PEGylation, successful approach to drug delivery

Francesco M. Veronese and Gianfranco Pasut

PEGylation defines the modification of a protein, peptide or non-peptide molecule by the linking of one or more polyethylene glycol (PEG) chains. This polymer is non-toxic, non-immunogenic, non-antigenic, highly soluble in water and FDA approved. The PEG-drug conjugates have several advantages: a prolonged residence in body, a decreased degradation by metabolic enzymes and a reduction or elimination of protein immunogenicity. Thanks to these favorable properties, PEGylation now plays an important role in drug delivery, enhancing the potentials of peptides and proteins as therapeutic agents.

The importance of proteins with structural, signaling or enzymatic functions has always been recognized but the importance of their post-translational modifications and their role in the pathological process was only recently demonstrated. Suitable investigations, possible now because of the advanced analytical tools, demonstrated how many proteins are present in the body in conjugated state and how diverse are the chemical reactions that modify the primary sequence: phosphorylation [1], acylation [2], methylation [3], glycosylation [4] and sulfation [5], just to cite the most known. Each of these modifications can involve different sites of a protein and they are exploited by nature to obtain different functions *in vivo*, where even a small modification creates a new entity that is recognized and might perform a different function compared with the native protein; a simple change in the protein structure can trigger functions, such as signaling, targeting, catalysis, catabolism, modification of circulation time in the body and immunogenicity.

What nature discovered and selected by evolution, we are now trying to replicate in the laboratory by using chemical tools to link synthetic molecules, generally polymers, to native proteins, yielding conjugates with more-favorable behavior. Among all advantages that a protein can achieve with this technique, there are decrease in immunogenicity and antigenicity, and increase in body-residence time and stability (Figure 1). The most successful strategy employs polyethylene glycol (PEG) as modifying polymer, a strategy termed PEGylation, which led to important results in therapy, organic biocatalysis and diagnosis.

PEGylation was first described in the 1970s by Davies and Abuchowsky and reported in two key papers on albumin and catalase modification [6,7]. This was an important milestone, because at that time it was not conceivable to modify an enzyme so extensively and still maintain its activity. Proteins were in fact considered very delicate entities and only few gentle modifications with low molecular-weight products were carried out, mainly to study SARs.

Since then, the procedure of PEGylation as initially described was expanded and developed tremendously and now a wide range of chemical and enzymatic

Francesco M. Veronese*
Gianfranco Pasut
Department of Pharmaceutical Sciences, Padua University, Via F. Marzolo 5, 35128 Padua, Italy
*e-mail: francesco.veronese@unipd.it

1359-6446/05/$ – see front matter PII: S1359-6446(05)03575-0

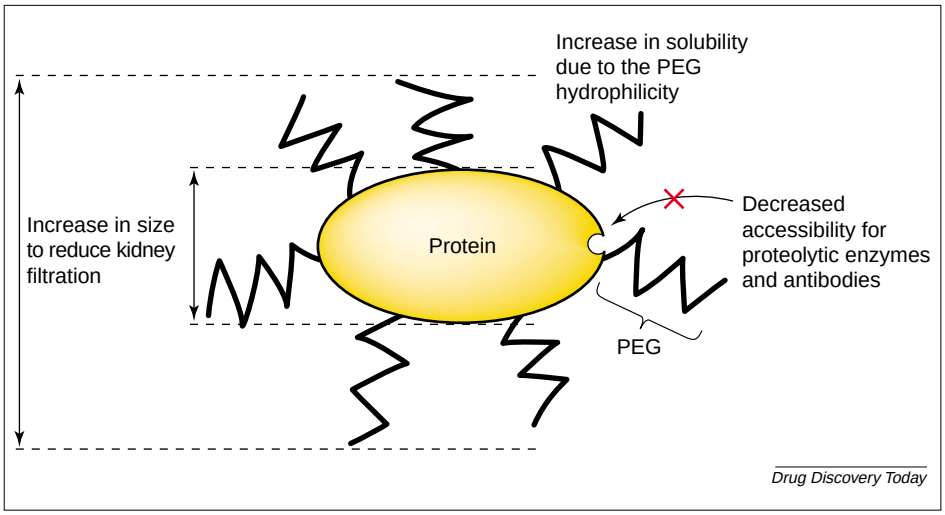
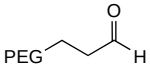
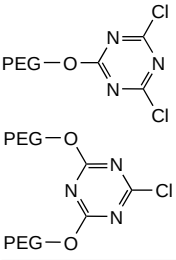
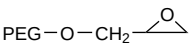


FIGURE 1

Main advantages of PEGylated protein. The figure represents a polymer-protein conjugate. The polymer, PEG, is shielding the protein surface from degrading agents by steric hindrance. Moreover, the increased size of the conjugate is at the basis of the decreased kidney clearance of the PEGylated protein.

TABLE 1

PEG derivatives that maintain the charge of the native protein in the final conjugate

Structure	Alkylating PEGs	Properties
	PEG–aldehyde (also in the form of more stable acetale)	A two steps reaction; the first product (a Shiff base) is reduced by $NaCNBH_3$. When the coupling reaction is carried out at low pH = 4.5–5, it labels only the α-amino group.
	PEG–tresyl or tosyl	Not much used because the chemistry leads to a mixture of products.
	PEG–dichlorotriazine or chlorotriazine	Now they are abandoned for therapeutic application because of their toxicity.
	PEG–epoxide	Slowly reactive, rarely used.

methods for conjugation are at hand [8]. This variety of modification procedures offers the possibility to address the requirements of different proteins. The choice of a better reactive PEG allows the modification of only the wanted amino acids in the sequence. Amino groups were the first target of PEGylation, by acylation or alkylation reactions, but now conjugation of PEG to thiol, hydroxyl or amide groups is also possible, by using several specific chemical or enzymatic methods.

Amino group modification

In the early days of PEGylation, researchers directed their attention towards the amino groups as suitable conjugation site, because they are the most represented groups in proteins, generally exposed to the solvent and can be modified with a wide selection of chemical strategies.

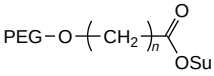
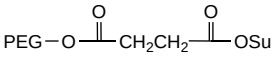
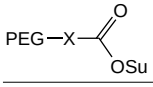
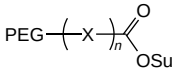
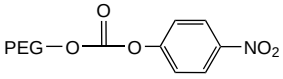
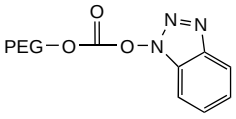
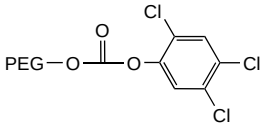
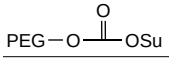
Several conjugation strategies are now available, such as alkylation, which maintains the positive charge of the starting amino group because a secondary amine is formed, or acylation, accompanied by loss of charge. Tables 1 and 2 show the most popular PEG derivatives along with some of their relevant properties.

Although amino conjugation represents so far the most common modification and often the first approach in many new PEG-protein projects, it is not devoid of limitations, because of the high number of isomers obtained. The purification of these mixtures is usually difficult, which complicates the needed disclosure of their composition for the FDA approval. However, the FDA will approve a mixture of isomers, if evidence for the reproducibility of the reaction is provided. This has been the case for the first two PEG conjugates on the market, PEG-asparaginase (Oncaspar®) [9], for the treatment of acute lymphoblastic leukemia and other lymphoid malignancies, and PEG adenosine deaminase (Adagen®) [10], for the treatment of severe combined immunodeficiency disease. Now, the requirements for the approval of new conjugates are more stringent and the characterization of each isomer, when it is possible, is compulsory. Examples are the two α-interferon conjugates, Pegasys® [11] and PEG-Intron® [12] (used to eradicate hepatic and extrahepatic hepatitis C virus infection), for which almost all the binding sites in the primary sequence were established.

The evolution of PEGylation chemistry allows also site-specific amino modification, thus helping the purification and the characterization procedures, because mixtures of PEGylated products are avoided. Furthermore, site-specific modification might lead to a better preservation of the native protein activity in the conjugate. A method devised by Kinstler [13] takes advantage of the lower pK_a of the N-terminal α-amino groups compared with that of the α-amino group in lysines [14]. The conjugation in this case was performed on granulocyte colony stimulating factor (G-CSF) by a reductive alkylation with PEG aldehyde, leading to the marketed Pegfilgrastim® [13], used to treat granulocyte depletion during chemotherapy. It is also feasible to protect the active site of enzymes or the recognition area of proteins by carrying out the PEGylation in the presence of an inhibitor, a substrate, or a specific ligand, with the aim to cover the reactive groups close to sensitive areas. These ligands might be free in the

TABLE 2

PEG derivatives that, after amino coupling, lead to a loss of positive charge in the final conjugate with respect to starting protein

Structure	PEG-carboxilates	Properties
	Several PEG derivatives with one or more CH_2 groups between the PEG and the carboxylic group	The carboxylic group is activated as N-hydroxy succinimidyl ester, imidazole or benzotriazole. The kinetic rate of conjugation depends on the numbers, and eventual ramification of CH_2 groups linked to the carboxyl group.
	PEG–succinimidyl succinate	The ester bond between succinic acid and PEG is easily hydrolyzed.
	PEG–amino acid–succinimidyl ester	Nle or βAla as amino acid moiety allows an easy quantification of the number of linked PEG chains by amino acid analysis.
	PEG–peptide–succinimidyl ester	The Met-Nle or Met-βAla allows the removal of PEG by CNBr treatment for an easy localization of PEGylation site. Lysosomal cleavable sequences, as H-Gly-Phe-Lue-Gly-OH, allow the release of the bound drug inside the cell.
Structure	**PEG-carbonates**	**Properties**
	PEG–*p*-nitrophenyl carbonate	Slowly reactive, yield a urethane linkage with amine.
	PEG–benzotriazolyl carbonate	Slowly reactive, yield a urethane linkage with amine.
	PEG–2,3,5-trichlorophenyl carbonate	Slowly reactive, yield a urethane linkage with amine.
	PEG–succinimidyl carbonate	Slowly reactive, yield a urethane linkage with amine.

Key: Su, succinimide; X, amino acid linker.

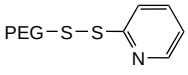
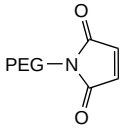
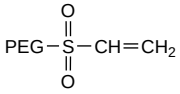
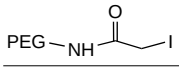
reaction solution or linked to an insoluble resin. In the latter case, not only the binding site but also its surroundings are protected from conjugation [15].

Thiol modification

PEGylation at thiol groups of cysteines not involved in disulphide bridges is one of the most specific methods because cysteines are rarely present in proteins or peptides.

TABLE 3

PEGs reactive towards a thiol group

Structure	Thioreactive PEGs	Properties
	PEG–pyridildisulphide	The most specific towards thiol but yields a cleavable linkage by a reducing agent also *in vivo*.
	PEG–maleimide	Gives stable linkage by double bond addition but can also react with amines at pH >8.
	PEG–vinylsulfone	
	PEG–iodo acetamide	Less reactive, not much used

Some selective thiol PEGylating agents are reported in Table 3. Unfortunately, as a result of its hydrophobicity, cysteine is often buried inside the protein structure and therefore only partially accessible to reagents.

Thiol modification by PEGylation is expanding its potential, thanks to genetic engineering, which allows the introduction of a cysteine residue almost anywhere in the protein sequence, by replacement of a nonessential amino acid. Many mutant proteins have been described, which were generated to PEGylate therapeutically important drugs, such as human growth hormone or G-CSF [16,17].

If a cysteine is present, but not accessible to reagents, it is still possible to perform PEGylation, as reported for β-interferon [18] or G-CSF [17]. In the former case, the buried and less-accessible cysteine 17 was modified with a double-step method. In the first step the cysteine was modified with a heterobifunctional low molecular-weight PEG, bearing at one end a thiol reactive group and at the other an azide group. This polymer can reach the cysteine because of its low steric hindrance. In a second step, a high molecular-weight PEG chain, with a group reactive towards azides, was specifically linked to the first PEG chain. A different approach was followed to modify the inaccessible cysteine 18 of G-CSF. This protein was partially denatured in 4 M guanidine chloride but maintained the

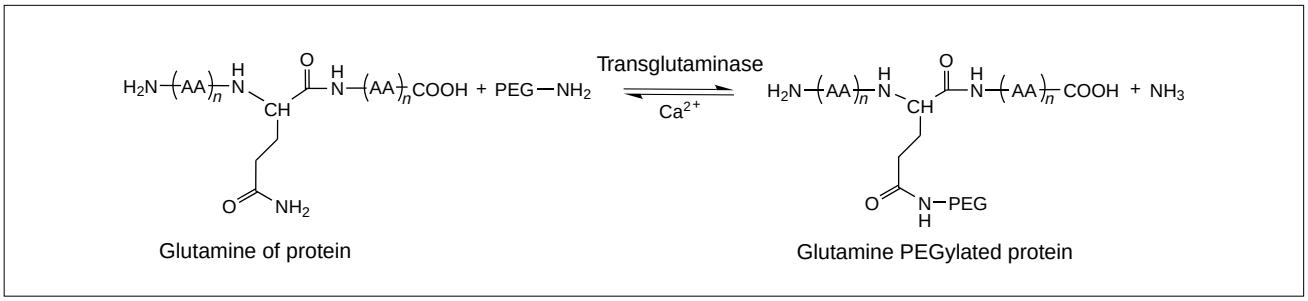


FIGURE 2

Schematic representation of enzymatic PEGylation conducted by transglutaminase. The enzymes catalyses the transglutamination between the PEG's amino group and the glutamine's amide, yielding a selective PEGylation at the level of preferential protein's glutamines.

native disulphide bridges, and then conjugated to thiol reactive PEGs. After renaturation, G-CSF regained structure and biological function [17].

A simple strategy, although less specific, relies on the reduction of protein disulphide bridges with the aim of exposing new thiol groups [19]. This strategy has been used for the PEGylation of antibodies, as the modification of amino groups is not suitable because of the marked loss of recognition that accompanies the procedure. However, disulphide bridges that link the IgG heavy chains can be cleaved, yielding to an active Fab moiety with new exposed thiol groups. These become sites of conjugation localized far from the antibodies recognition site [19].

Specific PEGylation by enzymes or by reversible protection

The specific conjugation of PEG to the amide group of glutamines or to the hydroxyl group of serines and threonines is only possible under mild conditions using enzymes. There are several naturally occurring enzymes that recognize glutamine as substrate, namely specific or non-specific transglutaminases. Recently, Sato [20] discovered that glutamine in proteins can be the substrate of the transglutaminase enzymes, if an amino PEG is used as the nucleophilic donor. Through a transglutamination reaction the enzyme links PEG to the protein at the level of the glutamine residue (Figure 2) [20].

A different strategy that exploits enzymes of the post-translational modification machinery was suggested by Neose Technology [21]. More precisely, *Escherichia coli*-expressed proteins (nonglycosylated) were first selectively glycosylated at the hydroxyl group of a specific serine and threonine by using a recombinant O-GalNAc-transferase to add a residue of *N*-acetylgalactosamine (GalNAc). The glycosylated proteins were subsequently PEGylated using the O-GalNAc residue as the acceptor site for a sialic acid PEG, a reaction selectively performed by a sialyltransferase. The sialyltransferase transfers a cytidine monophosphate (CMP) derivative of PEGylated sialic acid to the O-GalNAc residue of glycosylated proteins. Other enzymes have also been proposed for specific PEGylation, for example tyrosinase for conjugation to tyrosine [22].

An alternative to enzymatic methods is a recently developed two-step chemical procedure, which was implemented to link PEG to specific amines in peptides. This procedure involves the reversible protection of some residues that could be involved in PEGylation, with the aim of preventing their modification. Although this method was successfully employed on peptides, such as insulin or somatostatin analogues [23,24], it is not commonly suitable for proteins, because the protein's structure can be affected during the protection and deprotection reactions. The only example of a successful use of this method on proteins is the structurally stable superoxide dismutase [25].

Limitations in the use of PEG

PEG is obtained by chemical synthesis and, like all synthetic polymers, it is polydisperse, which means that the polymer's batch is composed of molecules having different number of monomers, yielding a Gaussian distribution of the molecular weights. This leads to a population of drug conjugates, which might have different biological properties, mainly in body-residence time and immunogenicity. Nowadays, because of the development of synthetic and purification procedures, PEGs on the market are less polydisperse than those employed initially, but the polydispersivity problem must be still taken into consideration, especially when dealing with low molecular-weight drugs, either peptide or non-peptide drugs, where the mass of linked PEG is more relevant for conveying the conjugate's characteristics, mainly those related to the molecular size.

A second problem for the use of this polymer relates to the excretion from the body. As for other polymers, PEGs are usually excreted in urine or feces but at high molecular weights they can accumulate in the liver, leading to macromolecular syndrome. It is not easy to extrapolate the kidney excretion limit of PEG by looking only at the kidney clearance threshold of protein (~60 kDa, albumin), because other factors play an important role. For example, the high water coordination of the polymer increases the PEG's hydrodynamic volume up to 3–5 times that of a globular protein having the same molecular weight, thus decreasing the polymer kidney clearance threshold and

TABLE 4

Approved PEG conjugates

PEG conjugates	Type of PEGylation	Year to market	Disease
With proteins			
PEG–asparaginase (Oncaspar®) [9]	Random, linear PEG	1994	Acute lymphoblastic leukemia
PEG–adenosine deaminase (Adagen®) [10]	Random, linear PEG	1990	Severe combined immunodeficiency disease (SCID)
PEG–interferon α2a (Pegasys®) [11]	Random, branched PEG 40 kDa	2002	Hepatitis C
PEG–interferon α2b (PEG–Intron®) [12]	Random, linear PEG 12 kDa	2000	Hepatitis C and clinical trials for cancer, multiple sclerosis, HIV/AIDS
PEG–G-CSF (pegfilgrastim, Neulasta®) [14]	Selective, linear PEG 20 kDa	2002	Treating of neutropenia during chemotherapy
PEG–growth hormone receptor antagonist (Pegvisomant, Somavert®) [50]	Random, linear PEG 5 kDa (genetic modified protein)	2002	Acromegaly
With oligonucleotides			
Branched PEG–anti-VEGF aptamer (Pegaptanib, Macugen™) [46]	Selective, branched PEG 40 kDa	2004	Macular degeneration (age-related)

the linear and flexible structure of PEG chains that help the polymer to cross the glomerular membranes by a 'snake-like' movements. Anyway, urinary excretion is dependent on molecular mass and a glomerular filtration threshold was calculated to be 30 kDa, whereas for higher molecular weights the excretion in the feces becomes predominant.

The chain length can also be reduced slowly *in vivo* by enzymes, such as cytochrome P450 [26] or alcohol dehydrogenase [27], and the branched PEG might lose one chain by an anchimeric-assisted mechanism of hydrolysis, where a specific neighboring group, in this case an amide, triggers the release [28]. So far, the higher PEG molecular weight used for protein conjugation is the 40 kDa branched form [11].

Improved protein drugs by PEGylation

Several classes of protein drugs, such as enzymes, cytokines and antibodies, are significantly improved by PEGylation [8]. Table 4 compiles the most important examples of protein conjugates, exploiting the advantages of PEGylation and leading to derivatives that are useful for therapy. In general, the improvements are an increased retention time in the body, a reduction of immunogenicity and increased stability towards metabolic enzymes.

Unfortunately, the PEGylation of proteins is often accompanied by loss of biological activity. However, this is compensated for by the prolonged body-residence time, as a result of the increased stability and higher hydrodynamic volume. A typical example is the PEGylated α-interferon Pegasys®, which retains only 7% of the antiviral activity of the native protein, but still shows a greatly improved performance *in vivo* compared with the unmodified enzyme because of improved pharmacokinetics [11]. This example demonstrates how difficult it is to extrapolate the *in vivo* behavior of the conjugates from *in vitro* data. A further instructive case comes from the PEGylation of the tumor necrosis factor (TNF) and its lysine-deficient mutant proteins, as recently reported by several laboratories [29–31]. It was demonstrated that body-residence time, PEG mass and shape, and stability of PEG–protein bonds are all important factors for the anti-tumor activity, probably because they play a role, in this specific case, in releasing the native protein [32] or in maintaining the activity of the protein inside the tumor mass. Therefore, if biological tests are needed to verify the advantages of PEGylation, they should always include a precise chemical investigation of the conjugate structure. For example, a detailed analysis was required to demonstrate the differences in biological activity of the two interferons (INF) studied by Hoffman-La Roche and Schering-Plough [11,12]. Both conjugates are monoPEGylated but in the former, a 40 kDa branched PEG conjugates of INFα–2a, 94% of the overall polymer attachment occurs at one of the four lysines (Lys^{31}, Lys^{121}, Lys^{131} and Lys^{134}). The latter is a more heterogeneous product, because there are 14 positional isomers of the mPEG12000-INFα–2b, with an unexpected conjugation at the level of histidine 34, which alone represents the 47.8% of all the isomers. The difference in linking sites is mainly due to the different pH values during the conjugation and to the different size and shape of PEG used [11,12]. The INF PEGylated with the 40 kDa polymer has a more prolonged activity because of its higher body-residence time, however, the mPEG12000-INFα–2b, with its uncommon PEGylation at the level of the histidine, possesses a faster but shorter therapeutic response, because *in vivo* the conjugate slowly releases the PEG chain, yielding the fully active native protein.

Antibodies and enzymes have been successfully studied as antitumor drugs because conventional chemotherapy and radiotherapy are ineffective in many circumstances, mainly because of unspecific activity. A disadvantage of mouse, chimeric and also humanized antibodies is their potential to induce immunogenic reactions, a limitation that is also common to other antitumor proteins, such as the metabolite-depleting enzymes obtained from microbial

cells (for example asparaginase, arginine deaminase and methioninase, which degrade tumor essential nutrients) [33]. The immunogenicity drawback, as well as the need of a prolonged body-circulation time, is now stimulating the use of PEGylation to improve the potency of many proteins and enzymes and also their safety after repeated administrations.

Small-drug PEGylation

Common problems encountered in the use of small drugs, especially the antitumor ones, are their low solubility, rapid excretion and untargeted biodistribution. All these factors could be addressed by PEGylation. Generally, the properties of PEG are conveyed to the conjugated drugs and their body fate reflects that of the polymer. Increased solubility, modification of pharmacokinetics and targeting have been described, after PEGylation, for important drugs such as taxol, camptothecin, *cis*-platinum and doxorubicin [8].

The conjugation chemistry for non-protein drugs faces fewer problems because of the reduced number of functional groups present on a small molecule, the absence of conformational constrains and the easier purification and characterization steps for the drug–polymer conjugates. For small drugs many researchers are exploiting PEGylation for conferring a passive targeting to solid tumors, by the enhanced permeability and retention effect [34], or simply for improving the drug's pharmacokinetic profile by slowing the drug clearance from the body. Usually the conjugates are considered macromolecular prodrugs, because the drug has to be released to exploit its activity. In recent years, methods have been developed to release the drugs from the conjugate in specific compartments under controlled conditions. For example, drug release inside the cell was achieved by using special linkers or bonds between the polymer and the drug. These might be hydrolyzed by the acidic pHs of endosome (i.e. *N*-*cis*-aconityl acid spacer and hydrazon linkages [35]) or by the lysosomal enzymes (i.e. H-Gly-Phe-Leu-Gly-OH or H-Gly-Leu-Phe-Gly-OH spacers [36]). A sophisticated strategy in the field of controlled-rate release of drug conjugates uses linkers that are designed to form double prodrug systems. In these, the drug linker is first released from the polymer by hydrolysis (first prodrug), then triggers a linker (second prodrug), releasing the free and active drug. Examples of these double drug-delivery systems are the 1,6-elimination reactions or the trimethyl lock lactonization [32,37].

A general problem encountered in PEGylation of small drugs is the low loading of this polymer, which possesses only one or two hydroxyl terminal groups that can be activated. To overcome this limitation, some research groups have proposed the construction of dendrimeric structures at the level of the polymer termini, obtaining PEG dendrons, called also forked PEGs. The implementation of the number of active groups is reached by using branching molecules, such as bicarboxylic amino acid, which allow the increase of the drug–polymer loading [38].

However, we must note that years of research on PEGylation of low molecular-weight drugs, although resulting in hundreds of interesting publications and patents, has not so far yielded commercially available PEG small-molecule products [39]. This missing point might be mainly due to the low loading of PEG itself and to the difficult synthesis of PEG dendrons in terms of cost and industrial production, compared with other multifunctional polymers, such as *N*-(2-hydroxypropyl)methacrylamide copolymer [40] and polyglutamic acid [41].

PEG as a diagnostic carrier

In vivo non invasive diagnosis is done by using tracers detected through magnetic resonance or radioactivity. Usually they are administered in a chelated form using compounds that can give specific biodistribution, stability or targeting. Among the chelators used, the macromolecular ones (e.g. polymers, antibodies and recognition proteins) are receiving increase interest. The properties of PEG have also been exploited in diagnostics. In fact, PEGylation increases the body-residence time of paramagnetic chelates that will be cleared more slowly than the unmodified molecules through the kidney or liver, thus allowing more detailed images by magnetic resonance [42]. Furthermore, the biodistribution pattern of radiodiagnostics is profoundly changed, as in the case of the C225 antibody–PEG–radiometal chelators [43], in which PEG acts also as linker between the targeting and diagnostic moieties. C225 is a monoclonal antibody directed against the epidermal growth factor receptor, which was conjugated to a heterobifunctional PEG bearing a radiometal chelator (diethylenetriaminepentaacetic acid, DTPA) at one terminus. The conjugate DTPA–PEG–C225 retained 66% of binding affinity, and, more importantly, when labeled with Indium-111 (^{111}In) it showed narrower steady-state distribution than the non-PEGylated ^{111}In–DTPA–C225, because of reduced nonspecific binding. Therefore, in case of protein targeted diagnostic, PEG could help to collect better defined images by limiting the background noise due to nonspecific protein–protein interaction.

Using PEG as macromolecular carrier for a phosphine chelating agent of technetium (Tc), the authors could recently discover an unexpected oxo-reduction reaction between the perthecnentate $^{99m}TcO_4^-$ (the ^{99m}Tc form as obtained from the ^{99}Mo/^{99m}Tc generator) and the phosphorus atom of the phosphine moiety to yield TcO^{3+}, the form needed for a proper chelation [44]. This reaction is simplifying the radiolabeling procedure because it avoids the need of an external reducing agent that later has to be removed, prior *in vivo* administration. As demonstrated, this oxo-reduction occurs only in the presence of a covalently linked PEG-phosphine because, with its amphiphilic behavior, it forms micelles after water dissolution, which catalyzes the reaction.

PEG oligonucleotides

Oligonucleotides, mainly antisense oligonucleotides and aptamers, are now under active investigation as new potential drugs because of their extremely high selectivity in target recognition. All of them, however, share the problems of short half-life *in vivo* because of either low stability towards the eso- and endo-nucleases (present in plasma and inside the cells) or their rapid excretion caused by their small size. Furthermore, their negative charge prevents an easy penetration into the cells.

A PEG molecule, bound to the hydroxyl group of a nucleic acid (directly [45] or through a spacer link [41]), was found to increase the stability towards enzyme degradation, prolong the plasma permanence and enhance the penetration into cells by masking the negative charges of oligonucleotides. A PEGylated aptamer, the 28mer oligomer aptanib, has already been approved by FDA for the treatment of age-related macular degeneration of retina. In this product, a branched PEG of 40 kDa was attached to the oligonucleotides through a pentamino linker [46].

Conclusions

Many studies and years of PEGylation development have given important theoretical and commercially useful results (Table 4), but many more applications can still be exploited. The products already approved by the FDA are a clear demonstration of the usefulness of PEGylation in the improvement of therapeutic value of drugs. The most relevant advantages are the prolonged body-residence time, which allows less frequent administrations, the increase in stability towards proteases or nucleases and the reduction of immunogenicity, particularly important in the case of nonhomologous proteins, such as asparaginase and adenosine deaminase. These advantages of PEGylation allowed this technique to create blockbuster products, such as PEG-Intron® and Pegasys®. In particular, the increasing use of PEGylation was possible because of the availability of PEGs with different molecular weights and activation forms (mainly from Nektar Therapeutics) needed to respond to the various drug-modification requirements.

In the future, the fields of non-protein drug and diagnostics PEGylation can be further explored with regard to the chemistry of binding as well as the targeting of these molecules. Another area of development will be the PEGylation of cells to create new drug-delivery systems, in-body bioreactors, or nonimmunogenic cells for safer transplantations. PEGylation of red blood cells (RBCs) at the level of membrane proteins, carbohydrates or lipid head groups was devised mainly for transfusion purposes, but also for the preparation of stealth RBCs useful as drug delivery system [47]. After encouraging results obtained in the PEGylation of white blood cells, Scott and Chen [48] PEGylated also the more challenging pancreatic islets (containing β cells that produce insulin). This latter modification is an interesting result, as the pancreatic cells have to retain, after PEGylation, not only the capacity to produce insulin but also the ability to fine tune the release of the hormone following glucose response.

PEG-based dendrimers could also open the way for new drug carriers, especially now that new monodisperse PEGs are commercially available [49]. Unfortunately, monodisperse PEGs are currently limited to molecular weights <1000 Da, but if higher molecular weights become available, the field of small-drug PEGylation will have greater advantages, as it will be possible to prepare conjugates with perfect homogeneity, a result that current polydisperse PEGs are not able to reach.

References

1 Hunter, T. (1995) Protein kinases and phosphatases; the yin and yang of protein phosphorylation and signalling. *Cell* 80, 225–236
2 Farazi, T.A. *et al.* (2001) The biology and enzymology of protein *N*-myristoylation. *J. Biol. Chem.* 276, 39501–39504
3 Wood, A. and Shilatifard, A. (2004) Posttraslational modifications of histones by methylation. *Adv. Protein Chem.* 67, 201–222
4 Varki, A. *et al.* eds. (1999) *Essential of Glycobiology,* Cold spring Harbor Laboratory Press
5 Hemmerich, S. *et al.* (2004) Strategy for drug discovery by targeting sulfation pathways. *Drug Discov. Today* 9, 967–975
6 Abuchowski, A. *et al.* (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem.* 252, 3578–3581
7 Abuchowski, A. *et al.* (1977) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* 252, 3582–3586
8 Pasut, G. *et al.* (2004) Protein, peptide and non-peptide drug PEGylation for therapeutic application: a review. *Exp. Op. Ther. Patents* 14, 859–894
9 Graham, L.M. (2003) PEGASPARAGINASE: a review of clinical studies. *Adv. Drug Deliv. Rev.* 55, 1293–1302
10 Levy, Y. *et al.* (1988) Adenosine deaminase deficiency with late onset or recurrent infections: response to treatment with polyethylene glycol modified adenosine deaminase. *J. Pediatr.* 113, 312–317
11 Bailon, P. *et al.* (2001) Rational design of a potent, long lasting form of interferon: a 40kDa branched poly-ethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. *Bioconjug. Chem.* 12, 195–202
12 Wang, Y.S. *et al.* (2002) Structural and biological characterisation of pegylated recombinant interferon α-2b and its therapeutic implications. *Adv. Drug Deliv. Rev.* 54, 547–570
13 Kinstler, O.B. *et al.* (1996) Characterization and stability of N-terminally pegylated rhG-CSF. *Pharm. Res.* 13, 996–1002
14 Wong, S.S. (1991) Reactive groups of proteins and their modifying agents. In *Chemistry of protein conjugation and cross-linking*, p. 13, CRC Press
15 Caliceti, P. *et al.* (1993) Active site protection of proteolytic enzymes by poly(ethylene glycol) surface modification. *J. Bioact. Comp. Polym.* 8, 41–50
16 Cox, G (1999) Bolder Biotechnology. Derivatives of growth hormone and related proteins, WO9903887
17 Berna, M. *et al.* (2005) Site-specific PEGylation of G-CSF by reversible denaturation. *32nd Annual meeting & exposition of the controlled release society*, 18–22 June, Miami, USA (Abstract No. 415)
18 El Tabir, N. and Roberts, M.J. (1999) Applied Research System. Polyol-IFN-beta conjugates, WO9955377.
19 Chapman, A.P. *et al.* (1999) Therapeutic antibody fragments with prolonged *in vivo* half-lives. *Nat. Biotechnol.* 17, 780–783
20 Sato, H. (2002) Enzymatic procedure for site-specific pegylation of proteins. *Adv. Drug Deliv. Rev.* 54, 487–504
21 De Frees, S. *et al.* (2004) Neose Technologies. Glycopegylation methods and proteins/peptides produced by the methods, WO2004099231
22 Chen, T. *et al.* (2003) Nature-inspired creation of protein-polysaccharide conjugate and its subsequent assembly onto a patterned surface. *Langmuir* 19, 9382–9386
23 Hinds, K.D. and Kim, S.W. (2002) Effects of PEG conjugation on insulin properties. *Adv. Drug*

Deliv. Rev. 54, 505–530
24 Morpurgo, M. *et al.* (2002) Selective alkylation and acylation of α and ε amino groups with PEG in a somatostatin analogue: tailored chemistry for optimised bioconjugates. *Bioconjug. Chem.* 13, 1238–1243
25 Hirano, T. *et al.* (1994) Synthesis of the conjugate of superoxide dismutase with the copolymer of divinyl ether and maleic anhydride retaining enzymatic activity. *J. Control. Release* 28, 203–209
26 Friman, S. *et al.* (1993) Hepatic excretion and metabolism of polyethylene glycols and mannitol in the cat. *J. Hepatol.* 17, 48–55
27 Kawai, F. (2002) Microbial degradation of polyethers. *Appl. Microbiol. Biotechnol.* 58, 30–38
28 Guiotto, A. *et al.* (2004) Anchimeric assistance effect on regioselective hydrolysis of branched PEGs: a mechanistic investigation. *Bioorg. Med. Chem.* 12, 5031–5037
29 Shibata, H. *et al.* (2004) Functionalization of tumor necrosis factor-alpha using phage display technique and PEGylation improves its antitumor therapeutic window. *Clin. Cancer Res.* 10, 8293–8300
30 Yoshioka, Y. *et al.* (2004) Optimal site-specific PEGylation of mutant TNF-alpha improves its antitumor potency. *Biochem. Biophys. Res. Commun.* 315, 808–814
31 Li, Y.P. *et al.* (2001) PEGylated recombinant human tumor necrosis factor alpha: preparation and anti-tumor potency. *Acta Pharmacol. Sin.* 22, 549–555
32 Greenwald, R.B. *et al.* (2003) Controlled release of proteins from their poly(ethylene glycol) conjugates: drug delivery system employing 1,6-elimination. *Bioconjug. Chem.* 14, 395–403
33 Morpurgo, M. *et al.* Pegylated proteins as cancer therapeutics. In *Delivery of protein and peptide drugs in cancers* (Torchilin, V. and Thon, E. eds), World Scientific/Imperial College Press (in press)
34 Maeda, H. *et al.* (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Control. Release* 65, 271–284
35 Kratz, F. *et al.* (1999) Drug-polymer conjugates containing acid-cleavable bonds carrier system. *Crit. Rev. Ther. Drug Carrier Syst.* 16, 245–288
36 Duncan, R. *et al.* (1983) Polymers containing enzymatically degradable bonds. 7. Design of oligopeptide sidechains in poly(*N*-(2-hydroxypropyl)methacrylamide) copolymers to promote efficient degradation by lysosomal enzymes. *Makromol. Chem.* 184, 1997–2008
37 Greenwald, R.B. *et al.* (2000) Drug delivery systems based on trimethyl lock lactonization: poly(ethylene glycol) prodrugs of amino-containing compounds. *J. Med. Chem.* 43, 475–487
38 Choe, Y.H. *et al.* (2002) Anticancer drug delivery systems: multi-loaded N^4-acyl poly(ethylene glycol) prodrugs of ara-C.: II. Efficacy in ascites and solid tumors. *J. Control. Release* 79, 55–70
39 Greenwald, R.B. *et al.* (2000) Poly(ethylene glycol) conjugated drugs and prodrugs: a comprehensive review. *Crit. Rev. Ther. Drug Carrier Syst.* 17, 101–161
40 Duncan, R. *et al.* (1983) Development of *N*-(2-hydroxypropyl) methacrylamide copolymers as carriers of therapeutic agents. In *Polymers in Medicine: Biomedical and Pharmacologicam Applications* (Chielline, E. and Giusti, P. eds) pp. 97–114, Plenun Press
41 Langer, C.J. (2004) CT-2103: a novel macromolecular taxane with potential advantages compared with conventional taxanes. *Clin. Lung Cancer* 6, 85–88
42 S. Duewell, R. *et al.* (1991) Non ionic poly(ethylene glycol)-ferrioxamine as a renal magnetic resonance contrast agent. *Invest Radiol.* 26, 50-57
43 Wen, X. *et al.* (2001) Poly(ethylene glycol)-Conjugated Anti-EGF Receptor Antibody C225 with Radiometal Chelator Attached to the Termini of Polymer Chains. *Bioconjug. Chem.* 12, 545–553
44 Visentin, R. *et al.* (2004) Highly efficient technetium-99m labeling procedure based on the conjugation of PEGylated *N*-[*N*-(3-Diphenylphosphinopropionyl)glycyl]cysteine ligand with poly(ethylene glycol). *Bioconjug. Chem.* 15, 1046–1054
45 Jaschke, A. *et al.* (1993) Automated incorporation of polyethylene glycol into synthetic oligonucleotides. *Tetrahedron Lett.* 34, 301–304
46 *The EyeTech Study Group* (2002) Preclinical and phase 1A clinical evaluation of an anti-VEGF pegylated aptamer (EYE001) for the treatment of exudative age-related macular degeneration. *Retina* 22, 143–152
47 Murad, K.L. *et al.* (1999) Structural and functional consequences of antigenic modulation of red cells with methoxypoly(ethylene glycol). *Blood* 93, 2121
48 Scott, M.D. and Chen, A.M. (2004) Beyond the red cell: pegylation of other blood cells and tissues. *Transfus. Clin. Biol.* 11, 40–46
49 Berna, M. *et al.* (2005) Novel monodisperse PEG-dendrons as new tools for targeted drug delivery: synthesis, characterization and cellular uptake. *32nd Annual meeting & exposition of the controlled release society*, 18–22 June, Miami, USA (Abstract No. 20)
50 Trainer, P.J. *et al.* (2000) Treatment of acromegaly with the growth hormone-receptor antagonist pegvisomant. *N. Engl. J. Med.* 342, 1171–1177