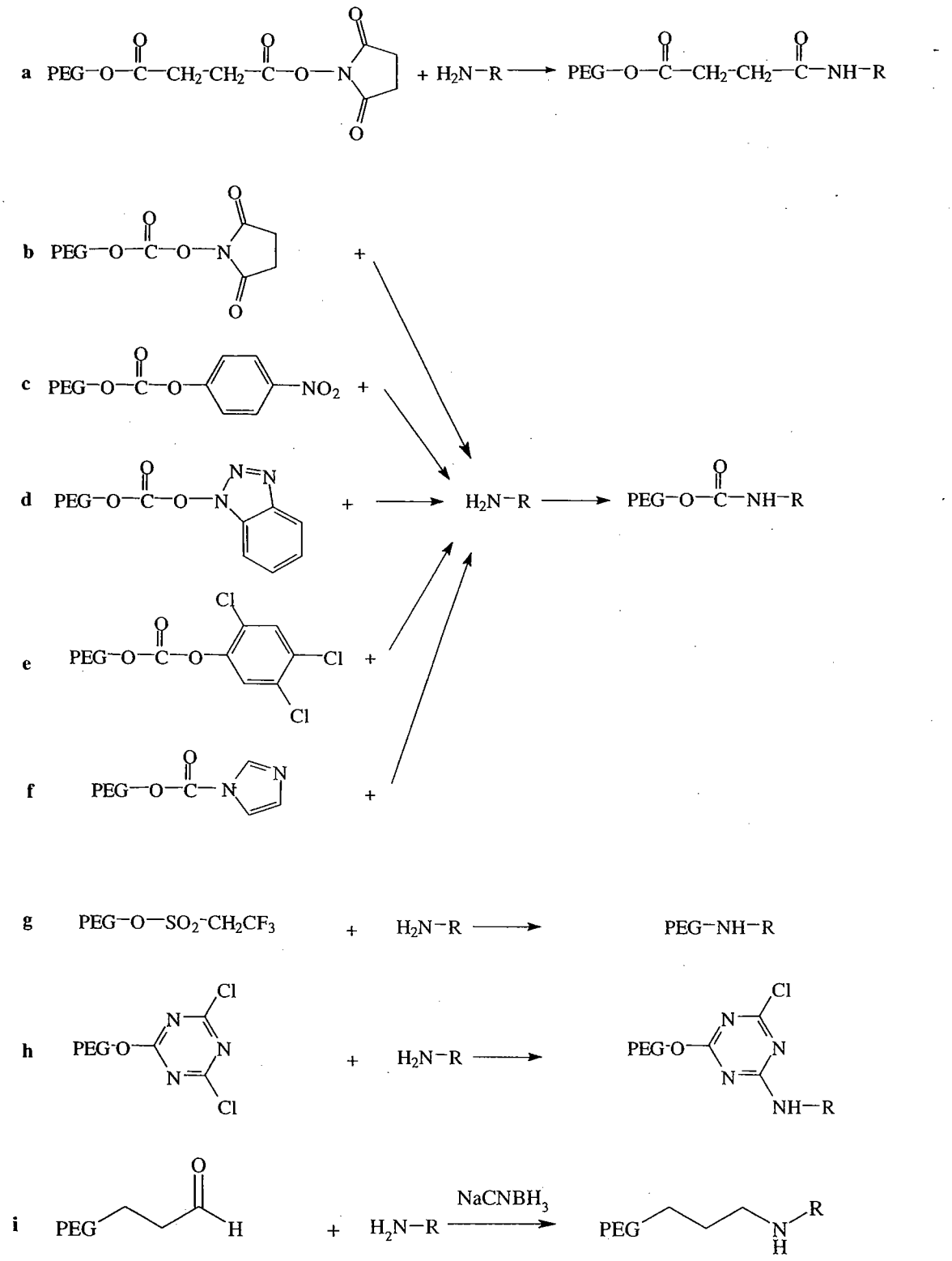
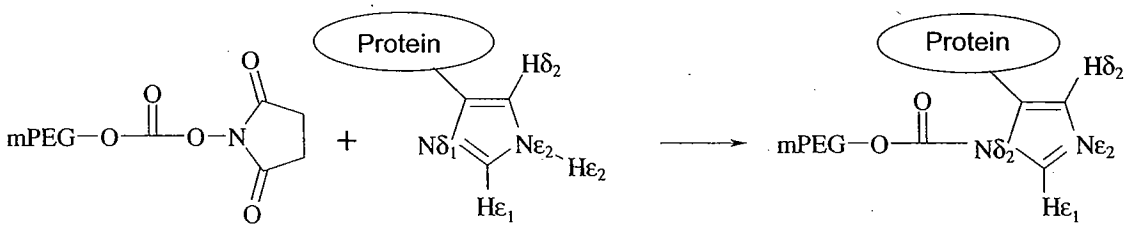


Fig. 6 Examples of activated PEG molecules reactive towards amino groups: (a) PEG succinimidyl succinate, (b) PEG succinimidyl carbonate, (c) PEG *p*-nitrophenyl carbonate, (d) PEG benzotriazol carbonate, (e) PEG trichlorophenyl carbonate, (f) PEG carbonylimidazole, (g) PEG tresylate and (h) PEG dichlorotriazine and (i) PEG aldehyde (AL-PEG).

equimolar protein/polymer ratio, led mainly to mono-PEGylated isomers and, in small amounts, to di-PEGylated conjugates and free interferon. Characterization of the conjugates indicated that lysine residues were the only site of PEGylation,[42] in agreement with the fact that lysine's amino groups possess higher reactivity than the N-terminal ones at alkaline pHs. The same PEGylation reaction, carried out in phosphate buffer at pH 6.5, demonstrated that derivatives with improved pharmacokinetic profiles and higher activity could be obtained by the conjugation of interferon α-2b with SC-PEG (12 KDa). This reaction gave a conjugate at histidine-34, representing approximately 47% of the total PEGylated species (Scheme 2).[43,44] The activity of this interferon preparation was related to the ability to release free and fully active interferon by slow hydrolysis of the labile His-PEG bond.[45] Although the *in vitro* potency of this PEG-interferon is only 1/4 of the free form, its serum residence time is about 6 times longer, allowing less frequent administrations to maintain an efficacy comparable to one of the unmodified interferon.[33,46] These studies brought to the market PEG-Intron® in the year 2000.

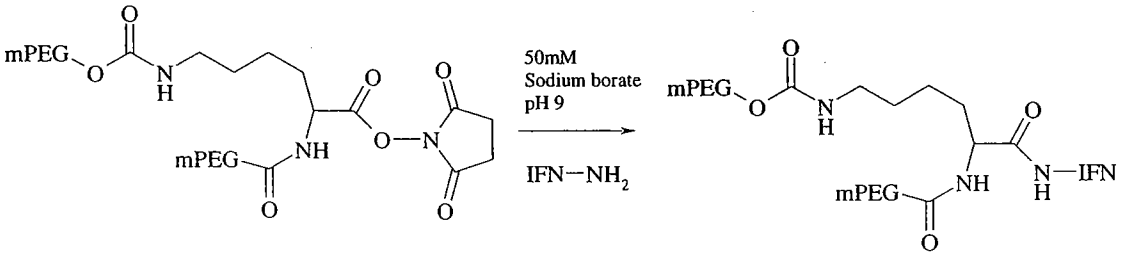
A different approach to interferon PEGylation exploited the special properties of branched PEGs. A high molecular weight branched PEG (PEG_2, 40 kDa) was chosen on the basis of several preliminary studies, disclosing that: (a) the protein surface protection with a single, long and hindered chain PEG is higher than the one obtained with several small PEG chains linked at different sites[1]; (b) branched PEGs have lower distribution volumes than linear PEG of identical molecular weight, and the delivery to organs such as liver and spleen is faster[47]; (c) proteins modified with branched PEG possess greater stability towards enzymes and pH



SC-PEG His-34 of IFN His of PEG-IFN

Scheme 2 Adduct formation at the level of His-34 in interferon α-2b using SC-PEG as PEGylating agent.

degradation.[23] The 40 KDa branched succinimidyl PEG (PEG_2-NHS) was linked to interferon α-2a using a 3:1 PEG/protein molar ratio in 50 mM sodium borate buffer pH 9 (Scheme 3).[34] PEGylation under these conditions led to a mixture containing 45–50% of mono-substituted protein, of 5–10% poly-substituted (essentially dimer) and 40–50% of unmodified interferon (Fig. 7). Identification of the major positional isomer within the mono-PEGylated fraction was carried out by a combination of high performance cation exchange chromatography, peptide mapping, amino acid sequencing and mass spectroscopy analysis. It was demonstrated that PEG was attached mainly to one of the following lysines: Lys-31, Lys-121, Lys-131



Scheme 3 PEGylation of interferon α-2a by branched $mPEG_2$-COOH (40 kDa).

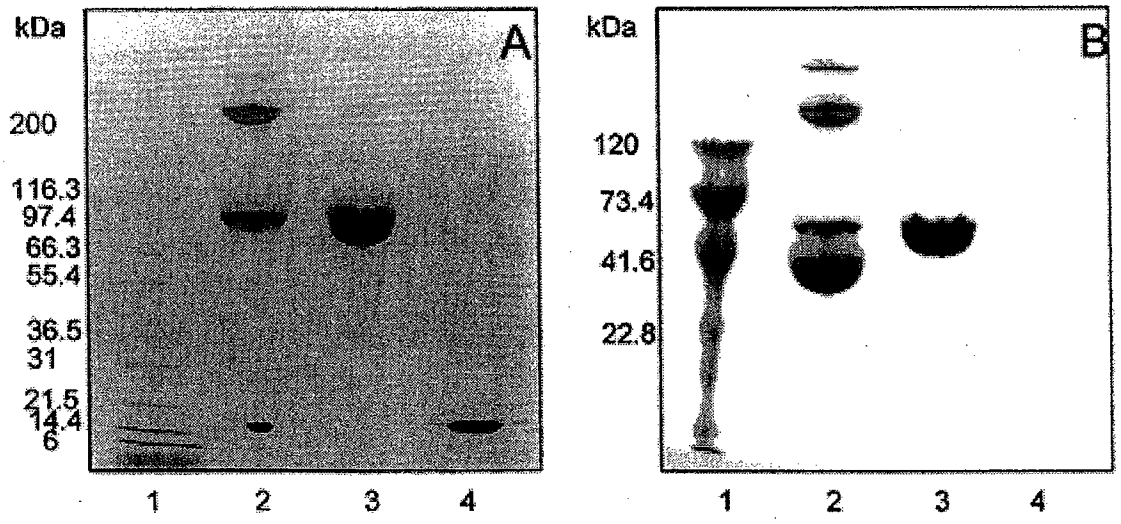


Fig. 7 SDS-PAGE analysis of the PEGylated interferon α–2a mixture. The conjugates were: (A) specifically stained for protein with Coomassie blue. Lanes: 1, molecular weight marker proteins; 2, PEGylation reaction mixture; 3, purified PEG_2-IFN; and 4, interferon –2a. (B) Specifically stained for PEG with iodine. Lanes: 1, molecular weight marker PEGs; 2-4, same as in Fig. 2A. Note that lane 4, containing interferon α–2a in gel B, is not stained by iodine.

Table 2 Pharmacokinetic properties of Interferon α-2a and its PEGylated form in rats.[35]

Protein	Half-life (h)	Plasma Residence Time (h)
Interferon α-2a	2.1	1.0
PEG2 (40 kDa)-interferon α-2a	15.0	20.0

or Lys-134.[34] Even though the *in vitro* antiviral activity for PEG2-IFN was greatly reduced (only 7% was maintained with respect the native protein) the *in vivo* activity, measured as the ability to reduce the size of various human tumors, was higher than that of free IFN. The positive result could be related to the extended blood residence time of the conjugated form as shown in Table 2. These studies brought into the market a blood long lasting interferon conjugate, Pegasys®, which is effective in eradicating hepatic and extrahepatic hepatitis C virus (HCV) infection.[35]

3.2. *Thiol PEGylation*

The presence of a free cysteine residue represents an optimal opportunity to achieve site direct modification, since its rare occurrence in proteins. PEG derivatives having specific reactivity towards the thiol group, namely MAL-PEG, OPSS-PEG, IA-PEG and VS-PEG (Fig. 4), are commercially available and allow thiol coupling in good yield, everyone presenting some differences in terms of protein-polymer linkage and reaction conditions. Even if the thiol reaction rate of IA-, MAL- or VS-PEGs is very rapid, some degree of amino coupling may also take place, especially if the reaction is carried out at pH values higher than 8. On the other hand, the reaction with OPSS-PEG is very specific for thiol groups, but the obtained conjugates may be cleaved in the presence of reducing agents as such simple thiols or glutathione (also *in vivo*). PEGylation at the level of cysteine allows easier conjugate purification and characterization, since the presence of only one derivatizable site (free cysteine) avoids the formation of positional isomers or products with different degree of substitution, problems common to amino PEGylation. The potential of thiol PEGylation may be further exploited by genetic engineering that allows the introduction of

cysteine residue wherever in a protein sequence by insertion or switching with a non essential amino acid.

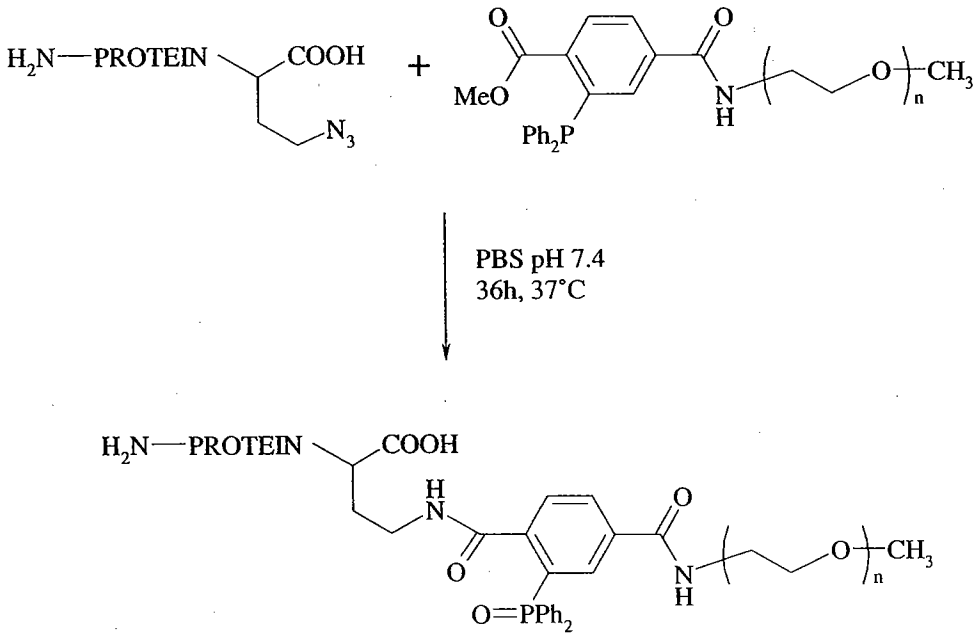
For example, *hGH* was extensively studied with this strategy; in fact, to overcome the common poly-substitution of amino modification, cysteine muteins were synthesized by recombinant DNA technology. Among all of the possible mutations described in literature and patents, the cysteine addition at the C-terminus of hGH leads to a fully active mutein that allows a site-specific PEGylation using the thiol-reactive PEG-maleimide (PEG-MAL, 8 kDa). It is necessary to treat the rhGH mutein with 1,4-dithio-DL-threitol (DTT) before the coupling step, to convert the carboxyl terminal cysteine in the reduced form and to prevent the formation of scrambled disulphide bridges. After the removal of DTT excess by gel-filtration, the conjugation leads to a mono-PEGylated derivative with over 80% yield.[48]

Another important example of thiol conjugation is the PEGylation of *hemoglobin* (Hb) that allows the prevention of its extravasation. After several unsuccessful attempts through random PEGylation, a site specific modification was performed at Cys-93(β) with maleimidophenyl PEG (MAL-Phe-PEG) (5, 10 and 20 kDa), leading to PEGylated Hb carrying two polymer chains per Hb tetramer.[49] The limit of native Hb is its vasoactivity as a consequence of its extravasation, which can be avoided by PEG coupling that modifies the colligative properties of the derivatives.[15] This product was found to be more efficient than polymerized Hb, the Hb-octamer or -dodecamer.

3.3. *Carboxy PEGylation*

PEGylation at the level of protein carboxylic groups needs a previous activation for reacting with an amino PEG. This procedure is not devoid of limitation since undesired intra or intermolecular cross-links may occur because the activated carboxylic groups, besides reacting with the PEG-NH_2, react also with the amino groups of the protein itself.

To circumvent this problem, it is possible to use PEG-hydrazide (PEG-CO-NH-NH_2) instead of the usual amino PEG. In this case, the protein's COOH groups are activated by water soluble carbodiimide at low pH and following reacted with PEG-hydrazide, where the protein amino groups are protonated and not more suitable for coupling, while hydrazide group, having a low p*Ka*, can still react.[50]



Scheme 4 Staudinger ligation leading to a C-terminal mono-PEGylated protein by reaction of a mutated protein, containing a C-terminal azido-methionine, with an engineered PEG derivative, methyl-PEG-triarylphosphine.

An alternative method for specific C-terminal PEGylation is based on the Staudinger ligation.[51] The protocol, settled for a truncated thrombomodulin mutant,[52] starts from the *E. coli* expression of a mutated protein containing a C-terminal azido-methionine. This reacts specifically with an engineered PEG derivative, methyl-PEG-triarylphosphine, leading to a C-terminal mono-PEGylated protein (Scheme 4). This method, however, implies the preparation of a gene encoding for a protein with a C-terminal linker ending with methionine. Expression in *E. coli* is induced when the transformed bacteria are suspended in a medium where methionine is replaced by the azido-functionalized analogue. Unfortunately, this method is applicable only in the rare case of proteins devoid of methionine in the sequence; otherwise, they will hinder the protein transduction because the azido-analogue does not permit the linking of the following amino acid.

4. Strategies in Protein PEGylation

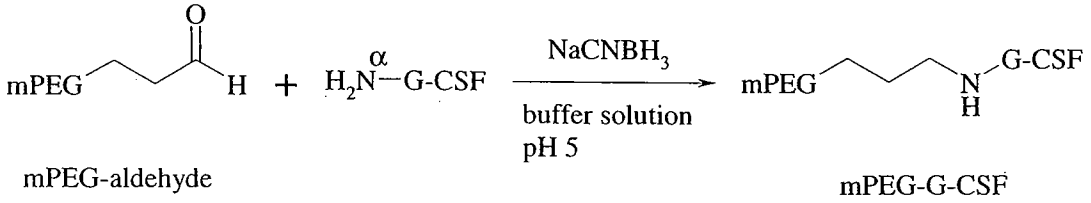
To better exploit the potential of PEGylation several strategies have been developed with the purposes of: (a) obtaining more homogenous products, (b) forming PEGylated conjugates with higher retention of activity,

(c) performing PEGylation in gentle chemical conditions more compatible with easily degradable proteins.

Site selective conjugation is preferable as it allows easier purification and characterization and, most important, a better maintenance of the protein biological activity. In fact, one of the major drawbacks in PEGylation is represented by in loss of protein biological activity, due to factors such as random PEGylation, steric hindrance by PEG chains (interfering with both protein-receptor recognition or enzyme active site accessibility) or changes in protein's secondary-tertiary structure (due to both PEG linkage or to partial denaturation during the PEGylation reaction).

Site direct PEGylation may be achieved by cysteine PEGylation, as described above. However, this is possible only when the amino acid is present in the reduced form in the native protein, a rare case that can be overcome by introducing a cysteine by genetic engineering. In other cases, site directed PEGylation may be obtained by taking advantage of the low p*K*a value of the α amino group, with respect to the lysine's one. As reported, the reaction conducted at neutral or mild acid condition, prevents the PEGylation at the level of lysine, but leaves the N-terminal amino group still able to react.[53] The most successful example of this strategy is the alkylation of r-metHuG-CSF with PEG-aldehyde, proposed by Kinstler. The reaction was carried at pH 5.5 in the presence of sodium cyanoborohydride to reduce the Shiff base initially formed (Scheme 5; Fig. 8).[37,54] The conjugate obtained with a molecule of PEG 20 kDa showed an improved pharmacokinetic profile mainly due to reduced kidney excretion. The PEG-G-CSF conjugate, Pegfilgastrim®, is on the market since 2002.

Preferential site PEGylation can also be achieved by exploiting the different accessibility of the protein amino groups, as reported for a truncated form of growth hormone-releasing hormone ($hGRF_{1-29}$). It was



Scheme 5 Mono-mPEG-G-CSF conjugates were prepared by reductive alkylation of the α-amino group of the N-terminal methionine residue of r-metHuG-CSF with mPEG-aldehyde.

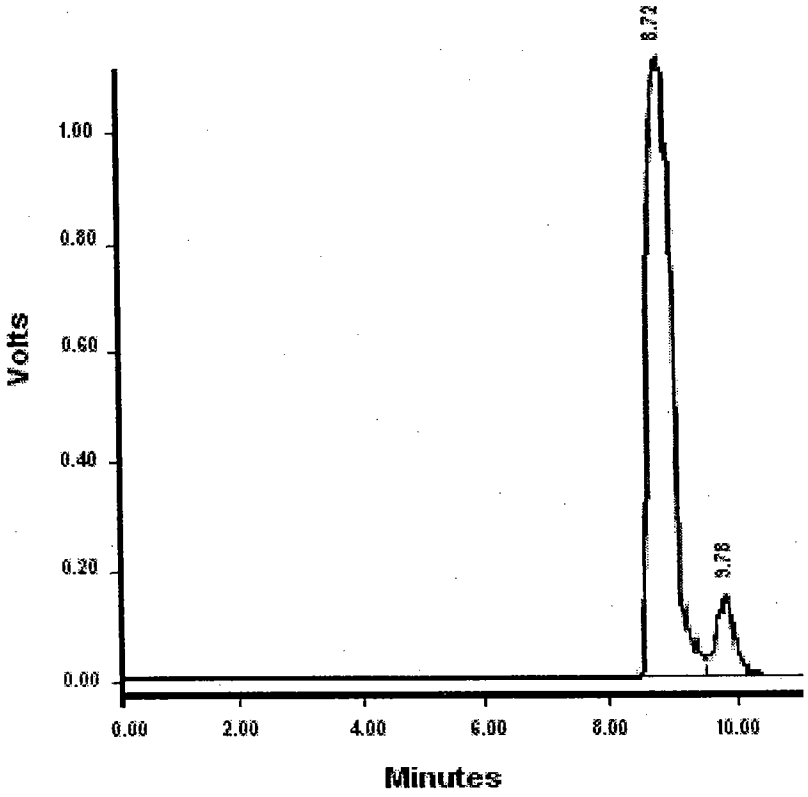


Fig. 8 Size-exclusion HPLC analysis (UV detector at 280 nm) of the reduced mixture of r-metHuG-CSF reacted with mPEG aldehyde (M_w = 6 kDa): *N*-Terminal mono-mPEG–G-CSF conjugate eluted at 8.72 min (92% of total area); Unreacted r-metHuG-CSF eluted at 9.78 min (8% of total area), as from Ref. 37.

demonstrated that, by using an appropriate solvent, it is possible to alter the accessibility and reactivity of the three available amino groups. In fact, NMR and circular dichroism analysis indicated that the percentage of α-helix in $hGRF_{1-29}$, only 20% in water, raises to 90% in structure-promoting solvents such as methanol/water or 2,2,2-trifluoroethanol (TFE), thus facilitating a region-selective modification. When PEGylation was performed in TFE, the monoPEGylated conjugate at the level of Lys-12 reached the 80% of the all PEGylated isomers.[55] Meanwhile, the same reaction conducted in DMSO yielded an almost equimolar mixture of mono-PEGylated Lys-12 and Lys-21 isomers.[56]

Alternatively, specific PEGylation may be performed by blocking some of the reactive groups with a reversible protecting group as reported for insulin. This protein is formed by two polypeptide chains, A and B, and its three amino groups (Gly-A1, Phe-B1 and Lys-B29) are all candidates for PEGylation. Hinds proposed a site-directed PEGylation procedure

involving the preliminary preparation of N-BOC (tert-butyl chloroformate) protected insulin.[57] For example, in order to synthesize $N^{\alpha B1}$-PEG-insulin the intermediate $N^{\alpha A1}$, $N^{\varepsilon B29}$-BOC-protected insulin was prepared prior to conjugation with PEG-SPA at the level of free $N^{\alpha B1}$. The final conjugate was obtained upon BOC removal with TFA treatment, forming the $N^{\alpha B1}$-PEG_{2000}-insulin conjugate with 83% of the native insulin activity.

In general, in the case of enzyme modification, a requirement for the preservation of activity is that the PEG chains do not have access to the active site and therefore the residues involved in catalysis are not modified. Many strategies have been developed for this goal: (a) the use of branched PEGs that, thanks to their higher hindrance with respect to linear polymers, have reduced accessibility to the active site (Fig. 3); (b) to perform PEGylation in the presence of a substrate or an inhibitor that blocks polymer access to the active site; (c) to conduct the modification after the enzyme is captured on an insoluble affinity resin by substrates or inhibitors linked on it. In the last case, the obtained conjugate is eluted from the resin by changing the pH or adding denaturants. The derivative will possess the active sites and its closer surroundings free of PEG chains (Fig. 9).[58]

A general strategy that may be applied to enzymes or signaling proteins, consists in linking the wanted mass of PEG by using few high molecular weight polymer chains, instead of a high number of low molecular weight ones. In fact, multipoint attachment of PEG on protein surface usually reduces or prevents protein recognition by shielding effect, more than a single PEG chain linked to one location in the protein. The effects of both number and mass of linked PEG chains on recognition and pharmacokinetic parameters are well documented in literature.[59]

A problem that may occur during a protein PEGylation is the low yield, especially when the modification is directed towards a buried or less accessible amino acid. This inconvenience is enhanced when the reaction is performed with high molecular weight PEG due to the high steric hindrance. In the case of *interferon beta* (IFN-β), conjugation at cysteine 17 could be achieved only with a low MW OPSS-PEG oligomer, but not with a high MW polymer.[60] On the basis of these results, modification with high molecular weight PEGs was successfully attempted via two-steps procedure: in the first step, the protein was modified with a short chain heterobifunctional PEG oligomer; and in the second step, the obtained conjugate was linked

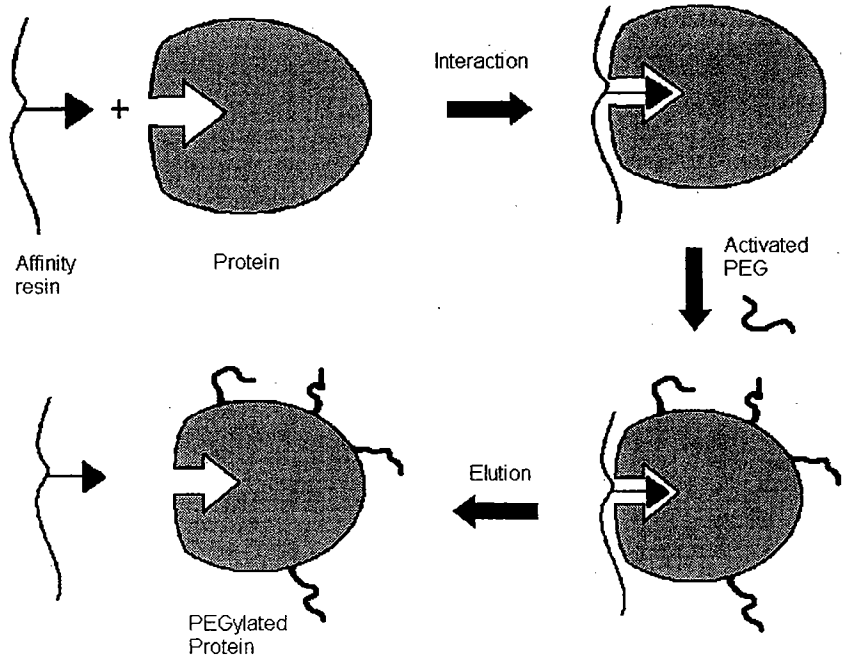


Fig. 9 Two phase PEGylation strategy for the protection of an enzyme active site from polymer conjugation. The enzyme is first loaded into an affinity resin functionalizated with appropriate ligand. The enzyme's active site binds the ligand, thus protecting the active site itself and the area close to it from PEG modification. After reaction in heterogeneous condition, the modified enzyme is eluted from the column.

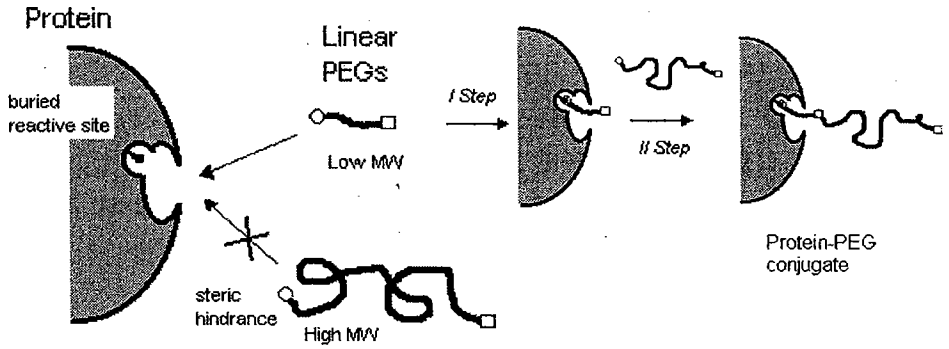


Fig. 10 Two step tagging PEGylation strategy for a buried SH group in protein. Smaller PEG molecules are more reactive than high molecular weight PEGs towards the buried cysteine.

to a higher molecular weight PEG, possessing specific reactivity towards the terminal end of the first oligomer (Fig. 10). The heterobifunctional PEG oligomer had a thiol reactive group at one end and a hydrazine group at the other (OPSS-PEG-Hz, 2 kDa). As mentioned above, hydrazine is

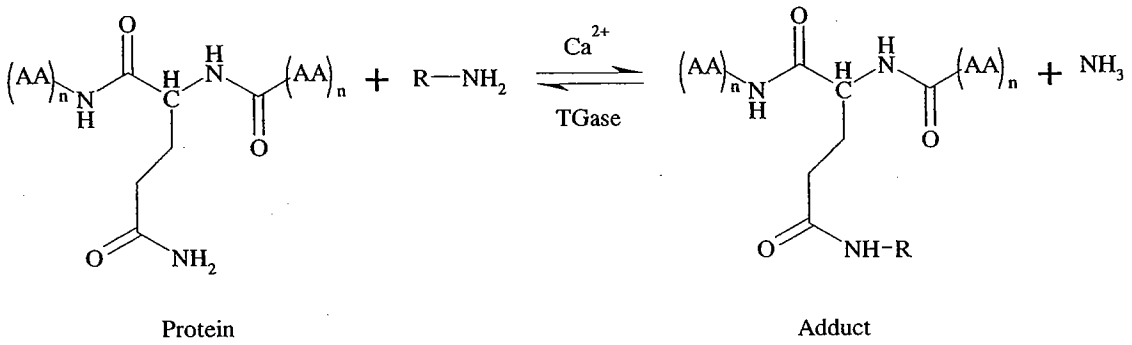
also reactive at low pHs, where protein's amino groups are usually protonated and not reactive, preventing unwanted amino PEGylation of the protein. The INF-SS-PEG-Hz conjugate could therefore be selectively modified with PEG-aldehyde (30 kDa) by reductive alkylation. The overall yield was higher than 80%.

A recent study demonstrated that specific PEGylation at the lone, but not accessible, thiol groups of G-CSF could be achieved upon its exposure in partially denaturant conditions. After modification with OPSS-PEG, the native conformation of G-CSF was recovered by removal of the denaturant.[61]

5. Enzymatic Approach for Protein PEGylation

Recently, as alternative solution to obtain homogeneous PEG-protein conjugates in mild reaction conditions, some researchers are investigating the possibilities to exploit the catalysis and specificity of enzymes in PEGylation. The aim is to simplify the purification steps and better preserve the protein activity than the common chemical methods. Since the first report proposed by H. Sato involving the enzyme transglutaminase (TGase),[62] other researchers developed interesting approaches with different enzymes. From the results reported so far, we can hypothesize that these procedures will lead to results of great interests and applicability.

Sato studied two methods for interleukin-2 (IL-2) PEGylation using transglutaminases (TGase) from guinea-pig liver (G-TGase) or from *Streptoverticillium sp.* strain s-8112 (M-TGase). Both enzymes catalyze the transfer of an amino group from a donor (for example PEG-NH_2) to a glutamine residue present in a protein (Scheme 6). The two enzymes differ for the required amino acid sequence neighboring the glutamine in the substrate. Several tailor made linear PEGs, differing in molecular weight and in the type of alkylamine at the polymer end, have been synthesized. Among all, the best reactivity was shown by polymers terminating with -$(CH_2)_6$-NH_2 group. IL-2 contains six glutamines but none of them is a suitable substrate for the more specific G-TGase. The problem was overcome by preparing a chimeric IL-2 proteins, having at the *N*-terminus, a peptide sequence known to be a good G-TGase substrate, the Pro-Lys-Pro-Gln-Gln-Phe-Met (called TG1) sequence, derived from Substance P[63] (to give rTG1-IL-2) and the Ala-Gln-Gln-Ile-Val-Met (called TG2) sequence,



Scheme 6 Reaction catalyzed by TGase between a glutamine residue in a protein and an alkylamine.

derived from fibronectin[64] (to give rTG2-IL-2) have been used. In the former case, a mono-PEGylated conjugate was obtained while a mixture of mono- and di-PEGylated forms resulted from the modification of rTG2-IL-2. The enzymatic coupling was carried out in the very mild conditions of 0.1 M Tris–HCl buffer, pH 7.5 at 25°C for 12 hr in the presence of $CaCl_2$ 10 mM.[65] The derivatives maintained the same activity of the native protein, whereas the classical chemical conjugation with mPEG-NHS yielded products with decreased activity (Table 3). Using the less specific M-TGase, $mPEG_{12000}$-$(CH_2)_6$-NH_2 could be directly incorporated into rhIL-2 at the level of Gln-74.[65] Compared to other site-specific chemical PEGylation, such as cysteine coupling or *N*-terminus modification at acidic pH by PEG-aldehyde, the enzyme method produces less undesirable products, namely

Table 3 Comparison of IL-2 conjugates activities between random PEGylation and site direct PEGylation by TGase.[62]

Proteins	% activity[a]
rhIL-2	100
PEG10-rhIL-2 (random PEGylation)	74
$(PEG10)_2$-rhIL-2 (random PEGylation)	36
rTG1-IL-2 (chimeric protein for enzymatic PEGylation)	72
PEG10-rTG1-IL-2 (enzymatic PEGylation)	69
$(PEG10)_2$-rTG1-IL-2 (enzymatic PEGylation)	72

[a]The amount of % activity was expressed as the percentage of residual bioactivity, compared to the rhIL-2.

the possibility to exploit the different isoelectric point of the isomers by ionic exchange chromatography is very useful; while the reverse phase chromatography was found to be less efficient where the gel-filtration separates only the species with different mass, and is unable to distinguish positional isomers.

Once the isomers are separated, it is necessary to identify the localization of the PEGylation site into the primary amino acids sequence. The classical approach involves enzymatic digestion of the polymer-protein derivative, purification of the peptides and their identification by mass spectroscopy or amino acid analysis. A good example is reported in the characterization of PEGylated interferon α-2a.[42] The comparison of the peptide fingerprint of the conjugated protein with that of the native protein allows the identification of the region where PEGylation occurred on the basis of the disappeared peptide signal in the peptide fingerprint. Besides the lengthy procedure, the conjugated polymer may interfere by steric hindrance with the proteolytic enzymes, resulting in an incomplete cleavage that complicates the interpretation of the peptide finger printing. To circumvent this inconvenience, a new approach has been recently developed based on the use of tailor-made PEGs, PEG-Met-Nle-COOH or PEG-Met-βAla-COOH, that possesses a chemically labile bond in the peptide spacer that can be cleaved at the level of methionine by the treatment with BrCN (Fig. 11), leaving only nor-leucine or β-alanine tag on the protein. These amino acid tagged peptides are more easily identified by standard sequence investigation methods or by mass spectrometry analysis in the enzymatically-digested mixture.[69]

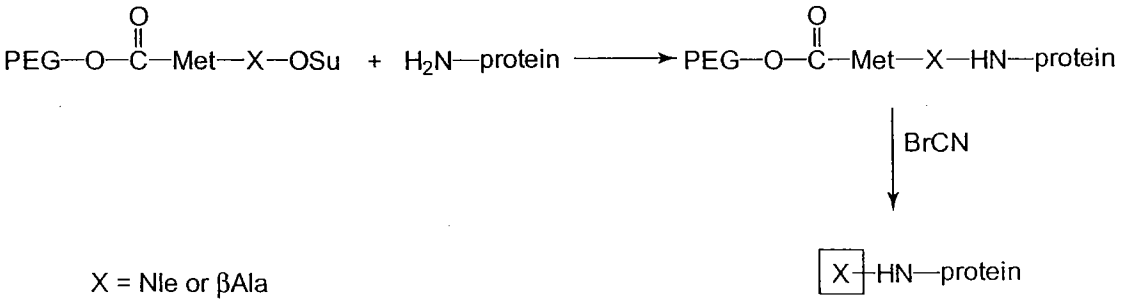


Fig. 11 Use of PEG-Met-Nle-OSu or PEG-Met-βAla-OSu to introduce a reporter amino acid at the PEGylation site: PEG conjugation and the release of PEG by BrCN. Nle or βAla that can be identified on the protein by a standard sequence analysis.

protein–protein dimers (due to cysteine oxidation) or εNH_2 lysine PEGylation (when *N*-terminus PEGylation is performed).

A two step enzymatic PEGylation, called GlycoPEGylation™, was developed. In this case, *E. coli*-expressed proteins were glycosylated at the level of specific serine and threonine amino acids with *N*-acetylgalactosamine (GalNAc) by *in vitro* treatment with the recombinant enzyme O-GalNAc-transferase. The obtained glycosylated proteins were subsequently PEGylated using the O-GalNAc residue as the acceptor site of a sialic acid-PEG, a reaction selectively catalyzed by a sialyltransferase.[66] This enzyme could transfer a PEGylated sialic acid, in the form of cytidine monophosphate derivative (CMP-SA-PEG), to an O-GalNAc residue of glycosylated proteins. The great advance of this technology is the possibility to PEGylate proteins produced in *E. coli* that mimic the mammalian ones, since the PEG chains replace the native sugar moiety at the precise site of glycosylation, forming conjugates that retain the correct structure for receptor recognition plus and the extended plasma half-life.

However, besides the new and interesting aspects of the enzymatic approaches, we have to consider the problems that will arise in an industrial scale production, since the studies so far were limited to a laboratory bench scale and the reactions had a low yield unfortunately.

6. PEGylated Protein Purification and Characterization

Theoretically, in a conjugation reaction conducted with an excess of PEG, one could expect that all of the reactive groups of the protein are modified, yielding a single product. However, many factors are involved in PEGylation, such as amino group accessibility and p*Ka*, and usually a mixture of multi-PEG conjugates is obtained. This is true when a lower amount of PEG is employed in order to avoid loss of biological activity. In this case, a mixture of positional isomers is always formed. This last situation requires special attention to maintain reproducibility of the mixture over different batches, since a mixture of products is still accepted by the Authorities, as long as the identification of all adducts is provided.[67,68] In this case, special skill is needed to fractionate the PEG isomers mixture. For this purpose,

7. Conclusion

Over the years, PEGylation has become a well developed technology and a wide range of possibilities to perform a protein modification are available these days. One may choose the best condition for the protein of interest and can design a method to obtain a conjugate with the wanted total mass of linked PEG, possibly to a selected amino acid. Furthermore, the combination of PEGylation with genetic engineering exploits new and smart strategies to direct the PEG link towards a preferred site, enabling it to obtain conjugates that better retain the activity of the starting molecule.

Davis and Abuchowsky[14,70] paved the way of this technology with their pioneering works. However, the PEGylation concept, initially proposed and developed for protein modification,[1,2,12] has since then been extended to peptide,[3,71] non-peptide drugs[72,73] and cells.[74]

A remarkable boost in this area came with the second generation of PEGs, when a considerable wide selection of derivatives in high purity and low polydispersity became available. An open problem in PEGylation is still the purification and separation of positional isomers formed during a protein modification. In fact, the similar chemical and physical characteristics of these isomers and their isolation may require the use of more than one chromatographic technique. Therefore, PEGylation methods that prevent the formation of positional isomers and products with a different extent of linked PEG chains are always awaiting new and original solutions. Advancements in this direction may be represented by the recently described PEGylation systems based on enzymatic coupling, in order to allow conjugation to unusual amino acid such as glutamine. Nonetheless, it is important to note that if one can show reproducibility and fine characterization of the conjugation reaction, the product may still be accepted by the Authorities as a well defined mixture of PEGylated isomers. A request that is still expecting satisfactory response is the availability of *in vivo* biodegradable PEG derivatives that release the native protein under controlled conditions. Lastly, the problem of monodisperse or at least very low polydisperse PEG, especially for high molecular weight polymers, still exists, even if the market is now offering only low molecular weight monodisperse PEGs that are below 600 Da.

For a complete summary on PEGylation, one may also be reminded that the technique is now expanding from the protein field to the non-peptide

drugs to solve problems beyond the immunogenicity as well as the short residence time in blood, as it was in the early stages of PEGylation. In the area of non-peptide drugs, attention is now dedicated to the potentials of the heterobifunctional PEGs which allow the combination of the advantages of polymer modification with the active targeting capacities of a second molecule linked to the PEG chain. The field of non-peptide drug conjugation is receiving increasing interest,[72] owing to the discovery of numerous new ligands targeted at specific tissues, organs or for the entrapment and the release of drugs into cells.

One can of course ask why is it that only PEG but not other polymers is chosen for protein modification. Actually, very few examples with other macromolecules are on the market, the most successful being the poly(styrene-co-maleic acid) derivative of neocarzinostatin (SMA-NCS), developed by Maeda.[75] The main reason for this situation resides in the mono-functionality of mPEG that avoids cross-linking reaction with the polyfunctionalized proteins, and in the favorable and unique characteristic of PEG. For instance, most of the usual natural or synthetic polymers present multiple points of attachment in the same molecule, as in the case of the natural polysaccharides or albumin, and also for the extensively studied poly(hydroxyethyl acrilamide) (HPMA)[76] that is very promising for small drugs conjugation, but not for proteins.

In conclusion, although PEGylation and conjugation with other polymers are emerging research methodologies, they have already yielded important results and a number of products have already hit the market.

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5

PEGylated Proteins as Cancer Therapeutics

Margherita Morpurgo, Gianfranco Pasut and Francesco M. Veronese

1. Introduction

In early days, the treatment of cancer could rely only on classic antiproliferative agents which act on the basic principle of killing fast replicating cells. These compounds lack tumor specificity and have several undesired side effects. Radiotherapy is another effective tool which can be used alone or associated with chemical treatment. In this case, localization can be achieved only by physically directing the radiation toward the target organ, thus radiotherapy is generally used to treat solid tumors and in the absence of metastasis.

The lack of tumor specificity by classic treatments has led to the search for more physiological strategies to fight cancer. Modern approaches are based on recent acquisitions on the molecular events associated with cancer initiation and progression. Implementation of natural body defence mechanisms and balancing deregulated processes involved in tumorigenesis, such as regulation of cell cycle progression, angiogenesis, and apoptosis provide rational targets for novel therapies. In most case, interfering with these mechanisms can be achieved by using biomolecules as the active