

Expert Opinion

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Protein, peptide and non-peptide drug PEGylation for therapeutic application

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For many years proteins have been investigated as therapeutic agents, but unfortunately their potential advantages could not be completely exploited. The main drawbacks are their intrinsic short life in the body, immunological adverse reaction and proteolytic digestion. Among all the approaches studied for overcoming these problems, PEGylation (the modification of molecules with polyethylene glycol [PEG]) achieved the most interesting results, leading to a novel series of products that have already reached the market, and hopefully other promising agents will soon be available. Since the first studies in this field, the conjugation of PEG to a protein has shown the possibility of improving the pharmacokinetic profile of a linked drug. In the last few years this technology, firstly developed for proteins, has been transferred to non-peptide drugs, opening a new area of investigation that is now receiving increasing interest. This leads to new opportunities for many therapeutic treatments as it is possible to use molecules that could not before be exploited due to limitations such as inadequate water solubility, high nonspecific toxicity and poor pharmacokinetic profiles. In this review the most recent achievements in PEGylation of protein, peptide and non-peptide drugs are described concerning the binding chemistry, and many examples from the literature are reported, in the fields of both protein therapeutics and non-peptide drugs.

Keywords: polyethylene glycol (PEG), PEG-drugs, PEG protein, PEGylation, polymer therapeutics

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1. Introduction

Polymer bioconjugation is receiving increasing interest in applied biotechnology, and polyethylene glycol (PEG), in particular, has been extensively used in the modification of different substrates of therapeutic interest.

It is usually common to divide therapeutic polymer conjugates into two main categories: conjugates with drugs having a peptide structure (so far the most studied area) and those with a non-peptide structure, in particular low molecular weight drugs such as anticancer drugs. The success of this technology is reflected not only by the many drug-polymer conjugates already available or under investigation but also by the growing number of publications and patents published each year.

Polypeptides and many low molecular weight molecules usually have shortcomings that restrict or even prevent clinical use. Common limits for many drugs are short *in vivo* half-life (due to enzyme degradation or rapid kidney clearance) and physicochemical drawbacks, for example low solubility or instability; polypeptides may present additional restrictions, such as immunogenicity and antigenicity [1-3]. These problems are often addressed by covalent linking of the pharmaceutically active molecule to polymers (Box 1), PEG being the most successful among those used [4-6]. Polymer conjugation, especially for small drugs, is also exploited to achieve an active or passive targeting, an improved biodistribution and a selected cellular uptake by endocytosis [7-9].

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Box 1. General advantages of bioconjugation in therapeutic application.

Stabilisation of labile drugs from chemical degradation
 Protection from proteolytic degradation
 Reduction of immunogenicity
 Decreased antibody recognition
 Increased body residence time
 Modification of biodistribution
 Drug penetration by endocytosis
 New strategies for drug targeting
 Increased water solubility
 Reduced toxicity

All protein and peptide drugs are candidates for alternative delivery methods. It is noteworthy that peptide drugs are worth > US\$10 billion of the world pharmaceutical market and represent a rapidly growing area. As drug delivery is closely tied with pharmaceutical manufacture, it is anticipated that its market will be worth an estimated US\$120 billion by 2007 and bioconjugation appears to be one of the most promising approaches to reach this goal.

Although polymer conjugation to proteins originated in the 1950s and 1960s using polysaccharide polymers, the real boost in this field was represented by the use of PEG, thanks to the pioneering studies conducted in the late 1970s by Professor Frank Davis at Rutgers University [10]. Since then many excellent reviews have been dedicated to different aspects of this technique, known as PEGylation, whereas this review focuses on more recent pharmaceutical applications of PEG with a particular attention to the patent literature.

2. General aspects of PEGylation

PEG, approved by the FDA for human use, has a variety of interesting properties such as the absence of immunogenicity, antigenicity and toxicity, and high solubility in water and in many organic solvents. These properties can be transferred to the final conjugates by PEGylation, obtaining modification of the pharmacokinetic and pharmacodynamic profiles of native drugs [11]. In general, PEGylated drugs, when compared with the parent molecules, show: i) reduced kidney excretion and altered biodistribution, mainly due to the increased molecular weight; ii) reduced degradation by proteolytic enzymes or hydrolytic media; iii) enhanced water solubility; iv) reduced reticuloendothelial (RES) clearance and v) reduced immunogenicity and antigenicity [12,13].

The evolution of protein PEGylation has been frequently described, and is divided into two generations:

- The first generation of conjugates refers to PEGs with low molecular weights (≤ 12 kDa) and with a relevant percentage of PEG diol impurities, a potential crosslinking agent originating from the synthesis of methoxy-PEG.

Furthermore, the chemistry employed often presented side reactions or led to weak and reversible linkages. Despite these initial difficulties, important products were created and some reached the market, such as Enzon Pharmaceuticals PEG-adenosine deaminase (Adagen[®], Enzon, Inc.) [14,201] for the treatment of severe combined immunodeficiency disease (SCID) and PEG-asparaginase (Oncaspar[®], Enzon, Inc.) [15,202] for the treatment of leukaemia.

- The second generation of conjugates was an improvement on the first, in particular as far as PEG purity is concerned; an important reduction in polydispersity and in diol amounts was achieved in industrial production and improvements are also made in selectivity of protein modification and in the range of available activated PEGs. Heterobifunctional PEGs were prepared in order to link a second molecule with a targeting role. Moreover, spacers between the polymer and the drug were studied to allow the release of bound drug under specific triggering conditions. Several products of this second generation reached the market, such as PEG-IFN- α 2b (PEG-Intron[®], Schering-Plough) [16,203], branched PEG (40 kDa)-IFN- α 2a (Pegasys[®], Roche Pharmaceuticals) [17,18,204], PEG-growth hormone receptor antagonist (Pegvisomant, Somavert[®], Pfizer) [19,205] and PEG-granulocyte-colony-stimulating factor (G-CSF) (pegfilgrastim, Neulasta[®], Amgen) [20,206] but many others are presently undergoing clinical trials and will hopefully be available in the near future.

3. Polyethylene glycol (PEG), a bioconjugation polymer

PEG, as obtained by ethylene oxide polymerisation, presents one or two terminal hydroxyl groups (mPEG-OH or HO-PEG-OH) depending upon the initiator of polymerisation: methanol or water, respectively. A branched form may also be obtained, as well as products with multiple reactive groups, at one or at both extremes (Figure 1). While the monofunctional polymers, linear or branched, are used for protein modification, those with multiple groups are indicated to enhance the loading of low molecular weight drugs.

The main characteristics of PEG, as compared to other polymers, are low polydispersity (M_w/M_n spanning from 1.01 for PEG < 5 kDa molecular weight and up to 1.1 for PEG as high as 50 kDa molecular weight), solubility in both aqueous and organic solvents and biocompatibility. The unique solvation properties of PEG are due to the ability to coordinate 2–3 water molecules per ethylene oxide unit and to the highly flexible the backbone chain [2]. These characteristics give PEG an apparent molecular weight 5–10 times higher than that of a globular protein of comparable mass, as may be verified by gel permeation chromatography (see [21] for a recent discussion) and this explains the protein-rejecting property, at the basis of the anti-immunogenicity and antigenicity conveyed to a conjugate [22]. This also explains the prolonged blood residence time and the decreased degradation by mammalian cells and enzymes [23].

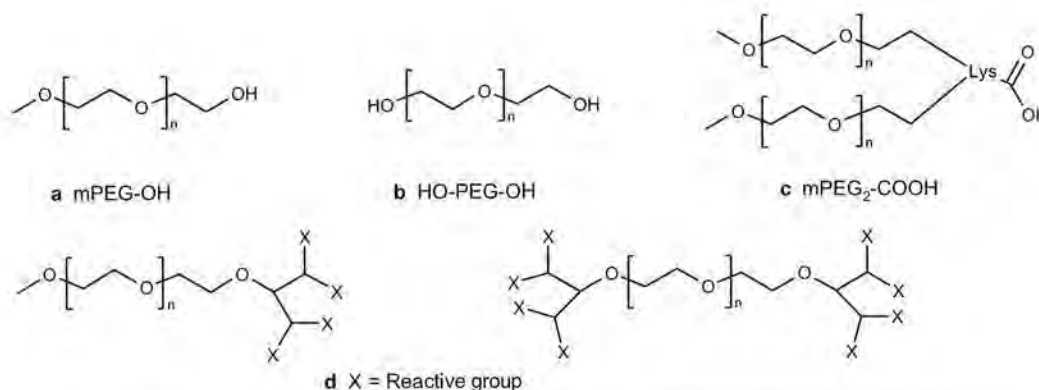


Figure 1. Different PEG structures. a) Linear monomethoxy PEG; b) Linear diol PEG; c) Branched PEG; and d) Multifunctional PEGs. PEG: Polyethylene glycol.

In vivo, PEG undergoes limited chemical degradation and its clearance depends on its molecular weight, < 20 kDa. PEG is easily secreted into urine, whereas for higher molecular weights it is eliminated more slowly into urine and faeces, up to the threshold of 40 – 60 kDa (a hydrodynamic radius of ~ 45 Å [24]), which represents the albumin excretion limit. Above this limit the polymer remains in circulation and is mainly accumulated in the liver. Alcohol dehydrogenase can degrade low molecular weight PEGs and chain cleavage can also be catalysed by cytochrome P450 microsomal enzymes [25]. Many years of the use of PEG as an excipient in foods, cosmetics and pharmaceuticals without toxic reactions are a clear proof of its safety [23].

Trends for new PEGs are directed towards: i) monodisperse polymer batches, not only in the case of low molecular weight PEG (≤ 600 Da), which have recently reached the market, but also for an extended range of higher molecular weights; ii) PEG polymers with specific functions tailored to provide conjugation with reactive groups on target molecules, also in view of a controlled release; and iii) high loading PEGs to increase the payload of active molecules conjugated to the chain, through the construction of dendrimeric structures or multi-arm forms at the polymer extremes [26,27,207].

4. Chemistry of polyethylene glycol (PEG) conjugation

The first generation PEGs were mainly designed for amine modification, as amines are a widely represented functional group in proteins. Among these, of particular note are: i) PEG succinimidyl succinate (SS-PEG); ii) PEG succinimidyl carbonate (SC-PEG); iii) PEG *p*-nitrophenyl carbonate (pNPC-PEG); iv) PEG benzotriazolyl carbonate (BTC-PEG); v) PEG trichlorophenyl carbonate (TCP-PEG); vi) PEG carbonylimidazole (CDI-PEG); vii) PEG tresylate; and viii) PEG dichlorotriazine (Figure 2).

Carbonate PEGs, such as pNPC-PEG, CDI-PEG and TCP-PEG, show slower reactivity than other PEG derivatives,

thus allowing a selective conjugation on the basis of reactivity of the different amino groups. It is noteworthy that PEG dichlorotriazine, PEG tresylate and PEG aldehyde (after sodium borohydride reduction) allow the same total charge of the native protein to be maintained because the conjugation, based on alkylation, leads to a secondary amine, whereas the coupling performed with the other PEGs, based on acylation, yields neutral acidic amide or carbamate linkages. Although, the primary amino group is the more reactive in proteins, PEGs such as SC-PEG, BTC-PEG and PEG dichlorotriazine can slowly react with hydroxyl groups (Ser, Tyr) and the histidine imidazole side chain to give hydrolytically unstable linkages. For example, one histidine residue of α -IFN was conjugated to SC-PEG or BTC-PEG under slightly acidic conditions [208]. PEG was also linked to hydroxyl groups of serine and tyrosine in the decapeptide antide and in the epidermal growth factor (EGF) [28,209], respectively.

The improvement of polymer synthesis and conjugation chemistry is now yielding a second generation of PEGs, mainly characterised by lower percentages of diol contaminant in polymer batches. This was achieved by the isolation of the monocarboxylic acid intermediate of PEG from the bicarboxylic intermediate, coming from the diol by ionic exchange chromatography [210]. Higher molecular weight polymers, with an improved pharmacokinetic profile and stability for non-peptide drugs, were also obtained. Among the new PEGs are reported:

- PEG-propionaldehyde, also in the form of the more stable acetal [211]: the reaction with the amino group leads to a Schiff base that is reduced by sodium borohydride, giving a derivative that maintains the same total ionic charge of the parent drug.
- PEG-succinimidyl derivatives: highly reactive towards amine group. The reaction rate of these derivatives may significantly change depending upon the extension and the composition of the alkyl chain between PEG and succinimidyl moiety [29,212].

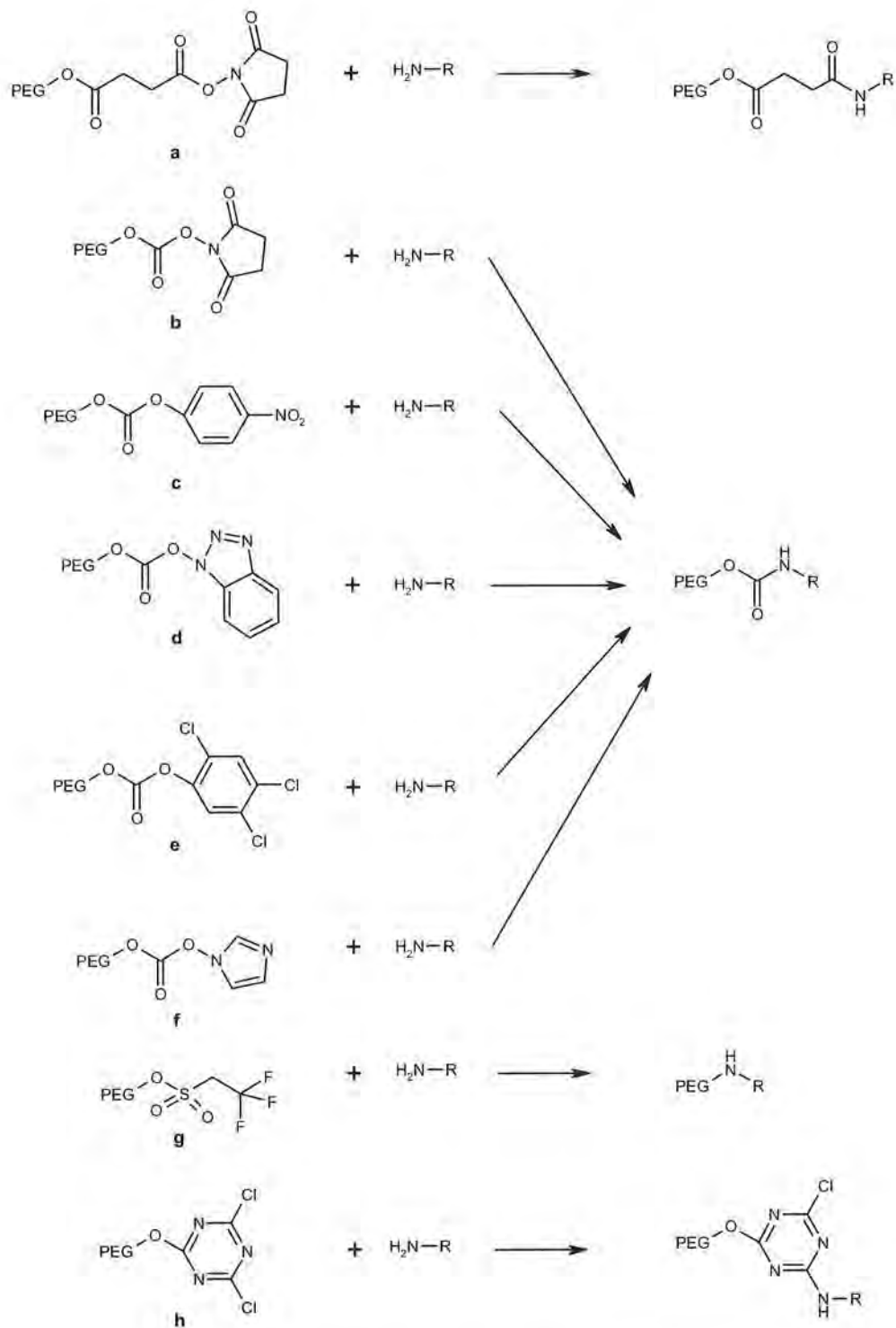


Figure 2. Examples of activated PEG molecules reactive towards amino groups. a) PEG succinimidyl succinate; b) PEG succinimidyl carbonate; c) PEG p-nitrophenyl carbonate; d) PEG benzotriazol carbonate; e) PEG trichlorophenyl carbonate; f) PEG carbonylimidazole; g) PEG dichlorotriazine; and h) PEG tresylate.
PEG: Polyethylene glycol.

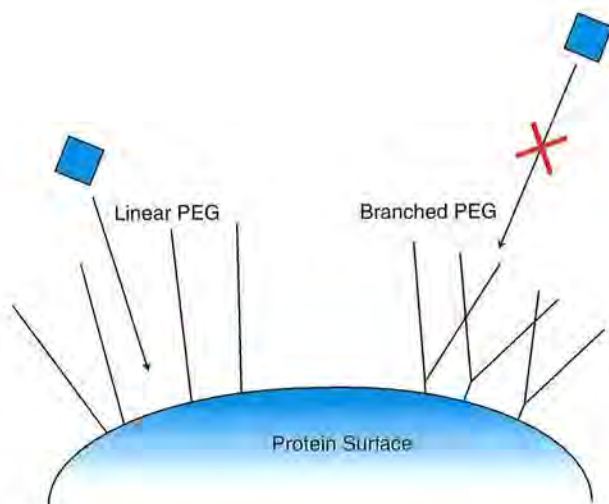


Figure 3. Structure of linear and branched PEG on the protein surface. The 'umbrella-like' structure of branched PEG explains its higher capacity for rejecting approaching molecules or cells compared to linear PEG.

PEG: Polyethylene glycol.

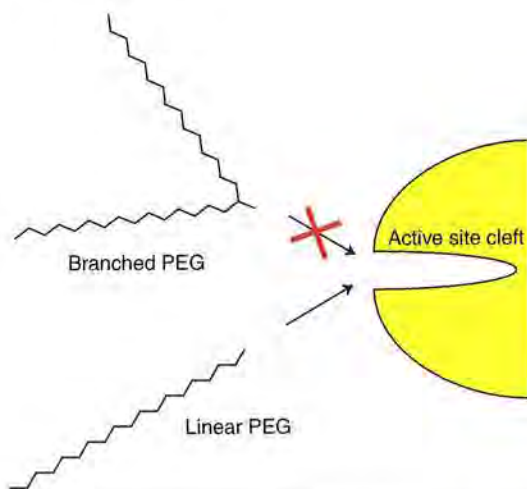


Figure 4. Effect of PEG hindrance on the enzyme active site. The high steric hindrance of branched PEG may be advocated to explain the lower inactivation of enzymes compared to linear PEG of the same size.

PEG: Polyethylene glycol.

- 'Y'-shaped branched PEG [30,213,214] (see Figure 1c): for its increased surface shielding (Figure 3), this PEG reagent is more effective in protecting the conjugated protein from degradative enzymes and antibodies. Moreover, enzymes modified with this PEG retain more activity with respect to the same enzyme modified by linear PEGs. This effect is probably due to the hindrance of the branching polymer that prevents the entrance of PEG inside the enzyme active site cleft (Figure 4).

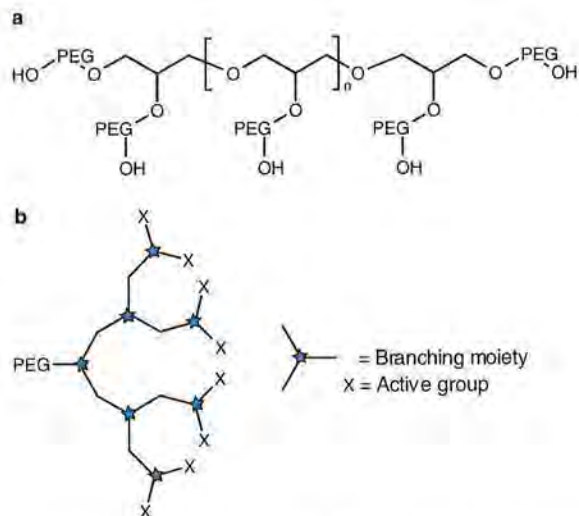


Figure 5. Different strategies to achieve multifunctional high loading PEGs. a) Multiarm PEGs. b) Dendronised PEGs, the branching moiety may be a bicarboxylic amino acid, lysine or other bifunctional molecule.

PEG: Polyethylene glycol.

- PEGs reactive toward thiol groups: PEG-maleimide (MAL-PEG) [215], PEG-vinylsulfone (VS-PEG) and PEG-orthopyridyl-disulfide (OPSS-PEG). Even if the thiol addition rate to the first two derivatives is very rapid, some addition to the amino group (mainly present in proteins) may also take place, especially at basic pHs. On the other hand, the reaction with OPSS-PEG is very specific for thiol groups but the conjugates may be reversed in the presence of thiols as reducing agents.
- Heterobifunctional PEGs [31,32,216]: these derivatives present two different functional groups, one for each extreme, which simplify the linking of different molecules to the same PEG chain. Therefore, it is easier to obtain conjugates that carry both a drug and a targeting molecule. Among the proposed and commercially available heterobifunctional PEGs, $\text{H}_2\text{N-PEG-COOH}$, HO-PEG-COOH and $\text{H}_2\text{N-PEG-OH}$ are the most used.
- PEG with linkers designed for a controlled release of the conjugated drug: one of the most exploited linkers is a peptide sequence, designed to be recognised and cleaved by lysosomal enzymes when the conjugates reach the intracellular compartment. Examples of such peptide linkers are $\text{H-Gly-Phe-Leu-Gly-OH}$ or $\text{H-Gly-Leu-Phe-Gly-OH}$ [33,34]. Alternatively, a linker may respond to pH changes or release the drug by a 1,6-elimination reaction or by a cyclisation reaction [35]. Moreover, the linker and the polymer together can form a double prodrug system, where the drug released is obtained after polymer hydrolysis (first prodrug) that triggers the linker (second prodrug), as reported for he drug delivery system based on trimethyl lock (TML) lactonisation [36,217].
- Multiarm or 'dendronised' PEGs (Figure 5): the former are compounds prepared by attaching linear PEG to a

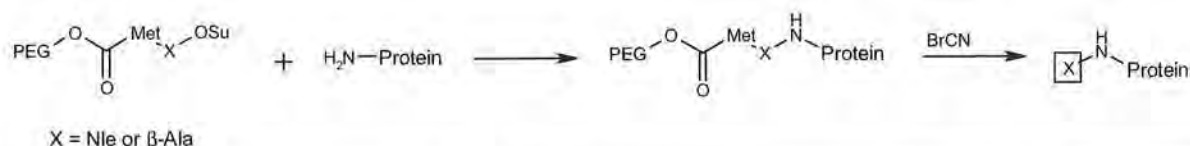
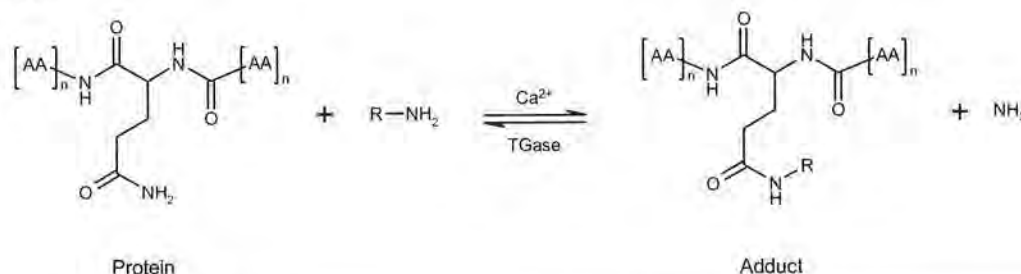


Figure 6. Use of PEG-Met-Nle-OSu or PEG-Met- β -Ala-OSu to introduce a reporter amino acid at the PEGylation site. PEG conjugation is followed by polymer moiety release by BrCN to leave Nle or β -Ala that can be identified on the protein by amino acid sequence analysis.

PEG: Polyethylene glycol.



Scheme 1. Reaction between a glutamine residue in a protein and an alkyl amine, catalysed by TGase.

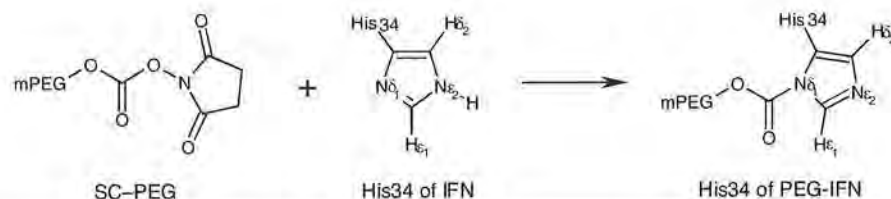
TGase: Transglutaminase.

multimeric compound [218], whereas the latter are linear PEGs with a dendritic structure at one or both chain extremes [26,37,207]. The aim of both derivatives is to increase the drug:polymer molar ratio, overcoming problems of high viscosity that may occur with monofunctional drug conjugate solutions, in particular for therapeutic treatments that require quantity of drug.

PEG and any polymer conjugate, especially when multifunctional drugs are involved, face great problems regarding reproducible synthesis, scaling-up, characterisation and methods of analysis, all issues that have been discussed in detail [38,39]. One additional problem in protein conjugation is the localisation of the PEGylation site into the primary amino acid sequence. So far the classical approach involves proteolytic digestion followed by Edman degradation, as reported for PEGylated IFN-2 α [40], where the PEGylated sites are determined by the missing amino acids. Beyond the lengthy procedure, the polymer may interfere with the analysis as cleavage by proteolytic enzymes may be incomplete due to steric hindrance. A more direct approach involves the use of two tailor made PEGs, PEG-Met-Nle-COOH or PEG-Met- β Ala-COOH [219], which possess a labile bond in the peptide spacer arm that can be cleaved by treatment with BrCN (Figure 6). After cleavage at the methionine level, the PEG chains are removed and the remaining nor-leucine or β -alanine tags, linked to the protein, are identified with standard sequence investigation methods. The tag can be revealed by amino acid analysis or mass spectrometry [41].

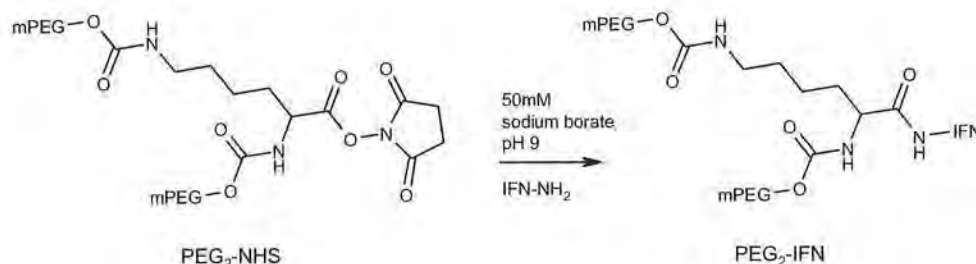
A PEGylation procedure that differs from all others was recently developed by Sato and involves the use of the enzyme transglutaminase (TGase) [42,220]. *In vivo* TGase catalyses crosslinking between a protein containing an acceptor

glutamine and another protein with an amino donor group, such as lysine [43,44]. This enzyme catalyses the attachment of PEG-alkylamine, which behaves as a new substrate of TGase to the glutamine residues of the protein (Scheme 1). This system provides new opportunities in polymer derivatisation; in fact, beyond the high homogeneity of the TGase-catalysed conjugates, recombinant DNA techniques, it may be used in combination to introduce amino acids sequences that are specific and more favourable TGase substrates. Amongst the several TGases isolated from natural sources, the guinea-pig liver TGase (G-TGase) and the microbial TGase (M-TGase) were reported for PEGylation procedures. The former possesses more stringent requirements on neighbouring glutamine amino acids for enzyme activity and transglutamination site flexibility; although there is no unique amino acid recognition sequence in proteins, G-TGase has been used for the preparation of site-specific PEGylated conjugates [45,46]. With TGase, the suggested strategy consists of the introduction of a specific sequence, comprising a glutamine residue, at the N terminus of proteins, in order to maintain the peptide backbone flexibility. Instead, M-TGase does not require high specificity in the substrate sequence, although an optimal accessibility to the glutamine site is required either through peptide flexibility or high solvation of the sequence [47]. On the other hand, the amino donors (i.e., PEG-NH₂) do not need strict requirements to allow TGase conjugation, and high affinity PEG-NH₂ has been designed to link the amino group to the PEG with a spacer chain of six carbon atoms (PEG-(CH₂)₆-NH₂ [48]). In the case of IL-2, the TGase-catalysed PEGylated form preserved a high degree of activity because the N terminus is, in this protein like many others, a flexible region and so is less involved in peptide recognition. The enzyme-catalysed coupling seems to yield more



Scheme 2. Adduct formation at the level of His34 using SC-PEG as PEGylating agent. (Reproduced with permission from [52]).

PEG: Polyethylene glycol; SC: Succinimidyl carbonate.



Scheme 3. PEGylation of IFN- α -2a by branched mPEG₂-COOH (40 kDa). (Reproduced with permission from [17]).

NHS: N-Hydroxysuccinimide; PEG: Polyethylene glycol.

homogenous derivatives than the chemical PEGylation that is known to often lead to a mixture of isomers.

5. Polyethylene glycol (PEG)–protein conjugates

Many proteins and peptides have short half-lives when administered *in vivo*, due to other proteolytic enzyme activity, fast kidney ultrafiltration and activation of immune system response. PEGylation may overcome all of these drawbacks thanks to the increased hydrodynamic volume of conjugates and the shielding effect of PEG towards enzymes and antibodies. Due to protein multivalence, the PEGs used for protein conjugation should have only one reactive group to avoid crosslinking reactions; for this reason, batches completely devoid or with minimal traces of PEG diol must be employed. Furthermore, to avoid protein denaturation, the conjugation chemistry should be gentle, yield stable bonds, avoid unmasking of epitopes or sites of proteolysis and, where possible, it should lead to homogeneous products or, at least, reproducible positional isomers. Far from addressing all and each of the above-mentioned requirements, several conjugates have been engineered showing interesting improvements over the starting materials, and some conjugates have already hit the market.

5.1 Interferons

Type 1 interferons (IFN- α , - β , - κ , - τ and - ω) are structurally and functionally related proteins belonging to a cytokine family. Their activity is mediated by IFN-inducible gene activation [49] that occurs when the IFN binds to a multimeric cell surface receptor. The activities of these proteins include a wide range of effects, mainly antiviral, antitumour and

immunomodulatory properties [50]. IFN- α was first approved for hairy-cell leukaemia therapy, then for the treatment of hepatitis B and C and later for various dermatological pathologies; INF- β was approved for the treatment of multiple sclerosis. The low molecular weight of interferons (~ 20 kDa) reflects the relatively short serum half-life of these proteins, which was improved by PEG conjugation.

The first study in this direction was the modification of IFN- α -2a with linear succinimidyl carbonate PEG (SC-PEG; 5 kDa) via a urea linkage. The coupling, performed at equimolar ratio of protein and polymer, mainly led to mono-PEGylated isomers and, in small amounts, di-PEGylated conjugates as well as free IFN [221]. Characterisation of the conjugates indicated that lysine residues were the site of PEGylation [40]. Although a once a week schedule was possible, while the unconjugated protein was usually given three times a week, the desired pharmacokinetic/activity profile was not yet achieved. After conjugation of PEG to IFN- α -2b via a carbamate linkage, Enzon researchers proposed a method that, by acidification of the conjugate aqueous solution to pH 3 or lower, allowed the removal of the few PEG chains that were attached to INF's amino acid residues (i.e., His) through unstable carbamate bonds [222].

Further investigations demonstrated that derivatives with better pharmacokinetic profiles and higher activity could be obtained by conjugation of IFN- α -2b with SC-PEG (12 kDa) in sodium phosphate buffer solution at pH 6.5. This reaction gave an unexpected conjugate at His34, representing ~ 47% of the total PEGylated species (Scheme 2) [51,203]. The stability of this species was studied by [¹H]-nuclear magnetic resonance (NMR) analysis following the ppm shift of H ϵ_1 of His34 during its conversion from the PEGylated to the

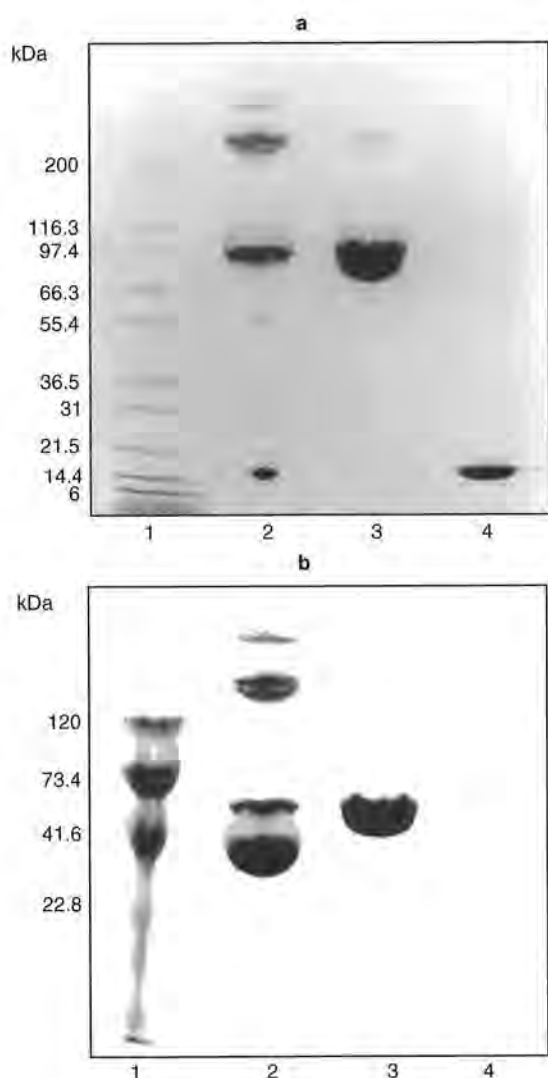


Figure 7. SDS-PAGE analysis of the PEGylated IFN- α -2a mixture. **a)** Gel specifically stained for protein with Coomassie blue, Lane 1: molecular weight marker protein; Lane 2: PEGylation reaction mixture; Lane 3: purified PEG₂-IFN; Lane 4: IFN- α -2a. **b)** Gel specifically stained for PEG with iodine, Lane 1: molecular weight marker PEGs; Lane 2: PEGylation reaction mixture; Lane 3: purified PEG₂-IFN; Lane 4: IFN- α -2a. Note that the IFN- α -2a is not stained by iodine. (Reproduced with permission from [17]).

PEG: Polyethylene glycol; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 1. Pharmacokinetic properties of IFN- α -2a and its PEGylated form in rats [18].

Protein	Half-life (h)	Plasma residence time (h)
IFN- α -2a	2.1	1.0
PEG ₂ (40 kDa)-IFN- α -2a	15.0	20.0

PEG: Polyethylene glycol.

nonPEGylated form [52]. The higher activity of this IFN preparation was related to the ability to release free and fully active IFN by slow hydrolysis of the His-PEG bond [208]. Further studies were also carried out to find adequate formulations to prevent loss of activity and denaturation of IFN-PEG during the conjugation and the lyophilisation procedure [223]. These investigations allowed marketing PEG-Intron in 2000. Although the *in vitro* potency of this IFN-PEG is only one-quarter of the free interferon form, its serum life is approximately six times longer, allowing for a less frequent administration schedule while maintaining an efficacy comparable with unmodified IFN [16,53].

A different approach to IFN-PEGylation exploited the special properties of branched PEGs. A high molecular weight branched PEG (PEG₂, 40 kDa) was chosen on the basis of several preliminary studies disclosing that: i) the protein surface protection at the conjugation site with a single, long chain PEG is better than several small PEG chains at different sites [4]; ii) branched PEGs have lower distribution volumes than linear PEGs of identical molecular weight and the delivery to organs such as liver and spleen is faster [54] and iii) proteins modified with branched PEG possess greater stability towards enzymes and pH degradation [30]. The 40 kDa branched succinimidyl PEG (PEG₂-NHS) was linked to IFN- α -2a using a 3:1 PEG:protein molar ratio in 50 mM sodium borate buffer pH 9 (Scheme 3) [17]. PEGylation under these conditions led to a mixture containing 45 – 50% monosubstituted protein, 5 – 10% polysubstituted (essentially dimer) and 40 – 50% unmodified IFN (Figure 7). Identification of the major positional isomer within the mono-PEGylated fraction was carried out by combination of high performance cation exchange chromatography, peptide mapping, amino acid sequencing and mass spectroscopy analysis, which showed that PEG was attached mainly to either Lys31, Lys121, Lys131 or Lys134 [17,204]. Even though the *in vitro* antiviral activity for PEG₂-IFN was greatly reduced (only 7% of residual activity was found), the *in vivo* activity, measured as the ability to reduce the size of various human tumours, 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 1. These studies brought into the market a long-lasting blood IFN conjugate, Pegasys, that is effective in eradicating hepatic and extrahepatic hepatitis C virus (HCV) infection [18].

A different cytokine, IFN- β , is approved for the treatment of multiple sclerosis in the US; like other cytokines, it suffers from a short blood residence time, again suggesting that a PEGylation strategy might be a solution to the problem. An exhaustive study conducted by Pepinsky and colleagues [55] resulted in a PEG modification of IFN- β -1a that exploited a reductive alkylation of an amine residue in phosphate buffer at pH 6 with an excess of 20 kDa PEG aldehyde and sodium cyanoborohydride to reduce the intermediate Schiff base. The conjugate, after extensive purification by gel-filtration and ion

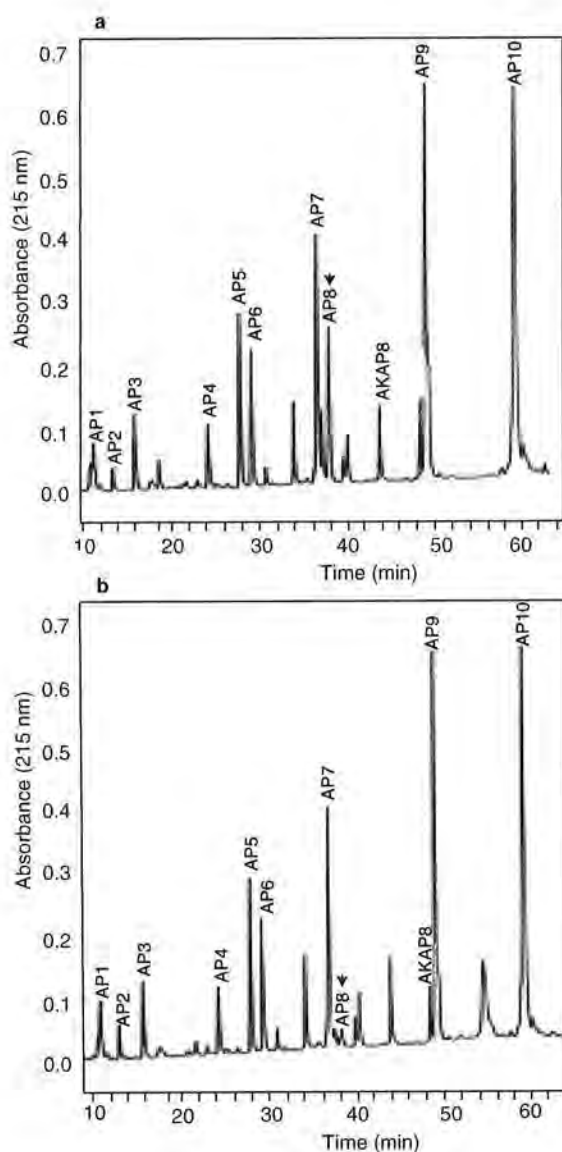


Figure 8. Localisation of the site of PEGylation by C4-HPLC peptide mapping following digestion with endoproteinase Lys-C. a) Unmodified IFN- β -1a. b) PEGylated IFN- β -1a. Arrowheads (pointing to peak AP8) mark the elution position of the N-terminal endoproteinase Lys-C peptide of IFN- β -1a containing amino acid residues 1–19, which disappears in the PEGylated protein in **b**. (Reproduced with permission from [55]).

HPLC: High performance liquid chromatography; PEG: Polyethylene glycol.

exchange chromatography, was identified as mono-PEGylated IFN modified at the N-terminal amine, as demonstrated by peptide mapping (Figure 8). Such a result could be achieved thanks to the high polymer molecular mass that prevented multiple conjugation due to steric hindrance and to the higher reactivity of the N-terminal amine with respect to the ϵ -amine of lysine in acid buffer. The modified protein retained the same potency of native IFN in activity assays,

while pharmacokinetic experiments showed a fivefold increase in serum half-life. A 20 kDa PEG was chosen, as the 5 kDa PEG led to a fully active conjugate but with no improvement in the pharmacokinetic profile, while the 40 kDa PEG derivative was completely inactive.

A different approach was followed by Shearwater and Sero researchers, taking advantage of a site-specific PEGylation at Cys17 of IFN- β [224] using a thiol-reactive PEGylating agent, OPSS-PEG. Conjugation could be specifically directed to Cys17 since the other two cysteines are, in the native form of IFN- β , involved in a disulfide bridge. Derivatisation was carried out with PEGs of different molecular weights but with a special and original strategy, well described in the patent. Low molecular weight PEGs are, in general, more reactive than those with a high molecular weight and in this special case the low molecular weight PEGs can overcome the steric hindrance around the thiol moiety at Cys17 IFN- β , yielding polymer-IFN conjugates. On the basis of this observation, a modification with high molecular weight PEGs was obtained via a two-step procedure: in the first step the protein was modified with a low molecular weight, heterobifunctional PEG oligomer; conjugation with a higher molecular weight PEG possessing specific reactivity towards the free terminal end of the first oligomer completed the procedure (Figure 9). This strategy implied the use of a special heterobifunctional PEG oligomer with a group reactive towards the free cysteine (OPSS) at one extreme and a hydrazine group at the other. Hydrazine was chosen since it is characterised by a very low acid dissociation constant ($pK_a = 3 - 4$), as compared to the protein amine residues ($pK_a = 7 - 9.5$). The PEG oligomer selected for this conjugation was therefore the OPSS-PEG-hydrazine (OPSS-PEG-Hz; 2 kDa) leading, in the first step, to IFN-SS-PEG-Hz. This was selectively modified with PEG-aldehyde (30 kDa) by reductive alkylation. The overall yield was >80% and PEG-IFN- β -1a from Sero has now completed Phase I clinical trials.

5.2 Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that controls proliferation, differentiation and functional activation of neutrophilic granulocytes [56]; it is already used to treat granulocyte depletion during chemotherapy [57]. Conventional PEGylation of G-CSF yielded a mixture of conjugates with prolonged blood residence time and resistance towards enzyme degradation. Kinstler proposed a reductive alkylation strategy with PEG-aldehyde/sodium cyanoborohydride in acidic buffer solution (pH 5, Scheme 4) to selectively label the low pK_a amino groups [225]. Under these conditions, modification limited to the N-terminal methionine of r-metHuG-CSF was obtained (Figure 10) [20,58]. The selective modification at the α -amino group with respect to the ϵ -amino residue of lysine can be explained on the basis of their differences in pK_a (7.6–8.0 and 10.0–10.2, respectively); at moderately acidic pH values the α -amino

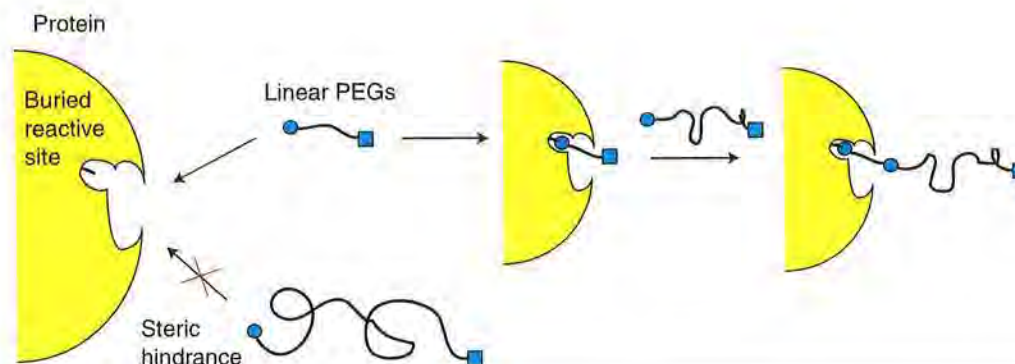
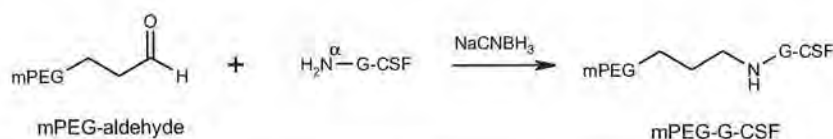


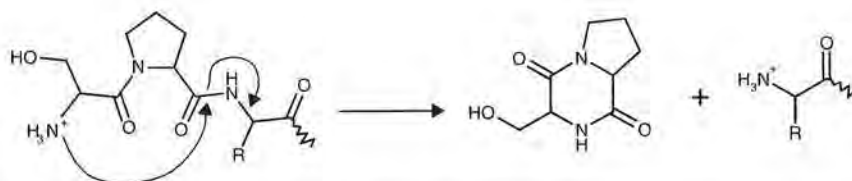
Figure 9. Two-step tagging PEGylation strategy for a buried thiol group in a protein. Smaller PEG molecules are more reactive than high molecular weight PEGs.

PEG: Polyethylene glycol.



Scheme 4. 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. (Reproduced with permission from [20]).

G-CSF: Granulocyte colony-stimulating factor.



Scheme 5. Chemical inactivation of rHuMGDF by cyclisation and cleavage of the first two N-terminal amino acids, Ser and Pro. The degradation leads to the formation of diketopiperazine and *des*(Ser,Pro)rHuMGDF. (Reproduced with permission from [66]).

MGDF: Megakaryocyte growth and development factor.

group is still reactive [59]. The PEG 20 kDa conjugate obtained presented an improved pharmacokinetic profile which is mainly due to reduced kidney excretion. The high clearance of G-CSF is also due to an internalisation process of the receptor–ligand complex in neutrophil cells and is in some way related to the number of circulating neutrophils. PEG–G-CSF, which remains in the bloodstream for a prolonged time, stimulates the proliferation of neutrophils and consequently causes its own clearance when the therapeutic target is achieved. The PEG–G-CSF conjugate pegfilgrastim has been on the market since 2002.

A number of PEGylation studies were also carried out on G-CSF mutants, a new class of genetic variants with higher activity with respect to the native human G-CSF. Several PEGs with different molecular weights and active moieties were investigated, the best one was the 20 kDa mPEG–succinimidyl propionic acid (mPEG–SPA) [226]. The *in vitro* activity of these PEG–G-CSF conjugates decreased with the increase of either the number of PEG chains or the PEG

molecular weight attached; on the contrary, the *in vivo* activity was directly proportional to the total molecular weight of PEG [60,61]. The extended blood residence time, of higher molecular weight conjugates, is the improvement that overcame the receptors reduced binding affinity after polymer conjugation. These findings indicate the difficulty in evaluating the success of a polymer–protein conjugate on the basis of *in vitro* assays only.

An interesting PEG modification was recently proposed for a multimeric single chain G-CSF [227], which is a covalent dimer of G-CSF. The dimer was obtained by a DNA recombinant technique using an expression vector encoding for two G-CSF polynucleotides sequences. The dimeric protein was also produced with one or more mutated sites in the amino acid sequence with the aim to improve the activity or to allow an easier PEGylation. PEG–SPA of different molecular weights (preferably 5, 12 and 20 kDa) was used in the modification, leading to conjugates with lower activity, as compared to G-CSF.

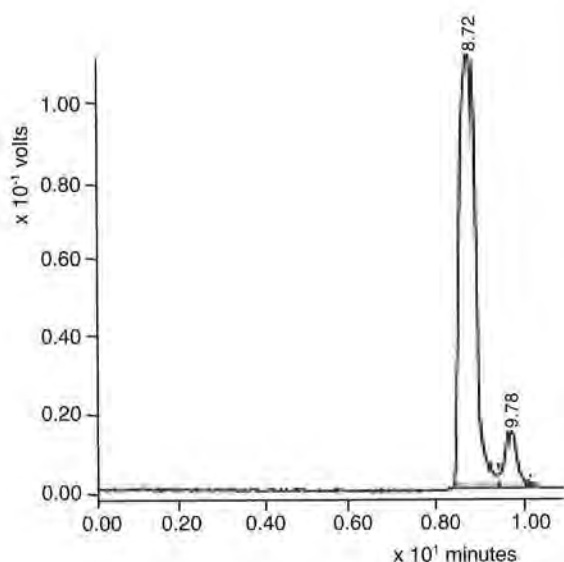


Figure 10. Size-exclusion HPLC analysis (UV detector at 280 nm) of the reduced mixture of r-MetHuG-CSF with mPEG aldehyde (molecular weight = 6 kDa). N-terminal mono-mPEG-G-CSF conjugate eluted at 8.72 min (92% of total area); unreacted r-MetHuG-CSF eluted at 9.78 min (8% of total area). (Reproduced with permission from [20]).

G-CSF: Granulocyte colony-stimulating factor; HPLC: High-performance liquid chromatography; PEG: Polyethylene glycol; UV: Ultraviolet.

5.3 Megakaryocyte growth and development factors

Thrombopoietin or megakaryocyte growth and development factor (MGDF) is a key regulator of thrombopoiesis [62] that acts on the expansion and maturation of megakaryocyte progenitor cells with the final result of an increase in platelet counts [63]. The level of thrombopoietin is thought to be self-regulated, mainly by internalisation, after binding to its cell surface receptors present in megakaryocytes and platelets [64].

Kinstler and co-workers synthesised and studied a recombinant truncated form of human MGDF, comprising the 1 – 163 sequence of the native protein (rHuMGDF), that exhibited a specific activity fivefold higher than the full-length hormone. Since the *in vivo* activity of the native form is extremely reduced due to its fast elimination with urine, a PEG-rHuMGDF was synthesised [228] with the same chemical strategy already proposed by the author in G-CSF modification [58]. As expected, the conjugation allowed an extended blood residence time [65], thanks to reduced kidney clearance and to the prevention of a specific non-enzymatic degradation of the protein. This chemical inactivation of MGDF is due to cyclisation and cleavage of the first two amino acids, leading to diketopiperazine and *des*(Ser,Pro)rHuMGDF (Scheme 5). Such a reaction could be avoided thanks to PEGylation of the N-terminal amine with PEG aldehyde [66]. When tested in increased platelet count

experiments, the PEG derivative was as active as the glycosylated full-length native thrombopoietin and much more active than both the free rHuMGDF and the non-glycosylated full protein [20]. Interestingly, in this research the truncated active form of the protein and not the native form was elected for PEGylation, obtaining a conjugate with comparable activity and improved pharmacokinetics with respect to the full-length hormone. This method could open new strategies and potentials for protein PEGylation.

5.4 Growth hormone, growth hormone-releasing hormone and growth hormone antagonists

Human growth hormone (hGH) is a protein that has several effects, including linear body growth, tissue growth, activation of macrophages, lactation, insulin-like and diabetogenic effects [67]. It exploits its action through receptor recognition and signalling that requires receptor dimerisation. Clinically, it is used to treat GH deficiency that causes short stature and both Turner's syndrome and cachexia in AIDS patients. A long-lasting form would be welcome because, due to its short *in vivo* half-life, hGH must be administered on a daily basis. One of the first studies in this respect involved a random PEGylation of hGH with low molecular weight PEG-*N*-hydroxysuccinimide (PEG-NHS; 5 kDa), leading to a mixture of derivatives. After purification it was possible to isolate hGH conjugates with up to seven PEG chains (Figure 11) [68]. Binding studies demonstrated the inverse correlation between the number of PEG chains and affinity, as PEG reduced the association rate for the receptor/adduct formation. On the contrary, the higher the number of PEG chains linked, the higher the *in vivo* potency, due to reduced kidney clearance. It was found that hGH conjugated to five PEG chains was a good compromise for therapeutic purposes, presenting tenfold higher potency than the unmodified hormone.

To overcome the polysubstitution issue, a different approach was investigated for hGH conjugation; it was based on the recombinant DNA technique that allowed the preparation of several monocysteine hormone muteins. Among all the possible mutations described in the literature and patents, the addition of a cysteine residue to the C-terminal amino acid of hGH allows the mutein to retain the full activity of the wild-type protein and permits an easy and site-specific PEGylation with a cysteine-reactive PEG-maleimide (PEG-MAL; 8 kDa). It was necessary to treat the rhGH mutein with an excess of 1,4-dithio-DL-threitol (DTT) before the coupling step, to convert this new C-terminal cysteine into the reduced form and prevent its involvement in disulfide bridges. After removal of DTT excess by gel-filtration, the conjugation afforded the mono-PEGylated derivative with a yield of > 80% [229].

An alternative treatment for GH deficiency involves the use of growth hormone-releasing hormone (GHRH) [69] or its analogues, commonly reported here as GRF. With its 44 amino acid sequence, GHRH controls the levels of expression and

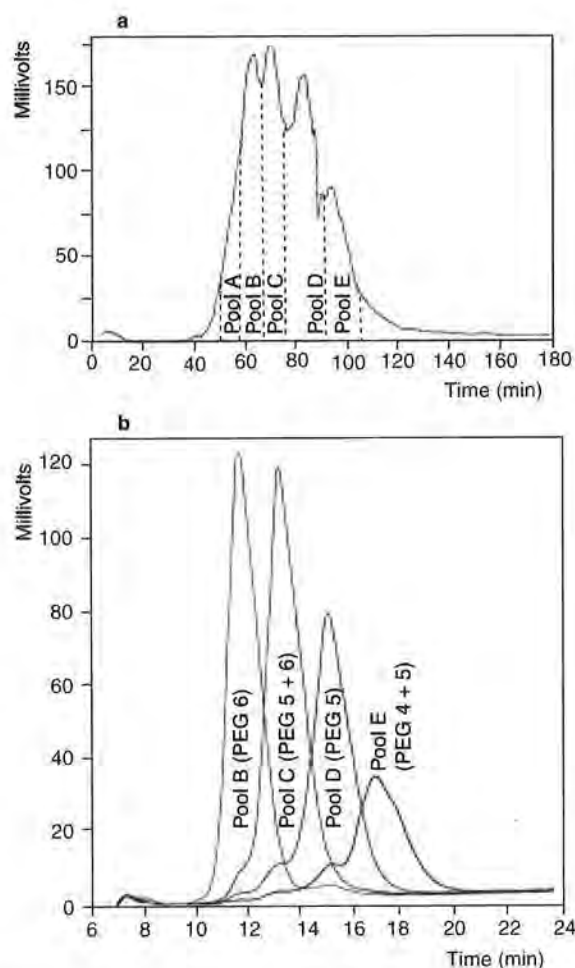


Figure 11. Preparative SP-Sepharose HPLC of a PEG₅₀₀₀-hGH reaction mixture. a) Fractions were pooled as shown on the chromatogram and analysed by electrospray mass spectrometry to determine the average number of PEG₅₀₀₀ groups attached per hGH. b) Purity of four of the five peaks was further assessed by analytical HPLC on a sulfopropyl TSK 5PW column. (Reproduced with permission from [68]).

hGH: Human growth hormone; HPLC: High performance liquid chromatography; PEG: Polyethylene glycol.

Table 2. Influence of total PEG mass on GRF conjugates activity, as assessed by specific reporter gene assay [71].

Product	EC ₅₀
Native GRF	0.18
GRF-PEG ₅₀₀₀	1.06
GRF-PEG ₁₀₀₀₀	2.8
GRF-PEG ₂₀₀₀₀	>1000
GRF-(PEG ₅₀₀₀) ₂	31.8
GRF-(PEG ₅₀₀₀) ₃	79.6

EC₅₀: Effective concentration for half maximum response; GRF: Growth hormone-releasing factor; PEG: Polyethylene glycol.

release of GH and is also naturally present in truncated active forms comprising only 40 or 37 amino acids. In addition, the 1–29 truncated peptide (hGRF_{1–29}), commonly prepared by chemical synthesis, retains the same activity, *in vitro* and *in vivo*, of the full-length hormone [70]. However, the clinical use of this truncated form of GRF is widely limited by the short biological half-life (10–20 min in humans), due to both fast kidney excretion and to N-terminal enzymatic degradation by endogenous aminopeptidases. PEGylation was therefore evaluated as it could act on both of these undesirable effects. Esposito and co-workers [71] coupled PEG-NHS (5–20 kDa) to hGRF_{1–29}, a peptide that has three available conjugation sites (Lys12, Lys21 and the N-terminal α -amine). PEG-NHS, with a molecular weight of up to 20 kDa, was used in the modification, and dimethyl sulfoxide (DMSO) was chosen as a solvent as, beyond offering more major stability and solubility to GRF than the aqueous media, it could induce a conformation of the peptide that lowers the amount of PEGylated isomers. The activity of PEG conjugates was found to be dependent on the total mass of linked PEG: the higher the PEG mass, intended either as number of polymer chains or as molecular weight, the lower the potency (Table 2). It was found that, under suitable reaction conditions and after chromatographic purification, PEG₅₀₀₀-OSu yielded to a solution with prevalent amounts of mono-PEGylated Lys12 and Lys21 isomers in an equimolar mixture (Figure 12) [72,230]. The mono-PEGylated mixture was purified from unmodified GRF and di-PEGylated conjugate by ionic exchange chromatography and it was characterised by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) analysis (Figure 13) [71]. The conjugates showed an improved pharmacokinetic profile which finally resulted in a more favourable pharmacodynamic response on the growth hormone-insulin growth factor 1 (GH-IGF-1) axis, as reported by the increased GH levels and the number of peaks in pig and rat plasma [73]. This work pointed out the importance of choosing the right solvent for the coupling reaction, which may reduce the number of PEG isomers by promoting structural rearrangement of the peptide. This could consequently lead to easier purification and higher yields. Investigation conducted by NMR and circular dichroism indicated, in fact, that the α -helix percentage in hGRF_{1–29}, which is only 20% in water, is raised to 90% in structure-promoting solvents such as methanol/water or 2,2,2-trifluoroethanol. In the last solvent the percentages of PEGylation at the level of Lys12 reached, with a different PEGylating agent, 80% of the total amount of PEGylated isomers [231]. This suggested that a highly regio-selective PEGylation could be achieved in these solvents.

Acromegaly is a disease caused by an overexpression of hGH. The therapy consists of the administration of a recombinant hGH mutein (B2036) with an antagonist effect. GH can bind two chains of its receptor thanks to two different binding sites, while B2036 can interact with only one receptor chain. B2036 was obtained by mutation

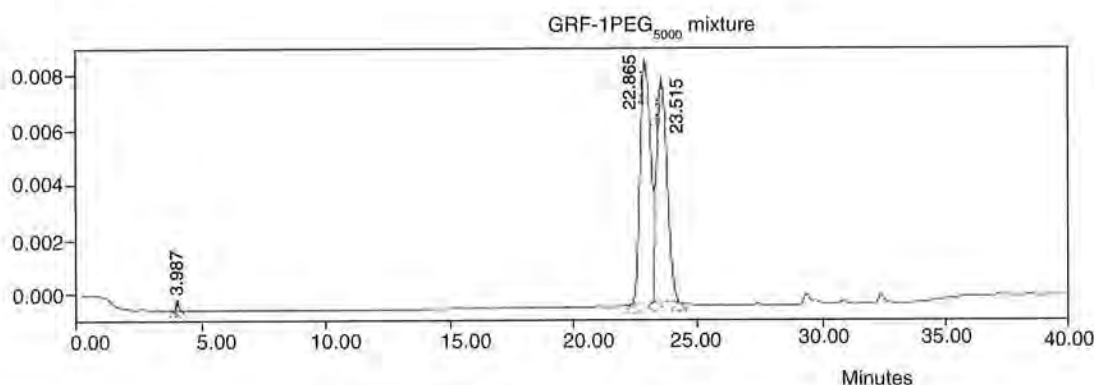


Figure 12. RP-HPLC C8 analytical chromatogram of the crude reaction mixture in DMSO before purification on the ion-exchange purification column. (Reproduced with permission from [72]).

DMSO: Dimethyl sulfoxide; GRF: Growth hormone-releasing factor; PEG: Polyethylene glycol; RP-HPLC: Reverse-phase high performance liquid chromatography.

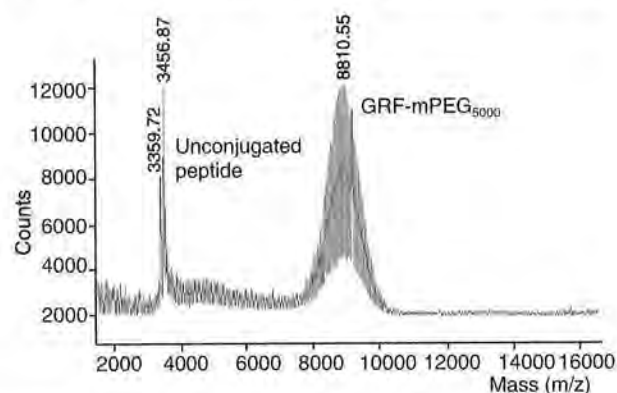


Figure 13. MALDI-TOF/MS investigation of GRF1-29 mono-PEGylated mixture. Signals at 8810 correspond to mono-PEGylated GRF and show the typical polydispersity of PEG. Minor signals for unmodified peptide were also detected. (Reproduced with permission from [71]).

GRF: Growth hormone-releasing factor; MALDI-TOF: Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry; PEG: Polyethylene glycol.

of hGH at both binding sites with the aim to retain the binding capacity of the first site and block the activity of the second, thus avoiding receptor signalling [74]. Furthermore, in view of PEG conjugation, a mutation was smartly performed to direct PEGylation to the second site only. To reach this goal a lysine residue in the first site was exchanged with a different, non-reactive amino acid, whilst a lysine was added to the second site. SPA-PEG (5 kDa) was employed for PEGylation, forming a mixture of derivatives with 4 – 6 PEG chains for each B2036 [205]. Further studies on the conjugate revealed a reduced binding affinity for the cellular receptor that was counterbalanced by a prolonged blood residence time [75]. This result may encourage the modification of proteins where the PEG moieties may enhance or decrease the protein binding

affinity, through direct steric entanglement, changing the final protein response.

5.5 Interleukin-2

IL-2 is a powerful immunoregulatory lymphokine [76] produced by lectin- or antigen-activated T cells that, like IFN, enhances their natural killer (NK) cell activity and therefore may find a role in the treatment of cancer. There is evidence that IL-2 can act as a GH for both B and T lymphocytes.

Goodson and collaborators [77] proposed an interesting modification strategy for a recombinant IL-2, where the native glycosylation site was substituted by a PEG chain. For this goal they introduced a cysteine residue at the glycosylation site, thus obtaining Cys3-rIL-2 that was later conjugated to PEG-MAL. The derivative retained the full activity of the parent protein and presented a fourfold-increased blood residence time. In this case PEG-Cys3-rIL-2 mimics the native IL-2, in fact the PEG chain replaces the sugar moiety. This method may better preserve the activity since the glycosylation site area, due to the steric bulk of the sugar moiety, is often not involved in receptor binding.

Sato [232] studied two PEGylation methods for IL-2, catalysed by different TGase, one coming from guinea-pig liver (G-TGase) and the other one from microorganism *Streptovorticillum* sp. strain s-8112 (M-TGase).

Both enzymes catalyse the transfer of an amino group from a donor (for example PEG-NH₂) to a glutamine residue present in a protein; the difference among the two involves requirement as far as the amino acid sequence for the transfer. G-TGase activity is more strictly dependent upon the amino acids surrounding the glutamine. For PEGylation, several PEGs of different molecular weight, terminating with a straight chain alkylamine at one end, were synthesised. Although IL-2 contains six glutamine residues, none of them is a suitable substrate for G-TGase, due to the unfavourable amino acid sequences in neighbouring glutamine. Chimeric proteins of IL-2 were therefore prepared by adding to the N-terminal sequence a peptide known to be a good

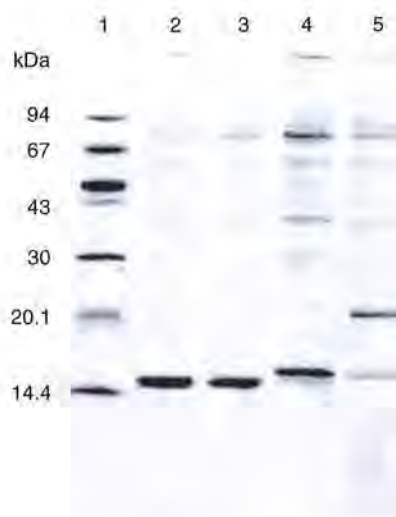


Figure 14. SDS-PAGE analysis of the G-TGase-catalysed incorporation of PEG-(CH₂)₆-NH₂ (PEG₃) into rTG1-IL-2 and rhIL-2 (migrating at ~ 15 kDa). Lane 1: molecular weight markers; Lane 2: reaction mixture of rhIL-2 and G-TGase; Lane 3: reaction mixture of rhIL-2, PEG₃ and G-TGase; Lane 4: reaction mixture of rTG1-IL-2 and G-TGase; Lane 5: reaction mixture of rTG1-IL-2, PEG₃ and G-TGase. The desired conjugate was observed only in lane 5. (Reproduced with permission from [42]).

G-TGase: Guinea-pig liver transglutaminase; PEG: Polyethylene glycol; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

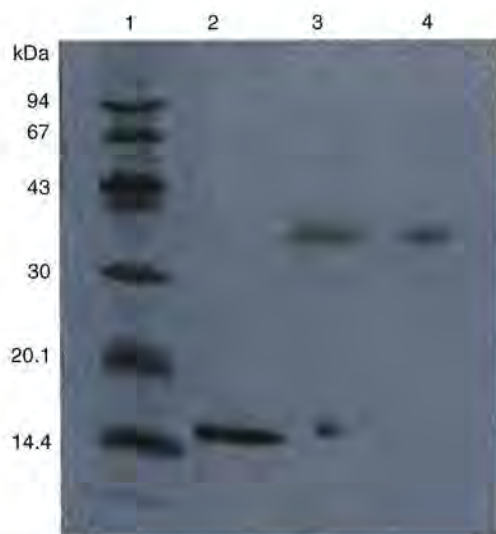


Figure 15. SDS-PAGE analysis of the M-TGase-catalysed PEG₁₂-modified rhIL-2. Lane 1: molecular weight marker; Lane 2: unmodified rhIL-2; Lane 3: rhIL-2, PEG₁₂ and M-TGase; Lane 4: purified PEG₁₂-rhIL-2. (Reproduced with permission from [42]).

M-TGase: Microbial transglutaminase; PEG: Polyethylene glycol; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 3. Comparison of the activity of IL-2 conjugates activities between random PEGylation and site-directed PEGylation by TGase [42].

Protein	% Activity*
rhIL-2	100
PEG ₁₀ -rhIL-2	74
(PEG ₁₀) ₂ -rhIL-2	36
rTG1-IL-2	72
PEG ₁₀ -rTG1-IL-2	69
(PEG ₁₀) ₂ -rTG1-IL-2	72

*The amount of activity was expressed as the percentage residual bioactivity as compared to rhIL-2.

TGase: Transglutaminase; PEG: Polyethylene glycol.

G-TGase substrate: Pro-Lys-Pro-Gln-Gln-Phe-Met (called TG1), derived from Substance P [78], to give rTG1-IL-2 or Ala-Gln-Gln-Ile-Val-Met (called TG2), derived from fibronectin [79], to give rTG2-IL-2. As expected the rTG1-IL-2 yielded one PEG chain conjugate and the rTG2-IL-2 led to two PEG chain conjugates (di-PEGylated form). The enzymatic coupling of mPEG-(CH₂)₆-NH₂ of 3 kDa molecular weight (PEG₃) to rTG1-IL-2 was carried out in 0.1 M Tris-HCl buffer, pH 7.5 at 25°C for 12 h in the presence of CaCl₂, 10 mM [42]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the reaction mixture (Figure 14) showed the prevalent formation of mono-PEGylated rTG1-IL-2, resulting in a band at 20 kDa (lane 5; Figure 14). It was demonstrated that intermolecular ε-(γ-glutamyl)lysine crosslinking does not take place for rhIL-2 and rTG1-IL-2 (lane 2, 4; Figure 14) and that the native rhIL-2 is not a substrate for G-TGase (lane 3; Figure 14). Modification of rTG2-IL-2, where double linkage is possible, incubated under the same conditions with mPEG-(CH₂)₆-NH₂ of 10 kDa molecular weight (PEG₁₀), resulted in the formation of a mixture of mono- and di-PEGylated adducts, together with unreacted rTG2-IL-2 [42]. Although a lower coupling yield was found, surprisingly G-TGase can also work with high molecular weight PEGs and can form di-substituted derivatives to the two neighbouring glutamines in the engineered sequence. Purified conjugates from the last modification, PEG₁₀-rTG2-IL-2 and (PEG₁₀)₂-rTG2-IL-2, were compared with the same conjugates obtained from classical mPEG-NHS (10 kDa; PEG₁₀) modification of rhIL-2, PEG₁₀-rhIL-2 and (PEG₁₀)₂-rhIL-2. The TGase PEGylation method gave derivatives that maintain the same activity of the native protein, whereas the rTG2-IL-2 random modification with mPEG-NHS gave conjugates with an activity that is inversely related to the linked PEG mass (Table 3).

On the other hand, the less specific M-TGase allowed the incorporation of mPEG-(CH₂)₆-NH₂ of 12 kDa molecular weight (PEG₁₂) directly into rhIL-2 [80]. SDS-PAGE electrophoresis demonstrated the formation of a single conjugate as a new band of 35 kDa (Figure 15). The apparent molecular weight of the conjugate was higher than expected,

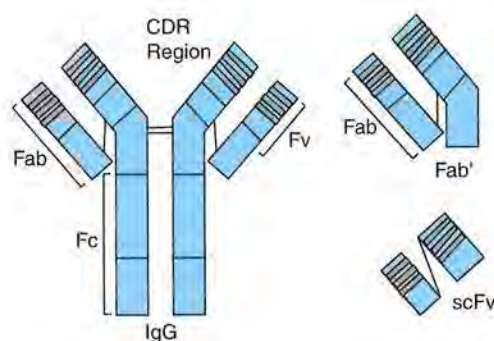


Figure 16. Schematic drawing of antibodies and antibody fragments.

CDR: Complementarity-determining region; SC: Single chain.

probably due to the bulkiness of PEG₁₂-rhIL-2. A characterisation carried out by liquid chromatography electrospray ionisation mass spectrometry (LC-ESI/MS) confirmed a mono-PEGylation at Gln74, which was proven to be the unique site of incorporation also when other different alkylamines were employed [80].

Compared to other site-specific PEGylation, such as thiol reactive PEG directed towards a cysteine mutain or N-terminal modification at acidic pH by PEG-aldehyde, this enzymatic coupling method produces less undesired products, namely the protein-protein dimers (due to cysteine oxidation) or the PEGylation at the εNH₂ lysine residues (when N-terminal PEGylation is performed). Nonetheless, the applicability of this revolutionary enzymatic method from the laboratory to the industrial scale production has still to be demonstrated.

5.6 Antibodies and antibody fragments

An increasing interest is centred on antibodies (see [81] for an overview) for the treatment of several diseases and ten products based on these entities have already reached the market [82], with more currently undergoing clinical evaluation [83]. PEGylation was initially applied to these proteins to reduce the high immunogenicity of murine monoclonal antibodies. Later, humanised and human antibodies seemed to be the solution to the problem, but the high production costs and the low expression levels limited their use to severe diseases, such as cancer. Recently *Escherichia Coli* expression of antibody fragments was proposed (Fab' and scFv; Figure 16), allowing cost cutting in the production and, consequently, a potential increase of the market of these proteins. However, since the major limitation is a short half-life, PEGylation was investigated widely. Many studies have been reported on random PEGylation (the reader is encouraged to refer to an extended review [84]) but almost all the conjugates showed a reduction of some degree in the binding affinity. PEGylation of anti-IL8 F(ab')₂ was an exception because coupling with PEG of 20 or 40 kDa did not heavily affect the binding affinity [85,233].

The reduced binding affinity of PEGylated antibodies is either due to modification in proximity of the antigen binding domain, generating steric hindrance, or to reduction of the number of free lysine residues involved in the ionic interactions that initiate the antigen binding. For example, a small modification of the Fc region may cause a dramatic loss in binding activity. The Fc region (see Figure 16) is the region that mediates the effector functions of the antibodies, either by complement activation or by cellular interaction after interaction with its Fc receptors. The high number of lysines in the Fc domain drives the PEGylation to this region, showing a high sensitivity; when the degree of lysine modification is > 15% the binding activity towards the Fc receptor is completely impaired [86]. PEGylation of antibodies as a means to reduce their immunogenicity has been overcome by the technical advances in the humanisation of murine antibodies or by the expression of human antibodies in bacteria. However, PEGylation still remains important for antibodies used in cancer therapy because of the positive enhancing effect of PEG on antibody tumour localisation and the lack of Fc effector functions.

A different approach used is based on engineered antibody fragments, such as Fab' (see Figure 16), with at least one free cysteine residue located far from the antigen binding site and reacting specifically with PEG-maleimide, leading to mono-PEGylated forms. Studies based on this strategy and employing a PEG of molecular weight up to 40 kDa demonstrated that the binding affinity was completely retained for all PEG-engineered Fab' conjugates, whereas the random PEGylation of Fab' NH₂ groups led to up to 50% loss of binding activity [87,234,235].

A further advance in engineered antibody fragments was the design of a shorter polypeptide sequence that still retains the binding activity towards the antigen, such polypeptides are called single chain Fv fragments (scFv; see Figure 16). The major limitation of these peptides is, however, a too short half-life (~ 0.7 h) that impairs any therapeutic application. Enzon researchers synthesised PEGylated scFv conjugates using several PEG derivatives (linear and branched SC-PEG and pNPC-branched PEG, among the others) [236]. These conjugates possess improved pharmacokinetics and lower immunogenic activity than a full-length antibody as, after PEGylation, the peptide is a small fraction of the total molecular weight.

As mentioned above, PEGylated antibodies and antibody fragments are still of great interest and are widely studied, mainly in the oncology field. These conjugates are used as targeted delivery systems for anticancer drugs, as several studies showed that PEGylation increases the accumulation of antibodies and antibody fragments in tumour but not in normal tissues. The advantages of these PEGylated derivatives over the unmodified antibodies may be summarised as follows:

Table 4. Molecular parameters of PEGylated haemoglobin [21].

Haemoglobin	Calculated mass (kDa)	Radius (nm)	Volume (nm ³)
Hb	64	3.12	127
(PEG ₅₀₀₀) ₂ -Hb	74	4.20	310
(PEG _{10,000}) ₂ -Hb	84	5.54	712
(PEG _{20,000}) ₂ -Hb	104	7.04	1436
Hb-octamer	128	4.12	293
Hb-dodecamer	192	5.56	720

Molecular radius measurements were made using light scattering
Hb: Haemoglobin; PEG: Polyethylene glycol.

- high tumour accumulation due to the enhanced permeability and retention effect (EPR), because most solid tumours have high vascular permeability allowing the extravasation of high molecular weight molecules that accumulate in the tissue due to inefficient lymphatic drainage [88];
- increased plasma half-life;
- decreased liver uptake [89].

The antibody fragments, with respect to full-length antibodies, possess higher tumour penetration potential [90], making them promising candidates for tumour-targeted delivery systems. The strategy was demonstrated by coupling the C225 monoclonal antibody, directed against epidermal growth factor receptor, to a heterobifunctional PEG having a radiometal chelator (diethylenetriaminepentaacetic acid, DTPA) at one end [91]. The conjugate DTPA-PEG-C225, with up to 60% modification of the C225 amino groups, retained 66% binding affinity and, more importantly, when labelled with Indium-111 (¹¹¹In) it showed narrower steady-state distribution than the non-PEGylated ¹¹¹In-DTPA-C225, due to reduced nonspecific binding. Another example is represented by derivatives where the anticancer drug is linked to a PEG chain conjugated to the monoclonal A33 antibody, leading to a conjugate useful for colorectal carcinoma treatment [237]. The A33 antigen is expressed in high amounts in some colon cancer cell lines, such as SW1222. Moreover, PEG-modified-Tc^{99m}-radiolabelled antibody fragments, useful for radioimmunodetection of tumours and infectious lesions are reported [238].

5.7 Other proteins

Insulin is one of the most well-studied proteins as far as PEGylation chemistry is concerned and its modification has been carried out through both random and site-specific methods. The protein is formed by two polypeptide chains, A and B, and its three amino groups (GlyA1, PheB1 and LysB29) are all candidates for PEGylation. Hinds, among others, proposed a site-directed PEGylation procedure involving the preliminary preparation of an N-BOC

(*tert*-butyl-chloroformate) protected insulin [92]. As an example, to synthesise N^{B1}-PEG-insulin the intermediate N^{A1}, N^{B29}-BOC-protected insulin was prepared and then conjugated to PEG-SPA. The Phe-B1 and Lys-B29 PEG conjugates, with respect to the unmodified insulin, showed a comparable *in vivo* potency, reduced self-aggregation and extended half-life. Similarly, the acylation of GlyA1 and LysB29 amino groups with the cyclic anhydride of a dicarboxylic acid, followed by PEGylation and deprotection under mild acidic conditions, afforded the PheB1 derivative [239].

Arginine deiminase from microbial origin has been studied as a potential anticancer enzyme as it degrades arginine, an essential nutrient for some tumours; however, its use is limited due to its short half-life and its high immunogenicity. The enzyme was modified with PEGs of various molecular weights and structures (branched or linear) and with different coupling chemistries [93]. It was demonstrated that 50% of the enzyme activity was maintained with up to 40% of amino group modification. With a PEG molecular weight up to 20 kDa, linearity was found between PEG weight, pharmacokinetic and pharmacodynamic properties; in fact, conjugates with a prolonged blood residence time, obtained using higher molecular weight PEGs, showed a better pharmacodynamic response. Reduced immunogenicity was also found for the PEGylated microbial enzymes. Arginine deiminase was additionally modified with SC-PEG (12 kDa) to obtain conjugates with 16–22 polymer chains for each enzyme subunit and a 20% residual activity [240]. Engineered arginine deiminases were designed to allow PEGylation far from the catalytic region. Conjugation with succinimidyl succinate PEG (SS-PEG) yielded a product with 70–80% of enzyme activity [241].

Haemoglobin (Hb) has been extensively investigated as an oxygen-carrying therapeutic agent but limitations for its clinical use resulted from its high vasoactivity, due to extravasation into interstitial spaces and scavenging of nitric oxide. PEGylation has been performed to prevent such Hb extravasation. After several unsuccessful random PEGylation attempts [242], a site-specific modification was made at Cys93(β) with maleimidophenyl PEG (MalPhePEG) (5, 10 and 20 kDa), leading to PEGylated Hb carrying two chains of the polymer per Hb tetramer [243]. The colligative properties of the derivatives [21] suggested that PEG helped to eliminate the Hb vasoactivity. This product was found to be more efficient than polymerised Hb, the Hb-octamer or the dodecamer. PEG₂-Hb conjugates also have higher oxygen affinity, independent of polymer chain length. A very detailed study demonstrated that the hydrodynamic volume and the molecular radius of the conjugates increase linearly with increase in PEG molecular weight (Table 4), while the viscosity and the colloidal osmotic pressure exhibited an exponential correlation with the same parameter. The authors concluded that the vasoactivity modulation is due to the surface shielding effect of PEG.

Erythropoietin (EPO) is a glycoprotein that increases the production of reticulocytes and red blood cells by stimulation of bone marrow cells. Recombinant (r)EPOs were prepared to

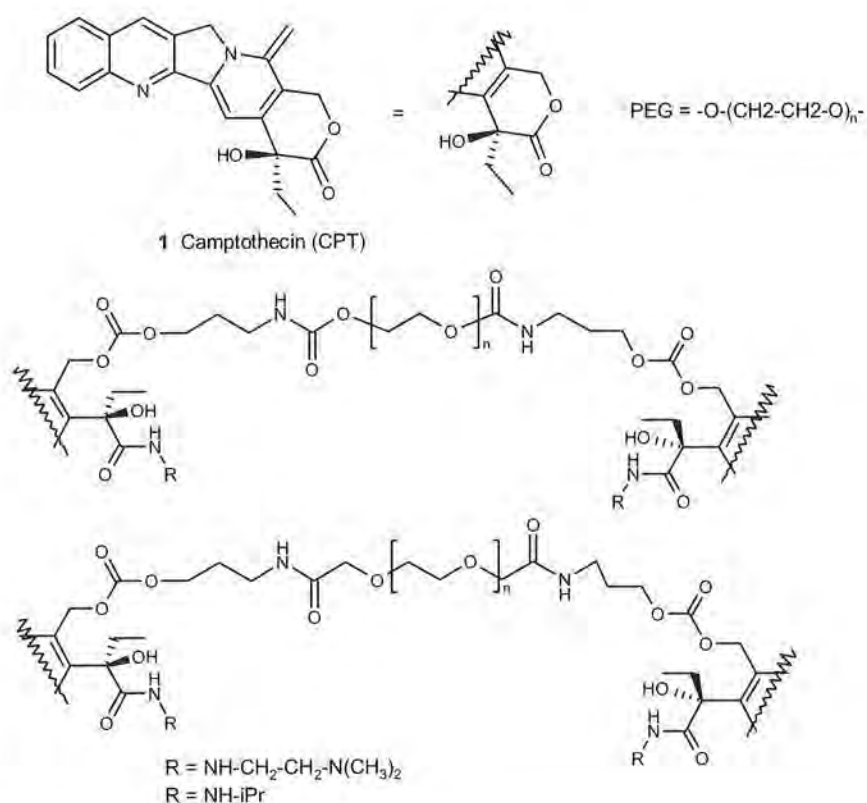


Figure 17. PEG–camptothecin dimers.

PEG: Polyethylene glycol.

increase the number of glycosylation sites and mono-PEGylated conjugates of the new rEPO showed *in vivo* potency higher than unmodified EPO [244,245]. The PEG conjugates are administered only once a week, instead of the three times a week for native EPO. A further study proposed a PEGylation of EPO at the Lys54 residue, through the optimisation of the molar ratio between polymer and EPO and the reaction time [246]. This controlled reaction led to a mixture of EPO conjugates with 1 – 3 PEG chains and the desired mono-PEGylated EPO at Lys54 was isolated by purification.

Leptin (Ob proteins) is primarily secreted from adipose tissue and plays an important role in body weight homeostasis by regulating food intake and energy expense. As a result, it could be used to treat obesity. Interestingly, the modification method of Ob involves a dual PEGylation of recombinant (r)Ob, where Arg78 is mutated in Cys78. Linking of two PEG chains, with different reactivity, in two protein sites is therefore possible. First MAL-PEG is coupled to Cys78 and later, in a second step, PEG-aldehyde reacts with the N terminus of the protein at acidic pH [247]. The conjugate has substantially higher activity and biocompatibility when compared to unmodified Ob. A modification of Ob protein with branched PEG2-NHS was also carried out and the purified mono-PEGylated form was tested in mice for its ability to reduce food intake and body weight [248]. Mono-PEG2-Ob

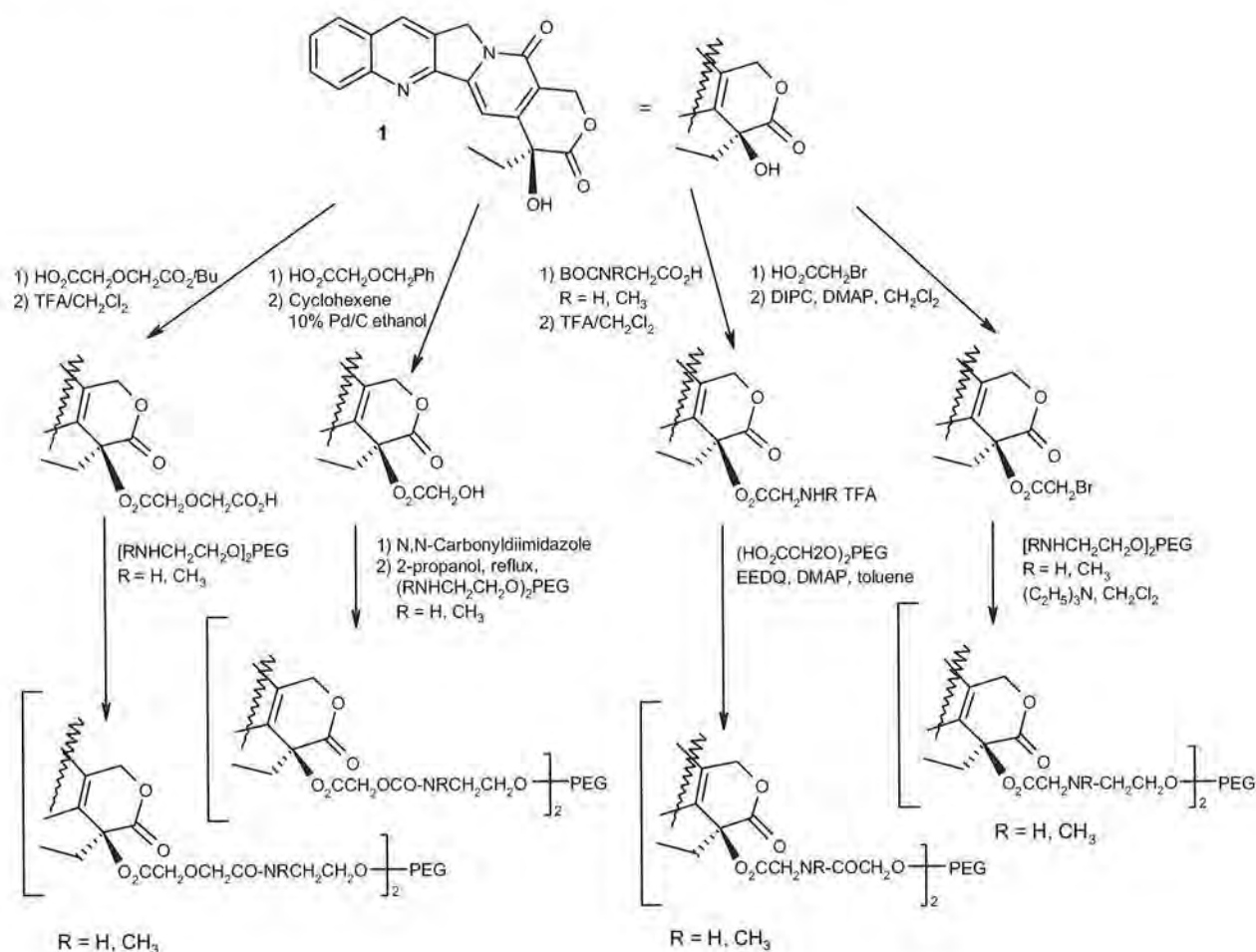
was found to be active over 3 days following injection, proving an extended blood residence time.

6. PEGylation of drugs

In this review, only the most recent findings regarding non-peptide drug modifications will be discussed. For a comprehensive historical overview of drug PEGylation previous to 2000, the reader is encouraged to refer to the authoritative reviews by Greenwald and co-workers [94,95]. In many aspects the chemistry involved in drug conjugation follows that devised for proteins, although the goal is, in this case, the release of the free, active drug and the development of reversible coupling techniques takes advantage of the experience in the field of prodrugs.

6.1 Camptothecins

Camptothecin (CPT; Figure 17), an alkaloid that was isolated and identified in the late 1950s from the Chinese tree *Camptotheca acuminata* [96], is the first member of a highly populated and very promising class of anticancer agents. The mechanism of action involves the inhibition of the nuclear enzyme topoisomerase I, overexpressed, according to many studies, in several types of solid tumours [97]. Several new, semisynthetic analogues of CPT were soon designed with improved pharmacological and pharmaceutical properties



Scheme 6. Synthesis of C-20 substituted camptothecin-PEG derivatives.

TFA: Trifluoroacetic acid; PEG: Polyethylene glycol.

while maintaining (or even increasing) the antitumour activity of the parent compound. However, the administration of these compounds for *in vivo* assays and efficient delivery to tumour cells remains difficult to achieve, due to their very poor solubility in the physiological medium.

The conjugation of anticancer agents to water-soluble, biocompatible polymers is one of the most recent and promising modifications used to improve the pharmacokinetic and pharmacodynamic properties of the cytotoxic drug. The high molecular weight prodrug carriers can lead to higher water solubility, reduced systemic toxicity, increased half-life, improved biological distribution and, possibly, higher therapeutic efficacy. Many polymeric derivatives of CPTs have been reported in the literature, including dextrans [98], poly-glutamates [99] and 2-hydroxypropyl methacrylate (HPMA) [100].

In 1998, Enzon researchers published a series of CPT-20-PEG ester transport forms based on an ester prodrug approach [101,102], using specific linkers (Scheme 6). Various synthetic strategies were developed to esterify the

20-OH moiety by means of dicarboxylic acids, BOC protected amino acids and ω -bromo acids, and the derivatives were conjugated to PEG-diamine or PEG-dicarboxylic acid to achieve the desired products. Anchimeric assistance was observed for those systems that had NH or $\text{NHC}=\text{O}$ moieties configured in such a way that a 3, 5 or 6-membered cyclic transition state could be formed and increase the rate of hydrolysis of the drug-ester bond.

In vitro and *in vivo* experiments reported in Tables 5 – 7 showed a significant increase in lifespan compared to camptothecin (compound 1); the compound with the glycine linker (compound 24) provided a transport form with higher pharmacokinetic parameters and higher efficacy compared to the ester 3.

The same authors reported, a year later, a series of PEG-CPT derivatives where the linker was represented by a single amino acid (Tables 8 and 9) [103].

Significant differences in kinetics (*in vitro* buffer and plasma) were observed for the various PEG-amino-acid-CPT esters and are probably due to a combination of steric factors and the hydrophobicity for a particular amino acid,

Table 5. Rates of hydrolysis and IC₅₀ values of various PEG–camptothecin derivatives.

Compound	Linker	IC ₅₀ (nM)	t _{1/2} (h)	
			PBS (pH7.4)	Rat plasma
1	–	7	–	–
3	-O-CO-CH ₂ -O-PEG	15	27	2
10	-O-CO-CH ₂ -O-CH ₂ -CO-NH-PEG	16	5.5	0.8
11	-O-CO-CH ₂ -O-CH ₂ -CO-N(CH ₃)-PEG	21	27	3
16	-O-CO-CH ₂ -O-CO-NH-PEG	7	0.2	ND
17	-O-CO-CH ₂ -O-CO-N(CH ₃)-PEG	18	28	5
24	-O-CO-CH ₂ -NH-CO-CH ₂ -O-PEG	12	40	6
25	-O-CO-CH ₂ -N(CH ₃)-CO-CH ₂ -O-PEG	15	97	10
28	-O-CO-CH ₂ -NH-PEG	24	12	3
29	-O-CO-CH ₂ -N(CH ₃)-PEG	42	102	> 24

ND: No data available.

IC₅₀: Median inhibitory concentration; PBS: Phosphate buffered saline; t_{1/2}: Half-life.Table 6. Activity* of PEG–camptothecin derivatives against P388/0 murine leukaemia *in vivo*.

Test compound	Linker	Mean time to death (days)	% ILS
Control	–	13.0	–
1	–	38.0	192%
3	-O-CO-CH ₂ -O-PEG	38.0	192%
10	-O-CO-CH ₂ -O-CH ₂ -CO-NH-PEG	17.4	34%
11	-O-CO-CH ₂ -O-CH ₂ -CO-N(CH ₃)-PEG	31.6	143%
17	-O-CO-CH ₂ -O-CO-N(CH ₃)-PEG	23.4	80%
24	-O-CO-CH ₂ -NH-CO-CH ₂ -O-PEG	35.0	169%
25	-O-CO-CH ₂ -N(CH ₃)-CO-CH ₂ -O-PEG	19.3	48%
28	-O-CO-CH ₂ -NH-PEG	30.6	135%
29	-O-CO-CH ₂ -N(CH ₃)-PEG	21.4	65%

**In vivo* efficacy study of the water-soluble camptothecin derivatives using the P388/0 murine leukaemia model. Compound 1 or prodrug derivatives were given daily (i.p. x 5), 24 h following an injection of P388/0 cells into the abdominal cavity with survival monitored for 40 days.

ILS: Increased life-span; PEG: Polyethylene glycol.

Table 7. Antitumour activity* against human colorectal xenografts (HT-29) *in vivo*.

Test compound	Dose (mg/kg)	Efficacy (%)	
		Week 5 (end of treatment)	Week 7 (two weeks post)
Control	–	+ 727	+ 1047
Topotecan	2.5	+ 78	+ 300
1	2.5	- 20	+ 62
3	2.5	- 89	- 98
11	2.5	- 60	- 73
24	2.5	- 80	- 96

**In vivo* efficacy study of the water soluble camptothecin derivatives using the HT-29 human colorectal xenograft. A subcutaneous injection of HT 29 cells was allowed to reach an average tumour volume of 250 mm³, prior to treatment. Mouse weight and tumour size were measured at the beginning of the study and weekly through to week 7.

Table 8. Rates of hydrolysis and IC₅₀ values (P388/0 cell line) of PEG–amino acid CPTs.

Amino acid spacer	IC ₅₀ (nM)	t _{1/2} (h) ^c Rat plasma	Human plasma
None (native CPT)	7	-	-
Glycine	34	9	4
Alanine	16	15	7
Phenylalanine	27	9	2
Methionine	7	6	3
Glutamate (OCH ₃)	16	21	9
Leucine	358	25	4
Proline	3000	113	6

CPT: Camptothecin; IC₅₀: Median inhibitory concentration; PEG: Polyethylene glycol; t_{1/2}: Half-life.

Table 9. Activity of PEG–CPT derivatives against P388/0 murine leukaemia *in vivo*.

Test compound	Mean time to death (days)	% ILS
Control	12.5 ± 1.3	-
CPT	8.1 ± 1.3	35.2
PEG-Gly-CPT	28.2 ± 15.3	125.6
PEG-Ala-CPT	21.7 ± 1.6	73.6
PEG-Phe-CPT	26.8 ± 8.6	114.4
PEG-Met-CPT	31.1 ± 10.0	148.8
PEG-Glu-CPT	19.7 ± 0.7	57.6
PEG-Leu-CPT	22.4 ± 1.0	79.2
PEG-Pro-CPT	13.1 ± 1.2	4.8

CPT: Camptothecin; ILS: Increased life-span; PEG: Polyethylene glycol.

Table 10. Activity of CPT-11, 10-amino-7-ethyl CPT sodium salt (SN-392Na) and PEG–Gly-Leu-Phe-Gly-SN-392 (compound 3) against Meth A fibrosarcoma in BALB/c mice [11].

Sample	Dose (mg/kg)	Inhibition rate (%)
Control	-	-
Compound 3	25	38.4
	50	55.5
	100	79.1
	25	62.3
	50	64.0
SN-392Na	100	69.3
CPT-11	25	49.5
	50	46.6
	100	52.7

CPT: Camptothecin; ILS: Increased life-span; PEG: Polyethylene glycol.

which impacts bond hydrolysis. All these derivatives were classified by the authors as possible tripartate prodrugs resulting from enzymatically mediated hydrolysis pathways.

The use of amino acid spacers with the aim of targeting the lysosomal enzymatic pool (cathepsins) has been extensively used in PEGylation technology, and PEG–CPT conjugates with tri- or tetrapeptide linkers have been shown to be highly stable in plasma but rapidly hydrolyse in the presence of lysosomal enzyme cathepsin B [104,249]. *In vitro* and *in vivo* results are encouraging and indicate that the polymer-bound CPT derivative SN-392 (10-amino-7-ethyl camptothecin) is less toxic than the free drug and the camptothecin analogue CPT-11 but is still highly effective against Meth A fibrosarcoma cell lines (Table 10).

From pharmacokinetic studies it was immediately evident that: i) the maximal plasma concentration (C_{max}) after administration of 10-amino-7-ethyl camptothecin is eightfold higher than that after PEG–SN392 injection; ii) although area under the curve (AUC) data are comparable, the mean resident time (MRT, a dose-independent measure of drug elimination expressing the average time a drug molecule remains in the body after rapid intravenous injection) after injection of compound 3 is threefold higher than those after injection of both forms of 10-amino-7-ethyl camptothecin. Both data indicate that compound 3 acts as a circulating reservoir of the antitumour agents (Table 11).

1,4-Benzyl elimination [95] is another mechanism that has been envisaged for release of a drug from a polymer carrier. The general mechanism involves a first step where the phenolic OH group is unmasked from its pro-prodrug form (usually an ester or a carbonate or a carbamate). The intermediate then promptly decomposes to release the free drug.

Among the many examples reported in a patent [250], and shown in Scheme 7, a PEG–camptothecin derivative is claimed. Recently, another patent [251] claims and introduces a new class of PEGylated CPT derivatives, where the E-ring lactone has been hydrolysed and the polymer is bound to the C20 carboxyl group (Figure 17). Although no pharmacological data are given, the compounds are claimed to control the conversion of the open E-ring lactone back to the biologically active form.

Many examples of high loading capacity PEG derivatives, based on several branching linkers, have been reported with CPT as an example of a carried drug. A comprehensive description of each and every polymeric derivative would be extremely difficult due to the proliferation of branching approaches that include structures depicted in Figure 18, taken as examples of PEG–CPT conjugates, as described in three patents [252–254].

6.2 Anthracyclines

Camptothecins are a paradigmatic example of conjugation to polymers in general, and PEG in particular, due to their extremely poor solubility in water that suggests their

Table 11. Pharmacokinetic parameters of 10-amino-7-ethyl camptothecin in rat plasma after intravenous administration of compound 3, 10-amino-7-ethyl camptothecin or its sodium salt.

Parameter		Compound 3		10-Amino-7-ethyl camptothecin	10-Amino-7-ethyl camptothecin sodium salt
		100 mg/kg (n = 2)	50 mg/kg (n = 2)	4 mg/kg (n = 2)	4 mg/kg (n = 1)
AUC _{inf}	(µg/h/ml)	1.89 (1.80, 1.98)	0.531 (0.507, 0.554)	2.08 (2.34, 1.65)	1.71
C _{max}	(µg/ml)	1.35 (1.20, 1.51)	0.436 (0.439, 0.433)	10.53 (14.09, 6.99)	10.50
t _{max}	(min)	2.00	2.00	2.00	2.00
MRT _{inf}	(h)	3.56 (3.52, 3.61)	3.31 (3.31, 3.30)	1.16 (1.22, 1.09)	1.09
CL _{tot}	(l/kg/h)	-	-	2.07 (1.71, 2.42)	2.42
Vd _{ss}	(l/kg)	-	-	2.37 (2.09, 2.65)	2.65
t _{1/2}	(h)	2.82 (2.78, 2.84)	2.69 (2.71, 2.66)	2.25 (2.24, 2.26)	2.26

AUC_{inf}: Area under curve, extrapolated to infinite; C_{max}: Maximal plasma concentration; t_{max}: Time of maximum peak concentration; MRT_{inf}: Mean resident time extrapolated to infinite; CL_{tot}: Total clearance; Vd_{ss}: Steady state volume of distribution; t_{1/2}: Serum half life.

incorporation in water soluble prodrug device systems. However, the list of anticancer drugs that have been bound to PEG also includes anthracyclines, methotrexate, cisplatin, Ara-C and others.

Acid-sensitive PEG conjugates of doxorubicin were prepared by Rodrigues and co-workers [255] by reacting maleimide derivatives of doxorubicin with α -methoxy-PEG-thiopropionic acid amide (molecular weight = 20,000 Da), α,ω -bisthiopropionic acid amide PEG (molecular weight = 20,000 Da) or α -tert-butoxy-PEG-thiopropionic acid amide (molecular weight = 70,000 Da) (Figure 19). The three maleimide derivatives differ in site (3' amino or 13-keto position) and stability (benzamide or benzoyl hydrazone bond) of the chemical linker between doxorubicin and the spacer molecule. As expected, the hydrazone derivatives showed good stability at pH 7.4 but a marked release at pH 5.0. Furthermore, release of drug was slower for PEG 70,000 than for PEG 20,000 conjugates, at pH 5.0.

Biological activity tests against T24 bladder carcinoma and LXFL 529 lung cancer cells returned IC₅₀ values higher than that of free doxorubicin. However, the authors conclude that preliminary data in nude mice show that PEG-doxo conjugates can be administered at far higher doses than the maximum tolerated dose of free doxorubicin (2 x 16 – 24 mg/kg as compared to 2 x 8 mg/kg).

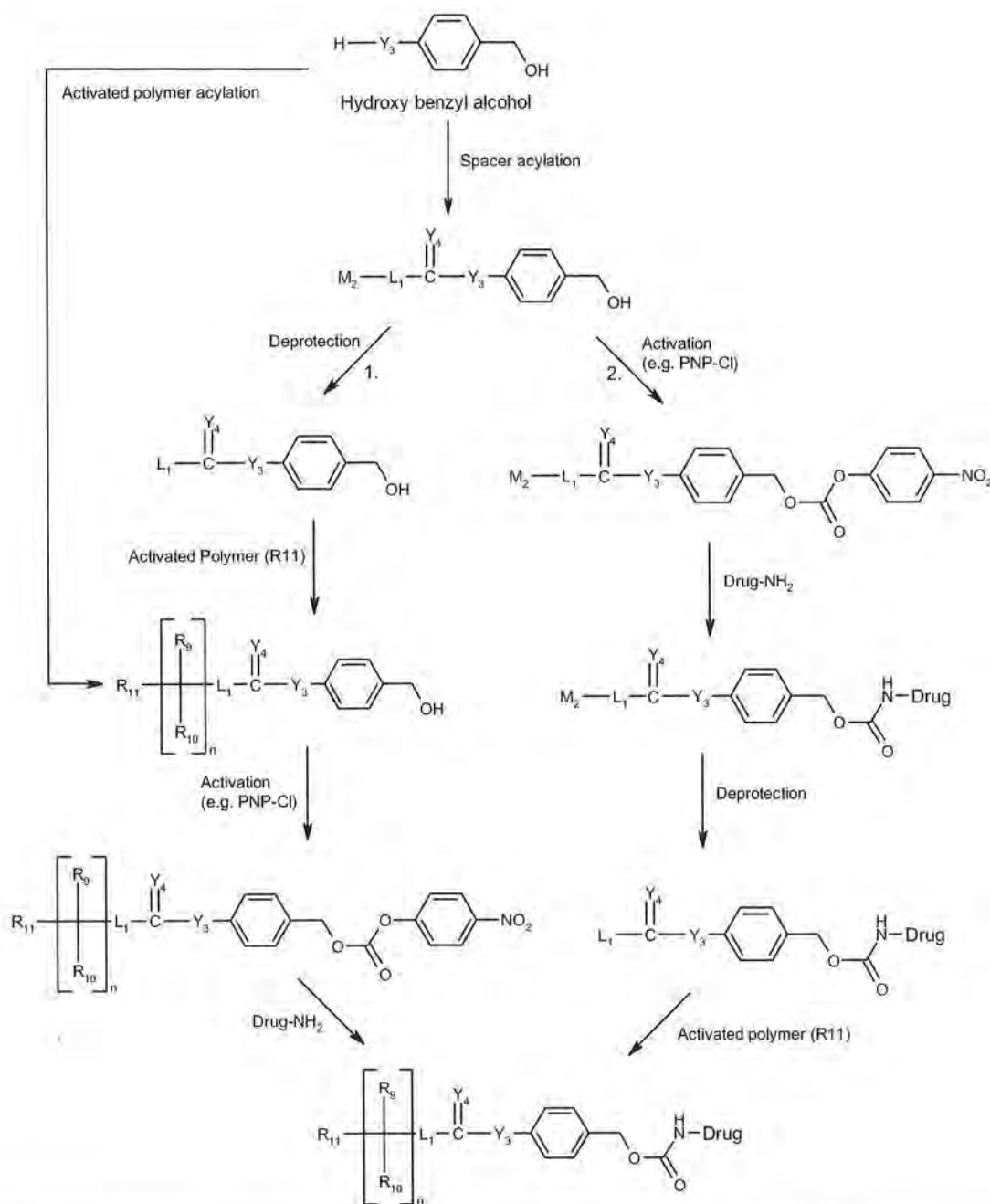
PEG-conjugated anthracyclines prodrugs based on 1,4- or 1,6-elimination reactions (benzyl elimination [BE] system; Figure 20) have been synthesised by Greenwald and colleagues. Such polymeric prodrugs have been demonstrated to be an effective means for delivery of anticancer agents and, in general, provide a practical approach for solubilising and transporting amino-containing antitumour agents as well as a variety of other drugs [105,250].

The TML lactonisation technology, developed by Greenwald and co-workers, has been applied among other drugs to daunorubicin [36]. A series of examples of the daunorubicin

(DNR)-PEG-TML double prodrug system, including amino acid esters, carbonate and carbamate triggers were prepared and demonstrated prolonged stability (> 24h) in pH 7.4 phosphate buffer at 37°C. The *in vitro* and *in vivo* results for the PEG conjugates are reported in Table 12 and confirm the relevance of the steric hindrance (proline causes a tenfold decrease in the hydrolysis rate compared to alanine) and the pro-prodrug trigger (carbonates hydrolyse slower than an ester, carbamates are extremely stable). Although the authors failed to correlate rat plasma half-life (t_{1/2}) with *in vivo* efficacy, the PEG-TML drug delivery approach ultimately resulted in the synthesis of a potent conjugate derived from daunorubicin that appeared more active in a SKOV3 xenograft model than the parent drug. Similar TML-based PEG systems but conjugated in the reported examples to doxorubicin and camptothecin, were patented by the same authors in 2003 [256]. Leu-doxorubicin was bound to PEG-TML systems and preliminary data against MX-1 (human mammary tumour) xenografts in nude mice indicated that PEGylated forms were as effective as the non-conjugated parent compound.

An ingenious coumarin-based pro-prodrug system, based on the esterase-mediated hydrolysis of a phenolic ester, lactonisation and release of the parent compound, has been developed by the same authors [257] to be applicable to a vast selection of amino- and hydroxyl-containing compounds (proteins, drugs etc.) although claims only refer to daunorubicin and doxorubicin together with mustards, Ara-C and gemcitabine (Figure 21).

PEG derivatives of anthracyclines with procoagulant properties have been synthesised by Trouet and Dubois [258] in order to obtain new anticancer compounds. Their polymeric systems consist of a procoagulant and heparin-binding polycationic linker and an anticancer agent bound to a PEG chain. While the polycationic linker, normally masked to prevent toxicity, reacts with heparin and promotes cloth formation within the neoangiogenic blood



Scheme 7. Synthetic pathways to PEGylated drugs with p-hydroxy benzyl spacer (1,4-elimination).

PEG: Polyethylene glycol.

vessels, the doxorubicin moiety is released and can reach the tumour exerting its activity.

6.3 Methotrexate

PEGylation of anticancer drugs with the aim of targeting tumours by the so-called EPR effect, therefore reducing systemic toxicity of the anticancer agent and optimising its pharmacodynamic properties, has also been applied to a

variety of other classical antitumour agents such as methotrexate, Ara-C and cisplatin.

Riebeseel and co-workers [106] reported a variety of PEG-methotrexate conjugates with molecular weight varying from 750 to 40000 Da, in 2002. The general synthetic scheme is reported in Figure 22; due to the presence of two reactive carboxylic groups, that cannot be discriminated, the PEGylated derivative is a mixture of the two possible α and

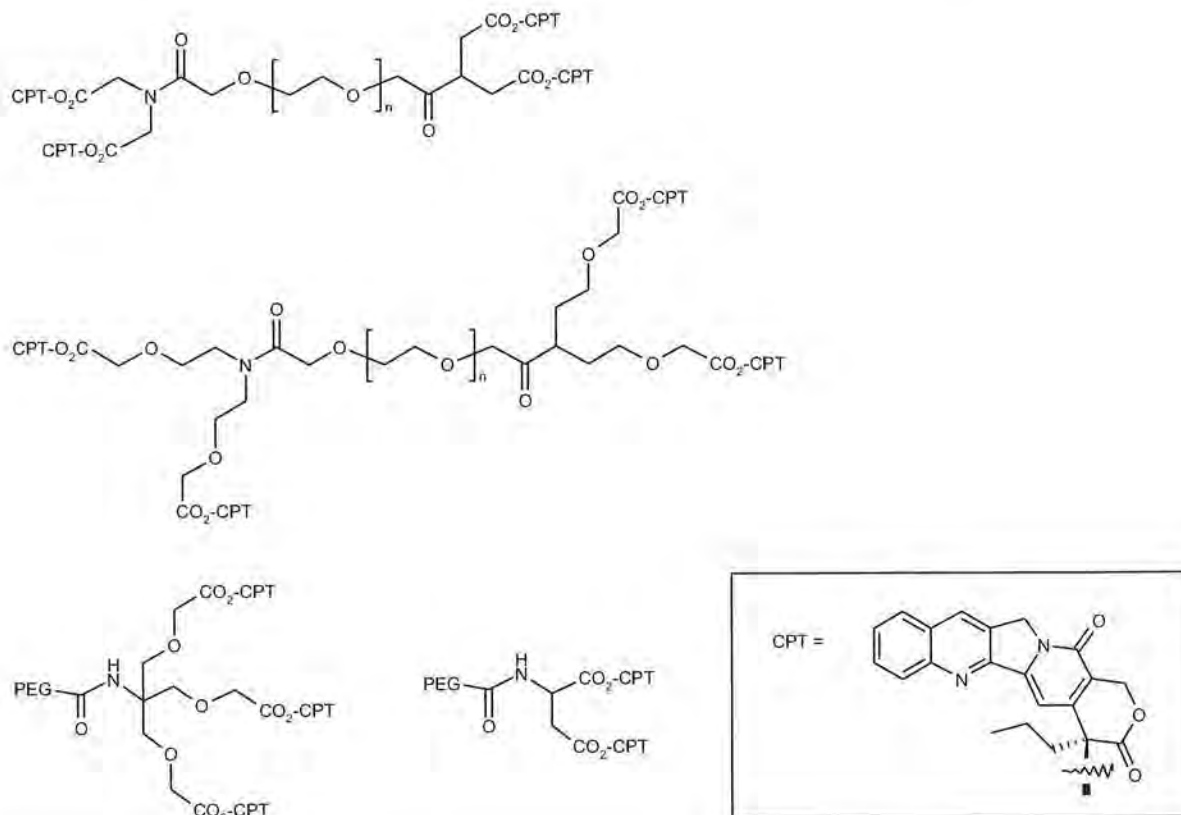


Figure 18. Multibranched PEG-CPT derivatives.

CPT: Camptothecin; PEG: Polyethylene glycol.

γ -isomers; moreover, the glutamate moiety racemes completely during activation, leading to a mixture of L and D-enantiomers. The partial derivatisation of the α -carboxylic group reduces the inhibition activity against the target enzyme dihydrofolate reductase but the MTX-PEG conjugates showed very similar IC_{50} values demonstrating that the length of the polymer backbone does not have a significant influence on the inhibition of the target enzyme. The activity of the conjugates has been tested against several cell lines (Table 13); it appeared evident that the molecular weight of the carrier molecule has a significant influence on the cytotoxic activity. Low-molecular weight conjugates (molecular weight < 50 kDa) exhibit an IC_{50} value of $\sim 0.5 - 3 \mu M$, while conjugates of higher molecular weight are less active. Free MTX is $\sim 20 - 1000$ -fold more active than the MTX-PEG conjugates. However, it is noteworthy that the PEG conjugate with the lowest activity *in vitro* (MTX-PEG 40,000) was the most active in subsequent *in vivo* experiments in a mesothelioma xenograft model.

6.4 Platinum complexes

Like most low molecular weight drugs, carboplatin has a short blood circulation time, which reduces tumour uptake and intracellular DNA binding. PEG conjugation of platinum complexes has therefore been attempted to efficiently deliver

the molecule to the tumour site, increasing the cytoplasmic concentration and subsequent DNA binding efficiency.

Veronese and co-workers prepared a series of PEG conjugates combining PEGs of different molecular weights and shapes (linear or branched), peptide linkers sensitive to lysosomal enzymes and the aminomalonato-Pt-(1,2-diaminocyclohexane) [DACH] active moiety (Schemes 8 and 9) [107]. When the stability of these conjugates was tested under aqueous physiological conditions, it was found that the active drug Pt-DACH was released with an initial fast kinetic rate regardless of the linker. After 2 h, the release rate was slower. Such behaviour could be related to the presence of two platinate isomers, Pt(O,O) and Pt(O,N) (Figure 23), revealed by ^{195}Pt -NMR. The Pt(O,N) isomer, thermodynamically more stable, was released slower than the Pt(O,O) one, which completely hydrolysed within 2 hours. Similarly, Aronov and co-workers [108] prepared a PEG conjugate binding the diamino diaqua Pt complex $[Pt(NH_3)_2(H_2O)_2]^{2+}$ to the polymer through a custom-made malonato linker (Scheme 10). The other extreme of the PEG molecule was functionalised with folic acid (FA), to promote the internalisation by folate receptor-mediated endocytosis. Although the cellular uptake of the folate-PEG-Pt construct was higher than the PEG-Pt analogue, the latter formed more DNA adducts and, as a result, scored higher IC_{50} values (Table 14).

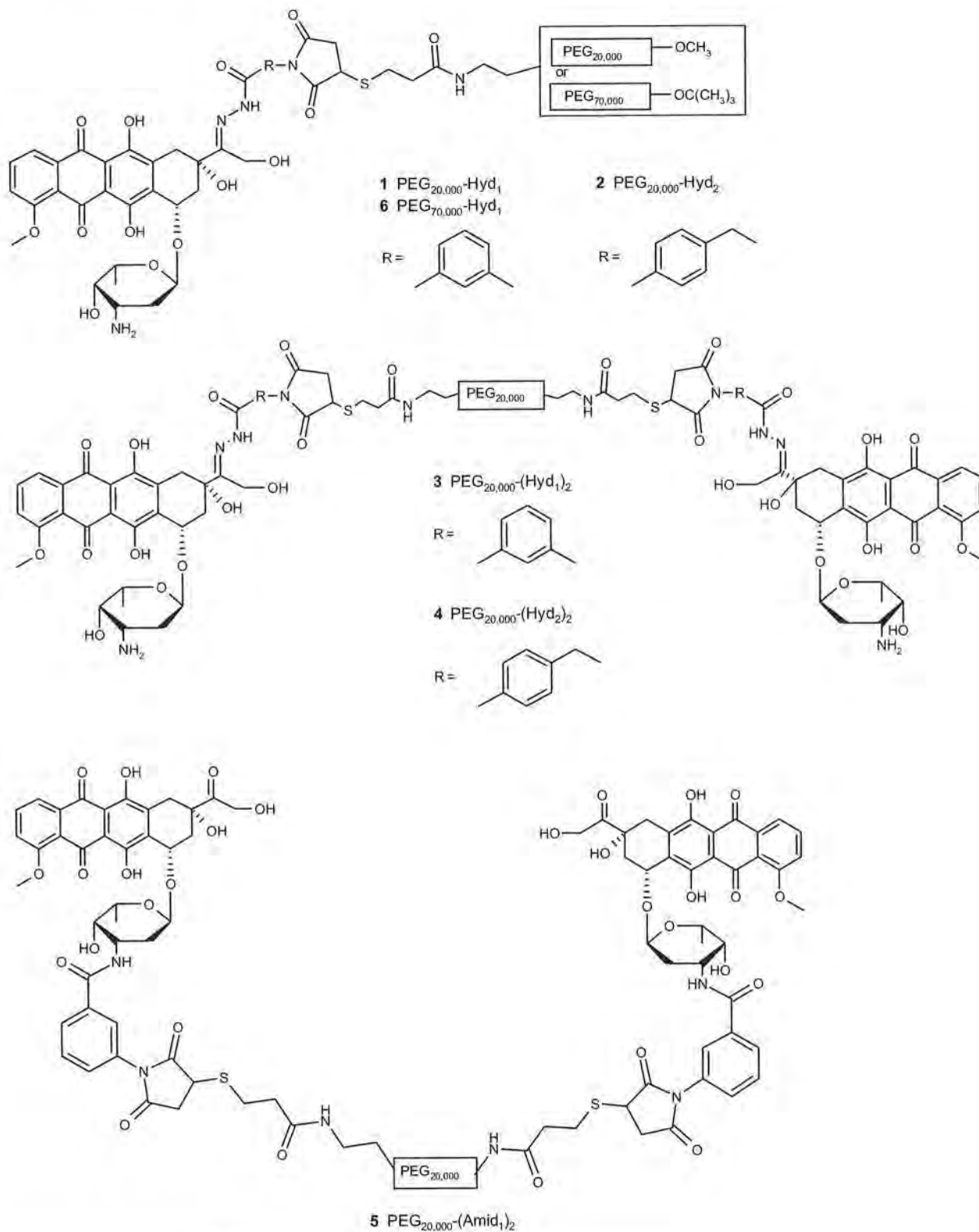


Figure 19. Acid-sensitive PEG conjugates of doxorubicin.

PEG: Polyethylene glycol.

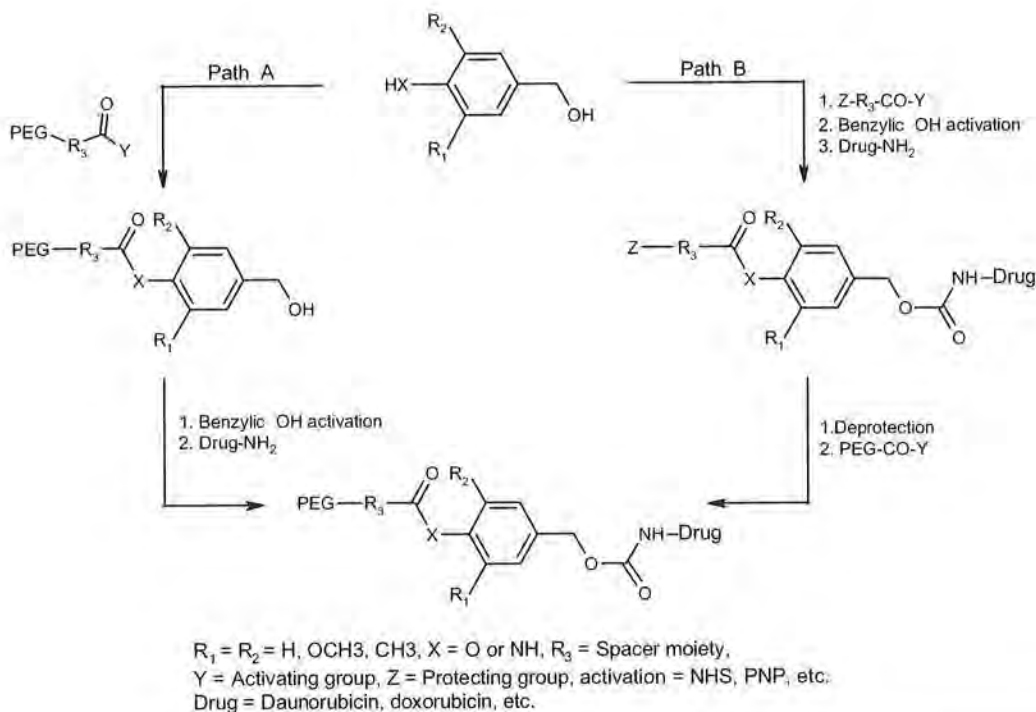


Figure 20. Synthetic preparation of PEG-anthracyclines prodrugs based on 1,4 and 1,6-elimination reactions (BE).

BE: Benzylic elimination; PEG: Polyethylene glycol.

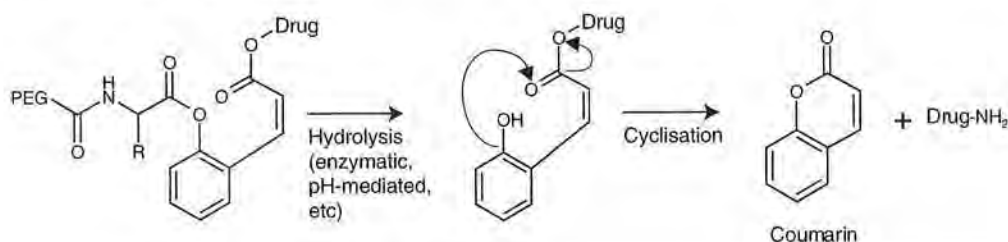


Figure 21. Coumarin-based prodrug system: mechanism of release of amino- or hydroxyl-containing compounds.

PEG: Polyethylene glycol.

6.5 Ara-C

A systematic study of N^4 amino PEG-prodrugs of Ara-C was recently reported by Greenwald and co-workers in two side-by-side papers [26,109]. Both amino acid spacers, BE-type spacers (1,4-elimination) and TML-type linkers (TML lactonisation approach) were used, and comparative *in vivo* tests on LX-1 tumour xenografts were performed (Table 15). N^4 derivatisation of ara-C was proven to prevent inactivation by cytidine deaminase and all the compounds, administered at one-fifth of the free ara-C dose, showed similar antitumour activity. As the low payload of drug carried was the major drawback of these monosubstituted PEG-araC compounds, the same authors prepared a series of tetra- and octa-substituted PEG conjugates, by means of a dendritic spacer based on glutamic or aspartic acid units. These dendritic conjugates showed the same correlation between *in vitro* plasma half life and cytotoxicity found for the monomeric derivatives and the

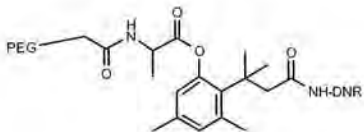
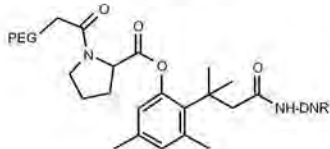
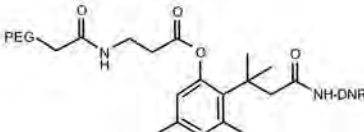
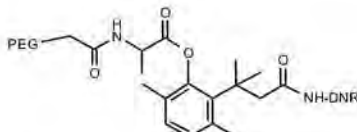
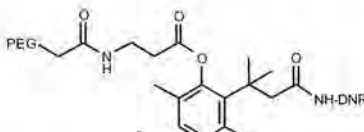
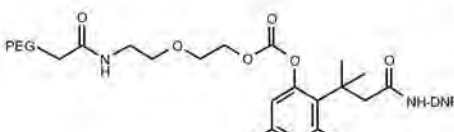
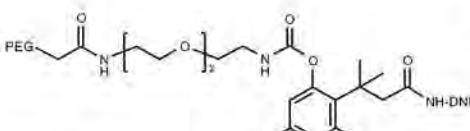
octameric derivative at 40 or 60 mg/kg/dose (based on ara-C content) demonstrated better tumour inhibition than unmodified ara-C or gemcitabine at 100 mg/kg/dose on an orthotopic pancreatic tumour model.

In the authors' laboratories, a series of multibranched PEG-AraC conjugates employing amino adipic acid as branching units were also prepared. This amino dicarboxylic acid was chosen on the basis of computer modelling to prevent steric hindrance. Two, four and eight Ara-C molecules were loaded to mPEG (10,000 Da) in high yield and the compounds were tested for cytotoxic activity and pharmacokinetic profiles. [27]

6.6 Taxanes

Like many other anticancer agents, taxanes are extremely potent agents but very poor drugs, due to their extremely low aqueous solubility. Attempts to formulate vehicles (e.g., Cremophor

Table 12. *In vitro* and *in vivo** results of PEG-TML prodrugs.

Compound	Number	$t_{1/2}$ (h)			IC ₅₀ [†] (nM) P388/O	M109 (%T/C [§]) i.p.	M109 (%T/C [§]) i.v.
		Buffer pH 7.4	Rat plasma	Cell Media			
Daunorubicin HCl	-	-	-	-	3	44.8	117.0
	11Aa	> 24	1.9	14	43	62.8	92.5
	11Ap	> 24	17	32	301	153.1	122.6
	11Aβ	> 24	0.2	80	203	101.2	63.7
	11Ba	> 24	21	36	389	153.6	72.5
	11Bβ	> 24	8	94	411	114.8	31.6
	21A	>24	1.1	38	142	57.1	118.4
	28A	>24	24	53	203	110.5	93.8

*To BALB/c mice bearing subcutaneous Madison lung carcinoma.

[†]Dose of drug required to inhibit tumour growth by 50% compared to controls.[§]Survival rate calculated as ratio between mean survival days of treated group and control group.

PEG: Polyethylene glycol; TML: Trimethyl lock

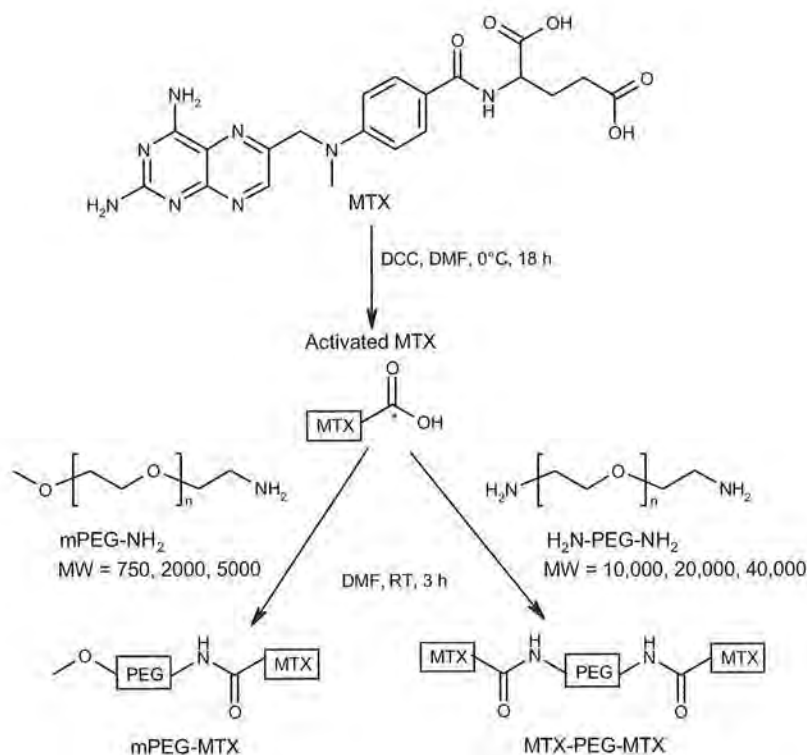


Figure 22. General synthetic scheme of PEG-methotrexate conjugates.

DCC: *N,N'*-Dicyclohexylcarbodiimide; DMF: *N,N'*-Dimethylformamide; MTX: Methotrexate; PEG: Polyethylene glycol; RT: Room temperature.

Table 13. *In vitro* cytotoxicity of methotrexate and MTX-PEG conjugates against various human tumour cell lines.

Compound	Suspension cell lines IC ₅₀ (μM)*		Adherent cell lines IC ₅₀ (μM)*		
	CCRF-CEM	THP-1	HL-60	MCF-7	DU-145
MTX	0.020	0.008	0.010	0.022	0.052
MTX-PEG ₇₅₀	0.57	ND	ND	ND	2.27
MTX-PEG ₂₀₀₀	0.64	ND	ND	ND	2.88
MTX-PEG ₅₀₀₀	0.69	0.64	0.84	0.93	2.08
MTX-PEG ₁₀₀₀₀	3.01	4.16	2.25	5.24	6.96
MTX-PEG ₂₀₀₀₀	1.95	1.97	2.44	1.85	5.11
MTX-PEG ₄₀₀₀₀	6.83	6.07	7.35	>10	>10

*Dose of drug required to inhibit tumour growth by 50% compared to controls.

MTX: Methotrexate; ND: No data; PEG: Polyethylene glycol.

plus ethanol) or designs for water soluble prodrugs have been proposed, including a PEG-paclitaxel conjugate [110].

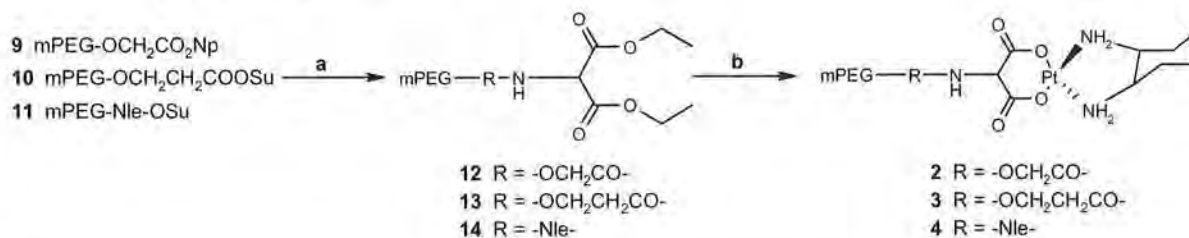
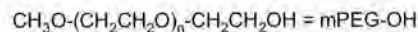
A series of disubstituted PEG-paclitaxel conjugates with amino acid spacers has been prepared by Feng and co-workers [111] and tested against MCF-7 human breast cancer, PG-49 human non-small lung cancer and L1210 murine leukaemia (Table 16). Authors stressed the importance of the amino acid spacer that influences the drug release rate and cytotoxicity.

6.7 Other drugs

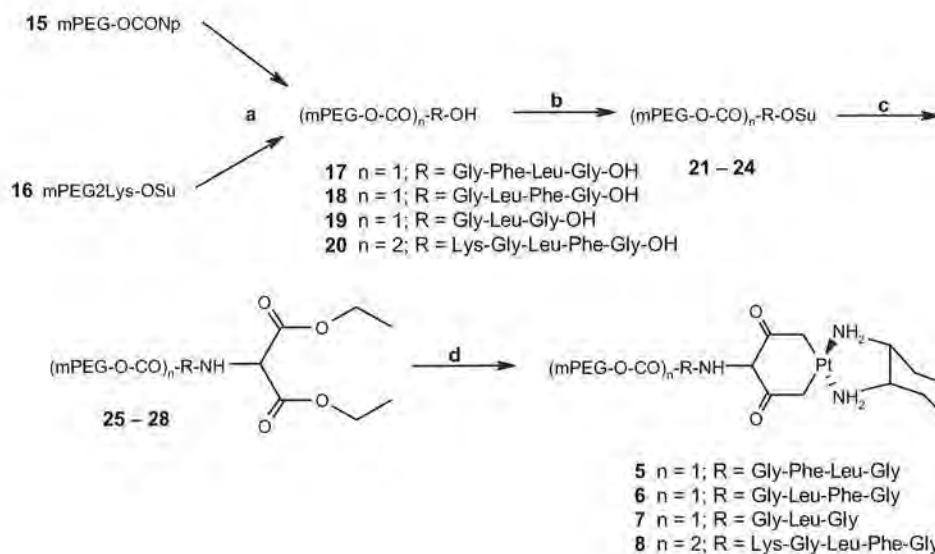
Among other drugs that have been conjugated to PEG in order to modify their pharmacological properties, HIV protease inhibitors, antimalarial and antiviral drugs have been recently reported. A common feature that these compounds share with the anticancer agents described above is the poor solubility in water, which jeopardises their therapeutic potential.

PEGylated HIV protease inhibitors (saquinavir, indinavir and nelfinavir) have been synthesised, together with a series of

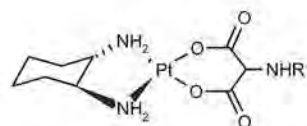
Protein, peptide and non-peptide drug PEGylation for therapeutic application



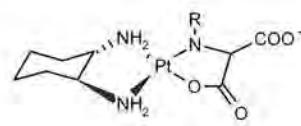
Scheme 8. Synthesis of mPEG-malonatoPtDACH. Reagents: **a**) diethyl aminomalonate, anhydrous DMF, (C₂H₅)₃N, RT, 18 h; **b**) 1) Ba(OH)₂·8H₂O, water, RT, 8 days; 2) cis[DACHPtCl₂], Ag₂SO₄, water, RT, 24 h, N₂ atm., in dark. DMF: Dimethylformamide; PEG: Polyethylene glycol; RT: Room temperature.



Scheme 9. Synthesis of mPEG-malonatoPtDACH. Reagents: **a**) peptide, (C₂H₅)₃N, 1:1 acetonitrile/water, RT, 12h; **b**) CH₂Cl₂ anhydrous, N-hydroxysuccinimide, DCC, 12h, RT; **c**) diethyl aminomalonate, anhydrous DMF, (C₂H₅)₃N, RT, 18h; **d**) 1) Ba(OH)₂·8H₂O, water, RT, 8 days; 2) cis[DACHPtCl₂], Ag₂SO₄, water, RT, 24 h, N₂ atm., in dark. PEG: Polyethylene glycol; RT: Room temperature.

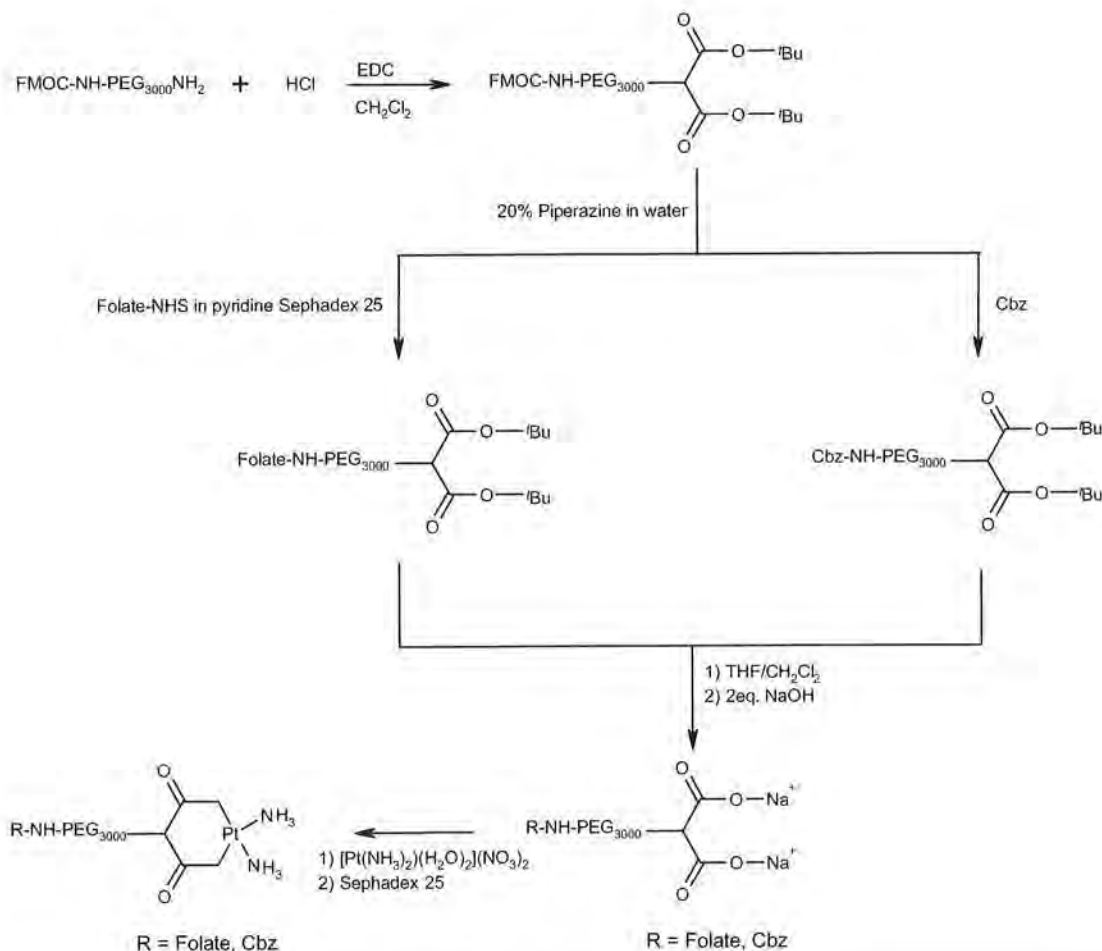


DACH-Pt-malonate (Pt O-O coordination, 6-membered ring)



DACH-Pt-malonate (Pt O-N coordination, 5-membered ring)

Figure 23. Malonato-Pt-DACH isomers (Pt(O,O) and Pt(O,N) forms).



Scheme 10. Synthesis of Folate-PEG-Pt conjugates.

PEG: Polyethylene glycol.

Table 14. Biological monitoring of PEG-Pt compounds.

Platinum compound	IC ₅₀ ^a (μM)	Cell uptake (ng Pt/10 ⁶ cells)		Pt-DNA adducts ng Pt/mg DNA	Calculation of Pt-DNA adducts/ whole cell uptake (%)	
		4 h incubation	20 h incubation		4 h incubation	20 h incubation
PEG-Pt	16.1 ± 2.7	6.7 ± 0.8	4.3 ± 0.3	9.7 ± 1.3	3.6	5.6
FA-PEG-Pt	27.7 ± 0.6	10.3 ± 0.4	6.7 ± 0.9	4.3 ± 0.8	1.0	1.6
Carboplatin	18.5 ± 1.2	25.2 ± 0.3	4.6 ± 0.4	3.7 ± 0.5	0.36	2

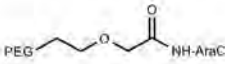
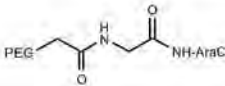
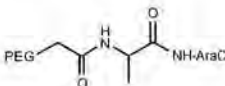
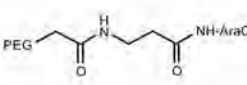
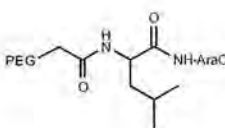
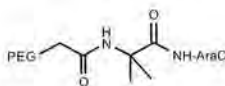
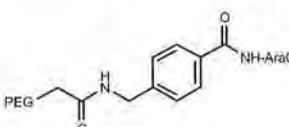
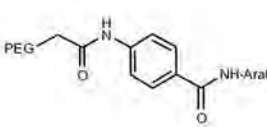
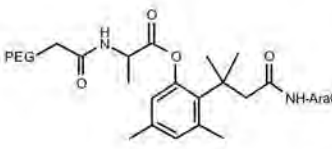
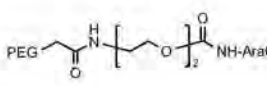
^aDose of drug required to inhibit tumour growth by 50% compared to controls.

D- and L-amino acid derivatives, in order to study the trans-epithelial transport kinetics in Caco-2 cell monolayers as a model of the intestinal barrier [112]. The hydrophilicity increase drastically reduced translocation across the cell membranes and PEG conjugates were administered intravenously (taking advantage of the protection offered by PEG against including high-loading dendrimers, has been patented by

degradation promoted by plasma proteins and the improved blood circulation time).

Artelinic acid is an antimalarial compound with low water solubility. Its sodium salt form is not highly soluble at physiological pH and higher pH is required for desirable solubility levels. A series of PEG derivatives (Figure 24), Shearwater [259].

Table 15. *In vitro* and *in vivo* (mice xenografts, LX-1 cells) results of PEG Ara-C derivatives.

Compound	$t_{1/2}^*$ (h, rat plasma)	$IC_{50}^\dagger$ (nM, P388/O)	%TGI [‡] (LX-1)
Ara-C	-	10	26.2
	3	15	50.5
	1.7	101	ND
	1.5	88	ND
	16	196	36.9
	14	41	38.7
	92	297	10.5
	65	122	12.3
	75	1190	0
	1.9	94	10.4
	38	226	23.8

*Plasma half life.

†Dose of drug required to inhibit tumor growth by 50% compared to controls.

‡Percentage growth inhibition was calculated from the quotient of the median tumour volume of the treatment group divided by the median tumor volume of the control group [(1-T/C) x 100].

PEG: Polyethylene glycol.

Table 16. Cytotoxicity of paclitaxel and prodrugs of general formula $\text{PEG}(\text{OCOCH}_2\text{CH}_2\text{CO-AA-2'-Taxol})_2$ in three tumour cell lines (IC_{50} [nM], continuous exposure time of 72h)

Compound	-AA-	MCF-7	PG-49	L1210
Paclitaxel		2.87	2.14	0.58
5a	Gly	3.51	3.15	0.87
5b	6-aminocaproic	0.46	1.79	2.32
5c	β -Ala	1.53	3.10	1.65
5d	Val	5.31	4.31	3.08
5e	Met	9.53	13.5	1.42
5f	Pro	1.53	2.00	2.22

IC_{50} : Median inhibitory concentration.

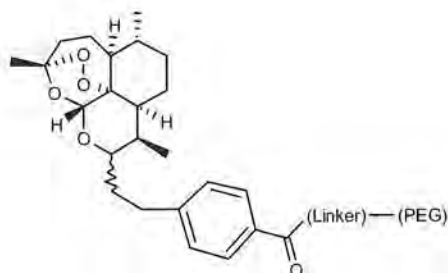


Figure 24. General formula of PEG-Artelinic acid conjugates.

PEG: Polyethylene glycol.

Some PEG–acyclovir and valacyclovir (Talavir[®]; Sigma Tau) conjugates have been proposed by Zacchigna and co-workers [113] with the aim of increasing water solubility and blood circulation time of the parent drug. A β -alanine linker was used to bind the acyclovir moiety to $\text{PEG}(\text{OH})_2$ (molecular weight = 1500 Da) and release studies in various media were performed. Valacyclovir conjugates were found to release the active drug slower than the acyclovir adducts that were completely hydrolysed in plasma within 24 h and at pH 1.2 buffer in 5 h.

PEGylation of amphotericin B (AmB), an antifungal polyene antibiotic virtually insoluble in water, has been proposed by Enzon researchers as a means to increase solubility and protect the amino group on the sugar moiety, essential for the antifungal activity [114]. One of their two platforms developed for PEG conjugates with controlled release (1,4- and 1,6-benzyl elimination and TML lactonisation) was used, namely the 1,6-benzyl elimination system and a comprehensive pharmacological study (*in vitro* and *in vivo* release, safety, circulatory retention) was performed. Conjugation of PEG to AmB produced prodrugs significantly less toxic than the free drug formulation but maintaining or even enhancing the *in vivo* antifungal effectiveness.

7. Expert opinion

Since the pioneering reports of Davis and Abuchowsky, described in the two most well-known Journal of Biological Chemistry papers [22,115], research on PEG conjugation has increased in academic institutions and in pharmaceutical companies. The PEGylation concept, initially proposed and developed for protein modification [4,5,10] was later extended to peptide [6,12], non-peptide drugs [94,95] and also to cells.

The first few years have seen an effort in improving conjugation strategies by taking into consideration even milder chemistries, compatible with the unstable structure of many ligands. Methods were also developed for conjugation of the most reactive groups in proteins, although some open problems still remain in this field, such as a satisfactory chemistry for specific binding to the guanidine group of arginine or to the indole group of tryptophan. An area that also needs further development deals with the purification techniques of the conjugates. A typical case is the removal of unreacted PEG from the desired conjugate in the case of low molecular weight drugs, when the small differences in weight and physicochemical properties between conjugate and unreacted polymer makes the separation difficult. Another case is when protein positional isomers are present in the conjugation mixture. Moreover, the formation of positional isomers during the conjugation reaction in proteins is another problem awaiting new and original solutions, although advances in this area were recently described to be based on enzymatic methods of PEGylation as patented by Sato *et al.* [232].

Last but not least, the problem of obtaining monodisperse or at least very low polydisperse PEG still exists, mainly with high molecular weight polymer species. In fact, LCC Engineering and Trading GmbH is now offering monodisperse PEGs but unfortunately this is still a limited solution as, so far, the company produces only PEG oligomers with a molecular weight < 600 Da.

With regard to more general considerations, the authors are pleased to observe that PEG bioconjugation is now expanding

greatly from proteins to drugs, and also in solving problems other than immunogenicity and residence time in the blood, the only properties investigated at first. In the area of non-peptide drugs in particular, attention is now dedicated to the potential of the heterobifunctional PEGs, which allow the combination of the advantages of polymer modification with the active targeting capacities of a second molecule. Thanks to the discovery of numerous new ligands for targeting [116] to specific tissues, organs, or for entrapment and release of the drug into cells, the field of non-peptide conjugation is receiving increasing interest. Of course this area will need new ideas on the chemistry of binding to expand the number of the already existing heterobifunctional products offered on the market, mainly from the Nektar Corporation.

An idea that will definitely receive further improvement and is already described in many patents is the combination of genetic engineering and PEGylation. This can lead

to conjugated muteins that have improved specificity or new properties in respect to native proteins or receptors. The patent filed by Genentech, Inc. [205] on GH receptor antagonists is a good example in this direction.

In conclusion, reviewing hundreds of papers and patents, of which a more recent selection is presented here, the authors have the clear impression that the field of PEGylation and conjugation, also through other polymers [117], is still a young and vigorous research area, although it is already considered, in many aspects, a mature technology. Therefore, it should not be surprising to see new and unexpected applications resulting from PEGylation in the near future.

Acknowledgements

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