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].

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 polydispersivity and in diol amounts was achieved in industrial production and improvements are also rmade 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-α2b (PEG-Intron®, Schering-Plough) [16,203], branched PEG (40 kDa)–IFN-α2a (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 polydispersivity (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].

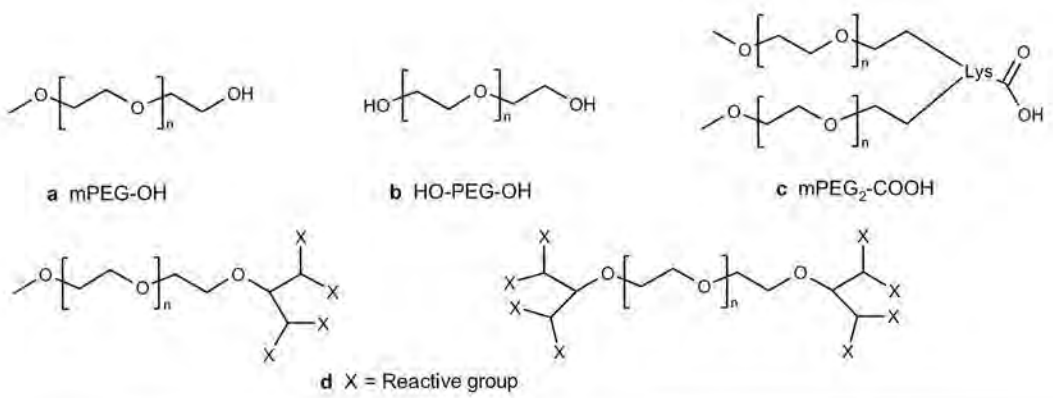

Figure 1. Different PEG structures. **a)** Linear monomethoxy PEG; **b)** Linear diol PEG; **c)** Branched PEG; and **d)** Multifunctional PEGs.
PEG: Polyethylene glycol.

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 multiarm 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 not 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].

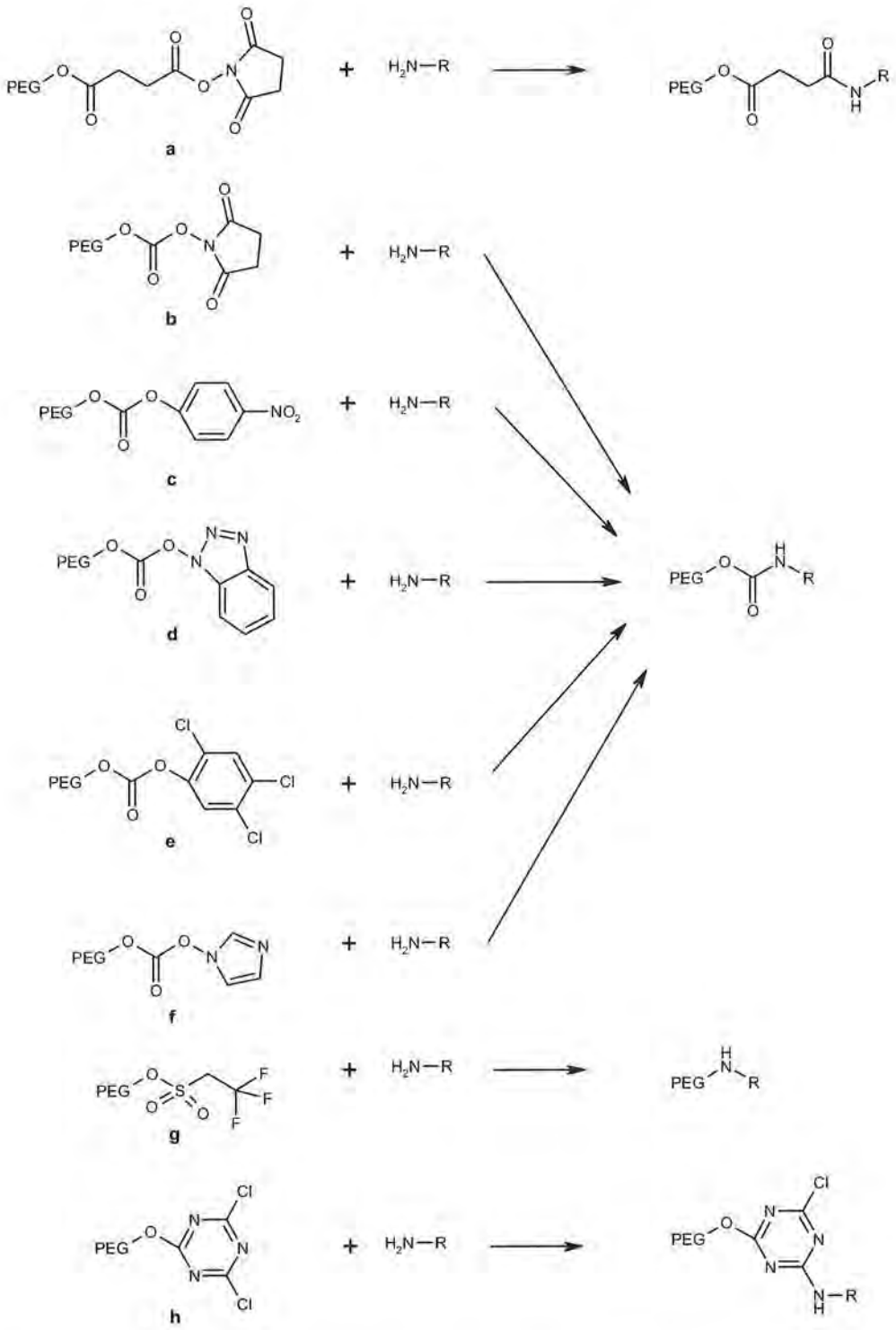

Figure 2. Examples of activated PEG molecules reactive towards amino groups. a) PEG succinimidyl succinate; **b**) PEG succinimidyl carbonate; **c**) PEG p-nitrophenyl carbonate; **d**) PEG benzotriazol carbonate; **e**) PEG trichlorophenyl carbonate; **f**) PEG carbonylimidazole; **g**) PEG dichlorotriazine;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

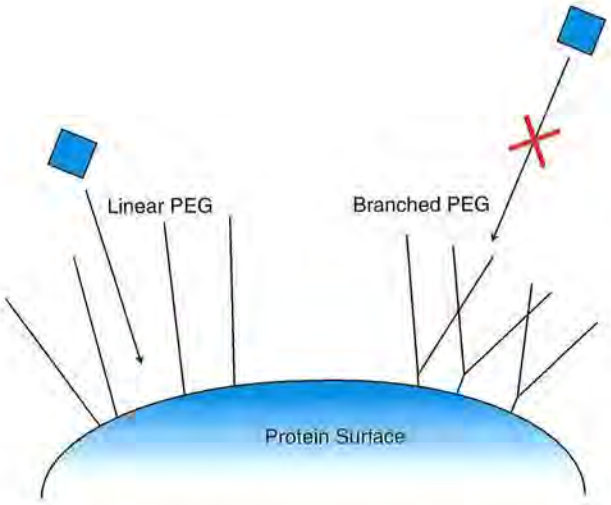

Figure 3. Structure of linear and branched PEG on the protein surface. The 'umbrella-like' structure of branched PEG explains its higher capacity for rejecting approaching molecules or cells compared to linear PEG.
PEG: Polyethylene glycol.

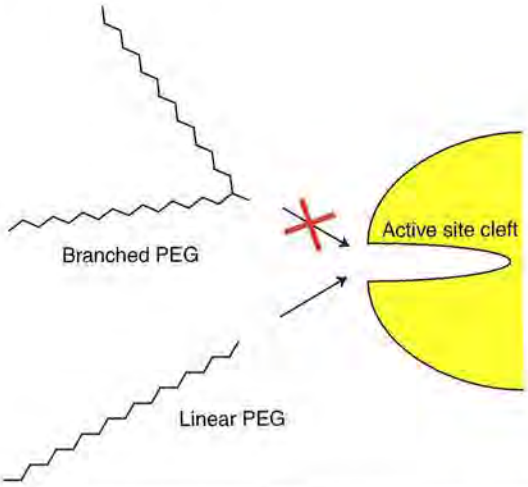

Figure 4. Effect of PEG hindrance on the enzyme active site. The high steric hindrance of branched PEG may be advocated to explain the lower inactivation of enzymes compared to linear PEG of the same size.
PEG: Polyethylene glycol.

- '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**).

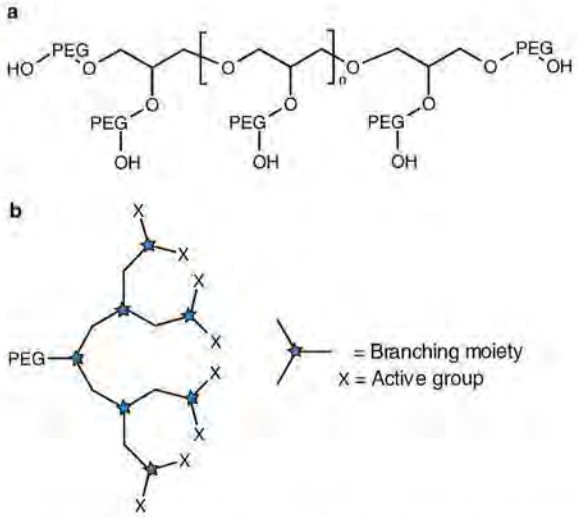

Figure 5. Different strategies to achieve multifunctional high loading PEGs. a) Multiarm PEGs. **b)** Dendronised PEGs, the branching moiety may be a bicarboxylic amino acid, lysine or other bifunctional molecule.
PEG: Polyethylene glycol.

- 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, H_2N–PEG–COOH, HO–PEG–COOH and H_2N–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 H-Gly-Phe-Leu-Gly-OH or 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

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