PEGylation of proteins and liposomes, a powerful and flexible strategy to improve the drug delivery

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Abstract:

PEGylation is one of the most successful strategies to improve the delivery of therapeutic molecules such as proteins, macromolecular carriers, small drugs, oligonucleotides, and other biomolecules. PEGylation increase the size and molecular weight of conjugated biomolecules and improves their pharmacokinetics and pharmacodinamics by increasing water solubility, protecting from enzymatic degradation, reducing renal clearance and limiting immunogenic and antigenic reactions. PEGylated molecules show increased half-life, decreased plasma clearance, and different biodistribution, in comparison with non-PEGylated counterparts. These features appear to be very useful for therapeutic proteins, since the high stability and very low immunogenicity of PEGylated proteins result in sustained clinical response with minimal dose and less frequent administration. PEGylation of liposomes improves not only the stability and circulation time, but also the 'passive' targeting ability on tumoral tissues, through a process known as the enhanced permeation retention effect, able to improve the therapeutic effects and reduce the toxicity of encapsulated drug.

The molecular weight, shape, reactivity, specificity, and type of bond of PEG moiety are crucial in determining the effect on PEGylated molecules and, at present, researchers have the chance to select among tens of PEG derivatives and PEG conjugation technologies, in order to design the best PEGylation strategy for each particular application.

The aim of the present review will be to elucidate the principles of PEGylation chemistry and to describe the already marketed PEGylated proteins and liposomes by focusing our attention to some enlightening examples of how this technology could dramatically influence the clinical application of therapeutic biomolecules.

Keywords: Drug delivery, EPR effect, Interferons, PEGylated liposomes, PEGylated proteins, PEGylation, Poly-(ethylene glycol)

Introduction

Over the last decades, important progress in pharmaceutical chemistry led to the discovery and design of modern pharmaceuticals and to the development of successful strategies to improve the clinical activity of new and old drugs. An important outcome of the latest technological advance was the development of recombinant DNA techniques, that enables large scale production of proteins and peptides suitable for clinical applications. Despite their apparent advantages, however, the practical clinical utilization of proteins and peptides presents significant limitation mainly due to their high molecular weights, poor solubility, and low physicochemical stability both during shelf storage and *in vivo* after administration. Parenterally administered proteins, actually, are either quickly cleared off from circulation by glomerular filtration or reticuloendothelial system (RES), and digested by circulating serum proteases, with the consequence of a rapid loss of their biological properties and the need of more frequent administrations. In addition, native proteins exhibit, very often, a significant immunogenicity and antigenicity that limits the opportunity of repeated administrations.

Many methods have been studied in an effort to overcome these inconveniencies and, more generally, to improve the stability and efficacy of all those drugs characterized by low stability and rapid clearance *in vivo*. Drug delivery can be improved either through a change in formulation or by a chemical modification of drug molecule. Innovative drug formulation such as liposomes, microspheres, nanoparticles or other colloidal systems are able to increase the circulating time by enhancing the stability and decreasing the clearance of the drug, but this strategy, that proved to be very useful for small molecules, is not easily applicable for proteins and peptides.

The most successful approach to enhancing protein delivery has been, so far, to modify the protein by linking one or more poly-(ethylene glycol) (PEG) molecules to it, through a technique called PEGylation.

The first successful application of this technology was described by Davies and Abuchowski in the 1977, when the authors achieved non-immunogenic, long-circulating versions of bovine serum albumin and bovine liver catalase through a modification of native proteins by covalent attachment of PEG [1, 2]. Since this essential discovery, PEGylation was progressively developed and exploited on a wide range of chemical and biochemical molecules, in order to improve the pharmaceutical application of bioactive proteins and small drugs [3-5]. PEGylation is able to enhance drug efficacy by reducing its clearance either through glomerular filtration (thanks to an increased weight of PEGylated molecule), and through RES or proteolytic degradation (thanks to the masking of protein surface). In addition, the ability of PEG to mask the protein surface drastically reduces the immune response instigated by heterologous proteins, minimizing thus either the production of antibodies recognizing and inactivating the foreign protein, and the risk of anaphylactic reactions for repeated administrations. Finally, PEGylation technology is versatile enough to allows a tailored-made modification of each protein, in order to address the requirement of different applications.

The advantages of PEGylation on drug efficacy were exploited also to improve other delivery technologies: a typical example of such application is represented by long-circulating liposomes. Classical, conventional, liposome consists of an aqueous core entrapped by one or more bilayers composed of natural or synthetic lipids. Liposomes composed of natural phospholipids are biologically inert and weakly immunogenic, and they possess low intrinsic toxicity. Further, liposomes are able to encapsulate drugs with different lipophilicities, since water-soluble molecules are entrapped into aqueous core, whereas lipophilic ones are incorporated into phospholipidic bilayer. Liposomes protect encapsulated drug from degradation and dramatically alter either its pharmacokinetics, by reducing drug clearance and volume of distribution, and its biodistribution, by a preferential accumulation of the drug in diseased tissue that have increased capillary permeability.

Liposomes were first described in the early 1960s, and immediately aroused a considerable interest among the researchers as drug carriers, particularly in the field of anticancer and antimicrobial treatment [6, 7]. Nevertheless, very soon the results of first *in vivo* experiments cooled down the initial enthusiasm, since conventional liposome formulations show

some important disadvantages that could limit their clinical utilization. After intravenous administration, in fact, liposome stability is rather low, since liposome particles are efficiently sequestered into the liver, spleen, kidneys and RES, and have a tendency to rapidly lose the encapsulated drug during the circulation. A number of different strategies was then tested, during the following years, in order to overcome the above-listed limitations. The best strategy was described in the early 1990s, when experiments carried out by several groups of scientists demonstrated that PEGylation of liposome surface was able to dramatically improve stability and circulation time of liposomes after intravenous administration: this kind of liposome was then named "Stealth®" because of his ability to evade interception by the immune system and RES, likewise the stealth bomber is able to evade radar [8, 9].

Since PEGylation was first applied by Davies and Abuchowski to improve *in vivo* protein bioavailability, the related technology has made considerable advances, thanks to the progress in the field either of protein structure and properties, and of organic and polymer chemistry. At present, PEGylation represents a powerful and flexible strategy that allowed the development of an ever growing number of pharmaceutical products still under investigation or already commercially available [3-5].

The aim of the present review will be to elucidate the principles of PEGylation chemistry and to describe some enlightening examples of how this technology could improve clinical application of proteins and antitumoral drug-containing liposomes.

Properties of PEG

In its most common form, PEG is a linear or branched polyether diol with many useful properties such as biocompatibility, solubility in aqueous and organic media, lack of toxicity, very low immunogenicity and antigenicity, and good excretion kinetics. These properties allow its use in a variety of applications, including the biomedical field, after Food and Drug Administration (FDA) approval for internal administration [10].

PEG is produced by linking repeated units of ethylene oxide to obtain a large number of different linear or branched configurations with different molecular weights (figure 1). Very useful, for PEGylation purpose, are monofunctional modifications of PEG as mono-methoxy PEG, on linear (mPEG) or branched structure (mPEG2), in order to avoid crosslinking during conjugation process (figure 1). The advantage of mPEG is mainly due to a chemically inert polymeric backbone that posses a terminal primary hydroxyl group available for derivatization with a number of different reactive functional groups [10].

In vivo PEG chains shorter than 400 Da are transformed to toxic metabolites by alcohol dehydrogenase, whereas longer PEG chains, used for PEGylation of proteins and liposomes, are not subjected to enzymatic degradation, but are eliminated through a mechanism which is dependent to its molecular mass: PEG molecules with a molecular weight below about 20 kDa are cleared by renal filtration, whereas PEG molecules with higher molecular weight are eliminated mainly by liver uptake [11, 12].

Rationale for PEGylation of proteins and liposomes

PEGylation produces alterations in the physicochemical properties of the parent molecule, including changes in conformation, steric hindrance, electrostatic binding properties, hydrophobicity.

PEGylation increase the size and molecular weight of proteins and peptides and improves their pharmacokinetics and pharmacodinamics by increasing water solubility, protecting from enzymatic degradation, reducing renal clearance and limiting immunogenic and antigenic reactions (figure 2). PEG polymer, linked to protein, associates with two or three

water molecules for each ethylene glycol subunit, and this feature creates a bulky hydrophilic shield able to efficiently mask the conjugated protein from enzymatic digestion and immunologic recognition. Besides, the shield created by PEG moiety with associated water molecules makes the conjugated protein more soluble and virtually five or ten times larger than a corresponding soluble protein of similar mass, thus decreasing considerably the renal clearance (figure 2) [10, 13]. This last property appears to be very useful especially for small proteins and peptides, that should be rapidly filtered by kidneys: PEGylation with a PEG mass of 40-50 kDa, achieved by conjugation either of a single large PEG molecule or of several smaller PEG molecules, is able to evade glomerular filtration prolonging thus the body-residence time.

The clinical utilization of therapeutic PEGylated proteins represents a considerable improvement, compared to unmodified native protein, since their high stability and very low immunogenicity results in sustained clinical response with minimal dose and less frequent administration, leading to improved quality of life for the patients. Non-PEGylated therapeutic proteins are administered usually every one or two days, with the consequence of fluctuations in its blood concentration with negative impacts to clinical activity; PEGylated proteins, on the contrary, are administered once a week as subcutaneous injection, and its blood concentration remains always near the optimal therapeutic level, since the PEG modification assures a sustained absorption from injection site that acts as depot.

Unfortunately, the PEGylation of proteins lead very often to a significant loss of biological activity, due to the chemical alteration of amino acid charge and protein surface, however this drawback is usually compensated*, in vivo*, by the longer half-life of PEGylated derivative. Furthermore, thanks to the high flexibility of PEGylation technology, it is possible to make an appropriate selection of different features of chemical binding protocol (*e.g.* PEG moiety length, configuration and modification; site of attachment on the protein chain; type of bond) in order to find the best compromise between body-residence time and preservation of *in vivo* biological activity. The two commercially available PEGylated derivatives of Interferon (IFN), Pegasys® and PegIntron®, represent an enlightening example of how different PEGylation strategies, applied on the same protein, could bring to different effects on pharmacokinetics, pharmacodynamics and biodistribution of PEGylated derivatives, each of which, however, still clinically effective.

PEG modification is a successful strategy also to improve the efficacy of liposome delivery by prolonging their blood circulation time: compared with classical liposomes, PEGylated counterparts (Stealth® liposomes) show increased half-life, decreased plasma clearance, and decreased distribution volume (V_d) along with a preferential accumulation on diseased tissues. PEG, incorporated into the lipid bilayer of the liposome, forms an hydrated shell that avoid the vesicle aggregation and, *in vivo*, protects liposomes from destruction by plasma proteins, avoiding a premature release of encapsulated drug during circulation. Furthermore, the PEG coating reduces the adsorption of opsonins, such as complement (C3a and C3b), fibronectin and immunoglobulin (mainly IgG), on liposome surface allowing Stealth® liposomes to evade interception and uptake by RES (figure 2) [8, 9].

PEGylation of liposomes improves not only the stability and circulation time, but also the 'passive' targeting ability on tumoral tissues: Stealth® liposomes, in fact, circulate for hours without loss of encapsulated drug and are small enough to extravasate through the highly permeable discontinuous endothelium of tumor vessels and passively accumulate in the interstitial fluid compartment due to the lack of functional lymphatic drainage. This process, known as the enhanced permeation retention (EPR) effect, causes the preferential accumulation of liposomes in tumors versus normal tissues: in this way drug release to tumor cells will mainly take place in the tumor interstitial fluid, where liposomes accumulate and behave as a sustained drug-release system (figure 3) [14, 15].

A further advantage of using small Stealth® liposomes in cancer therapy, is the minimization of toxic effects resulting from the extravasation of liposomes into healthy tissues, such as the heart or gastrointestinal tissues. In fact, microvessels of normal tissues have usually an intact endothelium, without gaps, and less permeable membranes than tumor tissues, and those characteristics avoid the extravasation of liposomes. Moreover, the molecules that should pass across this barrier,

may return to systemic circulation through an efficient lymphatic drainage, without accumulate in the interstitial fluid (figure 3).

Chemistry of PEGylation of proteins and liposomes

During the last twenty years, researchers described a number of different methods for PEG conjugation and, at present, this technology offers the possibility to answer the requirements of different applications for PEGylation of proteins, liposomes and other molecules [16].

In order to bind PEG to a molecule it is necessary activate the PEG moiety by preparing a derivative having a reactive functional group at one or both termini. The choice of functional group depends on the type of reactive group on the molecule that will be conjugated to the PEG.

Chemistry of PEGylation of proteins

The effect of PEGylation on protein physicochemical and biological properties are determined either by the protein and polymer properties and by the adopted PEGylation strategy: the first aim to achieving a successful PEG-protein conjugate is the elaboration of a binding protocol that will keep the highest protein biological activity. The polymer most widely used for protein PEGylation is linear mPEG, end-capped on one side with a methoxyl group and terminated with an hydroxyl group. A newer polymer form, branched mPEG2, is characterized by two polymer chain linked to one activated moiety and allows better masking and protection of the protein surface due to its "umbrella-like effect" (figure 1) [5, 17]. Additionally, branched PEG permits to double the mass of the conjugated PEG-polymer with the modification of only a single amino acid residue in the protein, decreasing thus the biological activity loss.

PEGylation of proteins is usually achieved by a chemical reaction between suitably activated PEG moiety and protein reactive groups that include amino acid side chains of lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine and tyrosine, or the N-terminal amino group and the C-terminal carboxylic acid [11].

Historically, the protein reactive group more frequently exploited for PEG-polymer conjugation was the ε-amino group on the side chain of lysine residues, through a reaction of alkylation or acylation. Alkylation maintains the positive charge of the original amino group because a secondary amine is formed (*e.g.* reaction with PEG-aldehyde or PEG-epoxide), whereas acylation produces a loss of charge (*e.g.* reaction with PEGs terminating with a carboxylic group reactive with N-hydroxysuccinimide). The disadvantage of this approach is mainly due either to the relative high number of lysine residues in protein sequences and to the low specificity of some PEGylation reagents that can react not only with lysine residues, but also with other protein nucleophiles (*e.g.* N-terminal amino groups, and the side chain of histidine, serine, threonine, tyrosine and cysteine residues). Although a strictly controlled reaction conditions (*e.g.* pH, temperature, reaction time, amount of PEG reagent and protein concentration) can avoid, to some extent, undesired derivatizations, this approach lead in any case to the production of an heterogeneous mixture of PEG isomers with different molecular mass and, often, different pharmacokinetics, biodistribution and biological activity. For this reason this "classical" PEGylation approach is often known as "random PEGylation" [12, 18-20]. At present, most of PEGylated protein commercially available has been produced by random PEGylation, despite the reproducibility of the reaction need to be proved by manufacturers for FDA approval of these mixtures (table 1).

More recently, new strategies have been developed, in order to avoid the formation of heterogeneous mixture of PEG isomers, by shifting from random to site-specific PEGylation reactions. Furthermore, site-specific modification might lead to a better preservation of the native protein activity in the conjugate [10, 11, 18]. One of the first example of site-specific PEGylation was described by Kinstler, that succeeded to selectively PEGylate the N-terminal α–amino group of some

proteins by a reductive alkylation with PEG aldehyde performed under acidic conditions (pH 5), taking advantage of the lower pK_a of the α–amine compared to other protein nucleophiles [21-23]. One of the proteins PEGylated by this method, the granulocyte colony stimulating factor (G-CSF), was approved by FDA in 2002 for the treatment of granulocyte depletion during chemotherapy and is marketed with the name of Neulasta® (table 1).

Another promising site-specific strategy is the PEGylation of thiol group of cysteines not involved in disulphide bridges, by selective thiol PEGylating reactives [11, 18]. This method offers the advantages of a relative low number of cysteine residues in protein sequences, that limit the number of different PEG isomers obtained, but its main disadvantage is the hydrophobicity of cysteine that make this amino acid often buried inside the protein structure and therefore hardly accessible for conjugation with bulky PEG moiety. A number of strategies have been investigated to overcome this limitation as, for example, to perform the PEGylation under transient light denaturating conditions able to expose partially buried cysteine residues, or to genetically replace a non-essential amino acid with a cysteine residue [24-26]. Another simple strategy is to reduce the disulphide bridges of a protein in order to expose new thiol residues. This approach proved to be very useful for the PEGylation of antibodies, since the modification of other amino acids could lead to a marked loss of the ability of antigen recognition [27].

Instead of a chemical site-specific reaction, Sato recently described an enzymatic site-specific method that take advantage of reaction catalyzed by specific or non-specific transglutaminases in order to selectively PEGylate glutamine residues with PEG-alkilamine reagents [28]. This method is suitable even for target protein that lacks reactive glutamine residues: in this case a short substrate sequence for transglutaminases can be genetically introduced at the terminus of the protein, allowing the PEGylation with a minimal alteration of protein structure and biological activity. The high homogeneity of the constructed conjugates and the ability to design conjugates with suitable incorporation sites, will improve the therapeutic indices of proteins PEGylated with this method.

The linkage between PEG and protein is generally stable, in order to ensure the long-term storage of PEGylated product and the *in vivo* high strength and long circulation time, very important for clinical applications. A stable linkage, however, can reduce the biological activity of the protein, decreasing the advantage of the improved pharmacokinetic profile: for this reason some researchers proposed degradable linkages in the attempt to increase the circulation half-life with a minimal loss of activity [16, 29-33]. Degradable linkages are designed to break and release over time the fully active native protein from PEG-conjugate through enzymatic degradation, hydrolytic cleavage or reduction. Often, these systems are based on ester bonds in which the rate can be tuned by changing the neighboring chemical groups. Proteins PEGylated with degradable linkages show, generally, higher *in vitro* biological activity, but shorter circulation half-life compared to stable PEG-protein conjugates. A clear example is obtained by the comparison between the two commercially available PEGylated IFN derivatives: PegIntron®, able to release the native protein, and Pegasys®, a stable PEG-IFN derivative [34] (table 2).

Chemistry of PEGylation of liposomes

PEGylation of liposomes don't lead the problems of bioactivity conservation or the need to site-specific modifications that are so critical for proteins. Moreover, the character of "synthetic compound" of liposome make the PEGylation chemistry easier compared to the chemistry required for protein modification.

PEGylation of liposomes can be achieved through different methods: by physically adsorbing the polymer onto the surface of the vesicles, by incorporating the PEG-lipid conjugate along with the other phospholipids during liposomes preparation, or by covalently attaching reactive PEG-derivatives onto the surface of preformed liposomes [9].

The most used method for preparation of clinically suitable liposomes is the anchoring of the PEG moiety to the liposomal membrane via a cross-linked lipid as, for example, the PEG-distearoylphosphatidylethanolamine [35, 36]. Important

parameters are the molecular weight and structure of PEG moiety to be bound to liposome. These can be properly chosen taking in mind that the behaviour of PEGylated liposomes depends on the characteristics and properties of the specific PEG linked to the surface, as well as the graft density, and the distance between graft sites. Usually, PEGylation with longer PEG chains give the greatest improvements in blood residence time comparing to the PEGylation with shorter PEG chains [35].

At present, in order to improve further the pharmacodynamic advantages of PEGylation, the most advanced research on liposome PEGylation concerns the designing of cleavable PEG-lipid derivatives. In fact, it was observed that liposomes modified with traditional PEG-lipid derivatives, after their accumulation at the target site through the EPR effect, could release very slowly the drug. This undesired behaviour is due to the high stability of the chemical bond between PEG and lipid. On the contrary, PEGylation with chemical bond cleavable under pathological condition only (*e.g.* decreased pH value in tumors), should be able either to prolong the circulation time of liposomes, promoting EPR effect, and to enhance the release of the drug at targeted location, after the destabilization of liposome structure by removal of PEG coating. Some examples of cleavable PEG-lipid bond potentially useful for clinical applications are a dithiobenzylurethane linkage between PEG and an amino-containing substrate (such as phosphatidylethanolamine) in which the PEG release process is based on mild thiolysis, and a PEG-peptide-lipid conjugate in which the PEG moiety can be removed from the carrier via cleavage of the specially designed peptide Gly–Pro–Leu–Gly–Ile–Ala–Gly–Gln by a matrix metalloproteinase specifically expressed in tumor tissues [37, 38].

Clinical application of PEGylated proteins

From 1990 to present, PEGylated proteins belonging to several classes (including enzymes, stimulating factors, cytokines and antibodies) were marketed for the treatment of chronic diseases such as hepatitis C, leukemia, severe combined immunodeficiency disease, rheumatoid arthritis, Crohn's disease, neutropenia and anemia (table 1). The majority of these already marketed PEGylated proteins were obtained by random PEGylation.

The first PEGylated protein approved by the FDA was the Pegademase (Adagen®, Enzon Inc.) that was obtained by multiple PEGylation of bovine adenosine deaminase with 5-kDa mono-methoxyPEG succinimides. Pegademase is used to treat severe combined immunodeficiency disease (SCID) with a better pharmacokinetic profile than its non-PEGylated counterpart [39].

Few years later, FDA approved the Pegaspargase (Oncaspar®, Enzon Inc.), a PEGylated derivative of L-asparaginase with 5-kDa linear PEG bound at multiple sites, that proved to be as effective as the native enzyme in treating patients with acute lymphoblastic leukemia, but demonstrated a longer half-life (357 compared to 20 hours) and a lower degree of immunogenicity [40].

Pegfilgrastim (Neulasta®, Amgen) represent the first protein derivative approved by FDA that was obtained by site-specific PEGylation. Pegfilgrastim was produced by conjugating a 20-kDa linear monomethoxy-PEG aldehyde with the already marketed recombinant methionyl human G-CSF (Filgrastim, Amgen) under acidic condition (pH 5) in the presence of sodium cyanoborohydride. This method permits a site-specific PEGylation at the N-terminal methionyl residue of G-CSF, with a good preservation of biological activity and an impressive improvement of pharmacokinetic properties. Both the drugs, Filgrastim and Pegfilgrastim, can be used to stimulate the proliferation, differentiation, and survival of neutrophils that are depleted during cancer chemotherapy, but, whereas Filgrastim requires a daily administration by sub-cutaneous injection for 2 weeks, for Pegfilgrastim it is enough only one sub-cutaneous injection per chemotherapy cycle [41, 42].

Pegvisomant (Somavert®, Pfizer Inc.) is a genetically engineered analogue of human growth hormone (GH) conjugated with 4-6 linear 5-kDa PEG chains by random PEGylation on amine residues, and was developed for treating acromegaly, a syndrome arising from an overproduction of GH due to a pituitary adenoma in which the excess of GH, binding to its receptor, activates a signal pathways that lead to insulin-like growth factor-1 (IGF1) overproduction that, finally, promote an abnormal soft-tissue enlargement. The genetic modifications of Pegvisomant were designed in order to increase its affinity for the GH receptor comparing to native GH, but, at the same time, to inhibit the activation of the signal pathways that lead to IGF1 production; furthermore, the PEGylation is able to prolong the half-life and allow a daily sub-cutaneous administration [43].

One of the last PEGylated proteins approved by FDA is the mono-mPEG-epoetin-β (Mircera®, Hoffman-La Roche Ltd.), also known as continuous erythropoietin receptor activator (CERA), that is a long-acting erythropoiesis stimulating agent used to normalize hemoglobin levels in patients affected by chronic renal failure. Mircera® is obtained by random PEGylation on amine residues of epoetin-β with linear 30 kDa PEG chains. PEGylation lead to a more prolonged half-life, in comparison with other proteins usually employed for the treatment of this kind of patients, allowing thus a monthly administration instead of the three injections a week required for the recombinant human erythropoietin, or weekly/biweekly injections required for the Darbepoetin-α [44].

Only one antibody PEGylated derivative has been marketed until now: the PEGylated Fab' fragment of the humanized anti-tumor necrosis factor (TNF)-α monoclonal antibody that binds and neutralizes membrane-bound or soluble TNFα (Cimzia®, UCB S.A.). Cimzia® is obtained by site-specific PEGylation of the thiol group of the C-terminal cysteine of the heavy chain with the maleimide group of a branched 40-kDa PEG chain to form a stable thioether linkage. Due to this single attachment of the PEG to the C-terminal of protein, very far to antibody recognition site, Cimzia® maintains the full binding activities of the native antibody fragment, while having a long circulation time and a reduced immunogenicity. Cimzia® was approved by the FDA in 2008 for patients with rheumatoid arthritis or with moderate-to-severe Crohn's disease who did not responded to conventional therapies [45, 46].

The two marketed PEGylated version of IFN, PegIntron® (Schering-Plough Corp.) and Pegasys® (Hoffmann La Roche Inc.), deserve a more detailed description, since they represent one of the brightest examples of the flexibility of PEGylation technology. Two different manufacturers, in fact, applied different PEGylation strategies on the same protein, achieving two derivatives, both clinically effective, but each characterized by a particular pharmacokinetics, pharmacodynamics and biodistribution.

IFNs were discovered in 1957 and represent, so far, the most effective drugs in inducing remission of chronic hepatitis C. Since the first FDA approval of two recombinant IFN-α (2a and 2b) preparations in 1986, many other IFN products have been developed in an attempt to improve their clinical efficiency [47]. First generation recombinant IFNs, however, are characterized by very short half-lives, as they persist in serum for only 4-8 hours: for this reason, they need to be administered at a dose of 3 million units three times a week, which leads to wide fluctuations in the serum IFN concentration (figure 4). Furthermore, standard IFN-α (Intron® A, Schering-Plough Corp.) administration every two days for 48 weeks was associated with viral rebound between injections, leading to a sustained virological response (SVR) rates only in 15-20% of patients, whereas daily injections, able to prevent the rebound, are poorly tolerated.

Effectiveness of the treatment with standard recombinant IFN-α is affected, actually, by protein characteristics that include poor stability, short half-life and high immunogenicity. The fluctuations in exogenous IFN concentration adversely affect the anti-viral efficiency of the drug, since a sustained drug concentrations at or near a target level for an extended period of time is mostly useful in antiviral therapy to prevent viral replication and restrain the emergence of resistant variants. In order to overcome these limitations, the researchers attempted very soon to develop a PEGylated IFN-α and, after several years of improvement, obtained PegIntron® (a PEGylated version of IFN-α2b) and Pegasys® (a PEGylated version of IFN-

α2a), approved in 2000 and 2002 respectively. Both the PEG-IFNs shows a number of favorable properties in comparison with the standard IFN-α molecules, such as an almost absent immunogenicity, a longer half-life and a sustained blood levels that enhances antiviral effectiveness and reduce adverse reactions, improving patient convenience (figure 4) [47-53]. Manufacturers of these two PEG-IFNs, however, have selected two PEGylation strategies that differ either on PEG-chain size and structure, and on the site of attachment and type of bond: these differences strongly affect the peculiar *in vitro* and *in vivo* behaviour of the two PEGylated products (table 2).

Both derivatives are a mixture of monoconjugate isomers obtained by random linkages of reactive PEG moiety. PegIntron® was obtained by covalently linking a 12 kDa linear mPEG chain, via an instable urethane bond, that slowly releases the free protein, to the IFN-α2b, mainly at the residue of His^{34} (about 50%), then to lysine residues (35%) and about 15% at other residues). Pegasys® was obtained by covalently linking a 40 kDa branched mPEG2 chain, by a stable amide bond, to the IFN-α2a at the lysine residues 31, 121, 131 or 134 (94% overall) (figure 5) [34, 54-56]. The different type of PEG chain and PEGylation bond strongly affect the *in vitro* specific antiviral activity of the two PEG-IFNs, since the smaller and releasable derivative PegIntron® retains 28% of the native activity, whereas the larger and stable derivative Pegasys® retains only 7% [48, 57]. The difference of *in vitro* specific activity demonstrates that increasing the size of PEG moiety is not always advantageous, in fact the steric hindrance of a larger PEG chain will severely restrict the antiviral activity of the IFN by masking somewhat the binding portion of the molecule interacting with the cell receptor, thus requiring higher doses to achieve the same physiologic effect. However, although a larger PEG moiety may be associated with lower *in vitro* specific activity, it may not be directly associated with lower clinical effect. Indeed, the lower efficacy of interaction with the receptor is often counterbalanced by a more prolonged residence in blood, or by a higher total drug exposure, as clearly proved by the half-life of the larger Pegasys® that results twice the smaller PegIntron® (80 and 40 hours respectively). Furthermore, it is worth also to note that, if on one hand the choice of an releasable bond for PegIntron® allows a slow release of the free, full active, protein, on the other hand it impairs the overall *in vivo* stability of the molecule, shortening the half-life and decreasing the total drug exposure. The type of bond between PEG and IFN molecules also influence the elimination route, since the PegIntron®, after an initial peak of PEG-IFN-α2b in plasma concentration, slowly releases the native IFN-α2b molecule that is cleared by kidneys, whereas Pegasys® requires metabolism via non-specific proteases (mostly in the liver) to be eliminated (table 2) [49, 50, 57-59].

Another important feature strongly affected by the size of the PEG chain is the biodistribution, since an higher PEG size prevents the free diffusion of the molecule to tissues and organs, as confirmed by the lower V_d of Pegasys® (8-12 liters, the approximate volume of the plasma and extracellular water) in comparison to the larger V_d of PegIntron® (69 liters, comparable to V_d of native IFN-α2b). Drugs with a high V_d have the best potential to infiltrate peripheral tissue, and this ability could be important for the treatment of hepatitis C. In fact, many studies demonstrate that hepatitis C virus (HCV) can be widely distributed in different extrahepatic sites such as peripheral blood mononuclear cells, renal cells, thyroid cells, and gastric cells, and that viral suppression in blood alone may not be sufficient, since reservoirs outside the blood and liver may play a significant role in both HCV persistence and reactivation of infection [57]. Furthermore, the size of PEG moiety affects indirectly the possibility to rapidly reduce the effects of adverse events by a dose-reduction or treatment discontinuation, since a larger PEG derivative (with a more prolonged half-life) will requires a more prolonged period of time to reduce the plasma concentration of the active molecule after a dose reduction [60].

Finally, the different stability of the two PEG-IFNs also lead to a difference in the formulation of the marketed derivatives: the releasable, less stable, derivative PegIntron® is provided as a lyophilized powder that should be administered immediately after the reconstitution, whereas stable derivative Pegasys® is provided as a ready-to-use solution, with the advantage of an easier use and less waste of product.

In conclusion, both the commercially available PEG-IFN-α2 derivatives confer enhanced therapeutic efficacy when compared with their IFN counterparts: PEGylation optimizes the IFN physiologic effect by conferring a number of advantages, such as sustained blood levels that enhance antiviral effectiveness and reduce adverse reactions, as well as a longer half-life and improved patient convenience with an administration once-weekly instead of three times a week. The comparison of pharmacokinetic behavior and biodistribution of non-PEGylated IFNs, PegIntron® (12 kDa linear PEG chain) and Pegasys® (40 kDa branched PEG chain) represents a clear example of how the length, shape and type of bond of PEG moiety are crucial in determining the effect on pharmacokinetic and pharmacodynamic properties of a PEGylated molecule.

The main challenge in designing a PEGylated drug, therefore, is to find the optimal balance between the loss of activity, due to the PEG derivatization, and the benefits of prolonged circulation times. The clinical activity of the two commercially available PEG-IFNs demonstrates that two different PEGylation strategies can achieve similar clinical results through two different ways: both PEG-IFNs, in fact, have been reported to yield comparable toxicities and a two-fold higher SVR rate than the corresponding standard IFN-α when administered alone of in association with ribavirine, even if the longer half-life of Pegasys® should indicates a more prolonged residence in blood allowing for continuous viral suppression, whereas the larger V_d of PegIntron® should suggests a more efficient action against the HCV persistence and reactivation of infection [61].

At present, a number of PEGylated proteins, obtained by either random or site-specific PEGylation, is under development, thus a huge increase of commercially available, clinically useful PEGylated proteins is expected in the next years [3, 16].

Clinical application of PEGylated liposomes

At present, the only Stealth® liposome on the market is the PEGylated liposomal doxorubicin (PLD; Doxil® by Alza/Johnson and Johnson in the USA and Caelyx® by Schering-Plough Corp. outside the USA), approved first in 1995 for treatment of Kaposi's sarcoma, then for treatment of resistant ovarian cancer and metastatic breast cancer also [62-64] Several studies are currently under way to investigate the anticancer activities of PLD in combination with other therapeutics for treatment of other tumors.

Doxorubicin (Adriamycin®, Pfizer) is one of the most widely prescribed and effective cytotoxic drugs, used in oncology. The main disadvantages of doxorubicin are the short half-life and a cumulative, dose-related, progressive myocardial damage that may lead to congestive heart failure. Both these limitations are related to the low size of this molecule (MW 580) that lead to a rapid clearance of the drug and to its diffusion and accumulation in normal tissue such myocardium, where toxic free radical species are generated. In order to overcome these pharmacokinetic and toxicological limitations, researchers developed two different liposomal formulations able to improve the half-life and reduce the toxicity of the drug: non-PEGylated liposomal doxorubicin (NPLD; Myocet® by Cephalon, approved only in Europe for the treatment of metastatic breast cancer), and PLD (Doxil®/Caelyx®). The comparison of pharmacokinetics, biodistribution and toxicity of these three different doxorubicin formulations (free drug and drug encapsulated in non-PEGylated or PEGylated liposomes) clearly explains how much the PEGylation could influence the *in vivo* behaviour of a liposomal drug: the encapsulation in conventional, non-PEGylated, liposomes already increase the half-life and total drug exposure, reducing the V_d and clearance, but only the PEGylation of liposomal carrier is able to raises dramatically those features (figure 6) (table 3) [8, 65-67]. In comparison with similar doses of free doxorubicin, in fact, administration of NPLD leads to a 5-fold increase of AUC and half-life, and a 2.5-fold decrease of clearance and V_d, but PEGylation results in an ever better improving of drug delivery, since administration of PLD leads to a 1000-fold increase of AUC, a 8-fold increase of half-life, and a 100-fold decrease of clearance and V_d (table 3) [67-70]. The very low V_d of PLD, comparable with the

approximate volume of the plasma, demonstrates that the majority of the doxorubicin (95-99%) remains encapsulated within the liposomes in the plasma compartment, since the PEGylation avoids the premature release of doxorubicin during circulation.

The high plasma stability and prolonged half-life of PLD promote the preferential accumulation of liposomes in tumour tissues by EPR effect, doxorubicin is therefore released from the liposomes into the tumor extracellular fluid, from where it can diffuse into tumoral cells, avoiding typical toxicity associated with free doxorubicin (cardiotoxicity, vesicant effects, nausea, myelotoxicity, vomiting and alopecia) (figure 3). PLD is characterized by distinctive forms of toxicity such as acute infusion reaction, mucositis and palmar plantar erythrodysesthesia occurring especially at high dose or by short dosing interval.

The ability of PEGylated liposomes to accumulate in tumour tissues with minimal diffusion into healthy tissues, such as the gastrointestinal tract and myocardium, was demonstrated by Harrington *et al.* and Koukourakis *et al.* with radiolabeled PEGylated liposomes [71-73], and was confirmed by other researchers that found a 10-fold higher concentration of doxorubicin into tumoral cells after administration of PLD in comparison with free drug, and a consequent 20-fold higher concentration of doxorubicin into tumoral cells comparing non-tumoral cells [67, 74].

The NPLD, on the contrary, shows a lower plasma stability and higher V_d in comparison with PLD, since the opsonins actively attach the surface of conventional liposomes, and lead either to the premature release of encapsulated drug during circulation, and the rapid sequestration of liposomes into the liver, spleen, kidneys and RES.

At present, other Stealth® liposomes, designed for the treatment of several tumoral diseases, are under development at the stage of clinical trial: two PEGylated liposomal cisplatin (SPI-077™ by Alza Corporation, and Lipoplatin™ by Regulon Inc.), and a PEGylated liposomal semisynthetic analog of camptothecin (S-CKD602, Alza Corporation) [75-78]. Finally, a PEGylated liposomal recombinant FVIII are presently under investigation for the treatment of haemophilia A proteins [79].

Conclusions

Since 1977, when Davies and Abuchowski succeed to obtain the first PEGylated proteins, an ever-growing number of applications of PEGylation technology have been described, in order to exploit the advantages of PEGylation to improve the delivery of therapeutic molecules. PEGylation, in fact, is able to modify either the pharmacokinetic and pharmacodinamic properties, and biodistribution of the molecule, improving the solubility and stability, prolonging the body-residence time, assuring a sustained drug concentrations and decreasing the immunogenicity. PEGylated molecules, therefore, can be regarded as true new pharmaceutical entities, in comparison with theirs non-PEGylated counterparts. The above mentioned features have been exploited, during the last thirty years, to overcome the main drawbacks of therapeutic proteins, leading to tens of PEGylated proteins currently at the stage of clinical trial, and eight already on the market: Adagen®, Oncaspar®, PegIntron®, Pegasys®, Neulasta®, Somavert®, Mircera® and Cimzia®.

In the last years, PEGylation chemistry has evolved considerably, allowing the researcher to pass from a “random” to a “site-specific” PEGylation strategy, with huge improvement in protein therapeutic efficiency. The first marketed proteins, in facts, were produced using rather non-specific PEG derivatives, able to react with different amino acid side chains, with the consequent production of an heterogeneous mixture of PEGylated isomers characterized by different molecular weight, pharmacokinetics, biodistribution and biological activity. The development of more specific PEG derivatives, able to react with selected groups of the protein by site-specific chemical or enzymatic reaction, along with the designing of new PEG moiety with branched structure, allowed the production of well characterized novel PEGylated protein. At present, researchers have the chance to select among tens of PEG derivatives characterized by different molecular weight,

shape, reactivity, specificity, and type of bond, in order to design the best PEGylation strategy for each particular application. The introduction of PEGylation technology and its current considerable development revolutionized so much the production of new therapeutic proteins, that, at present, every new protein, obtained by recombinant DNA technique and potentially useful for clinical application, is always PEGylated before starting any trial.

Although proteins and peptides have been the first targets for PEGylation, the power and versatility of this technology also improve the surface behaviour of liposomes, micelles and nanoparticles and the delivery of many other classes of molecules, such as small drugs, oligonucleotides, cofactors, and saccharides, useful for therapeutic and diagnostic purpose. PEGylation, in fact, proved to be a powerful strategy to improve the activity of liposomal or nanoparticulate carriers not only by prolonging the half-life of encapsulated drugs, but also by improving their preferential accumulation in the target tissue by EPR effect, thus enhancing the clinical efficacy and decreasing the toxicity of antitumoral drugs.

At present, the new frontier in liposome or nanoparticle PEGylation is represented by bifunctional PEG chains, able to react with particle surface, from one side and with a targeting agent (*e.g.* small molecule, peptide or antibody fragment) on the other. In this new type of conjugates the PEG moiety, along with the "classical" function of prolonging half-life and promote EPR effect, acts as spacer arm for the targeting agent, in order of increase the accumulation of carrier at the target site not only through the EPR effect, but also through an active targeting mechanism [80-83].

Finally, a promising new application of PEG derivatives is the PEGylation of dendrimer, a new class of polymers able to efficiently deliver drugs across cellular barriers, in order to reduce the cytotoxicity and immunogenicity of this emerging new type of carrier, currently at the stage of preclinical trial [84].

For years to come we expect that the progressive development of the PEGylation chemistry, along with the application of this strategy to different classes of molecules, will lead to an impressive increase of the number of commercially available PEGylated products in a broad range of therapeutic and diagnostic areas.

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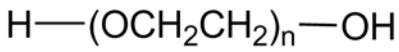
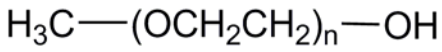
Figures

Linear PEG



Linear mPEG



Branched mPEG2

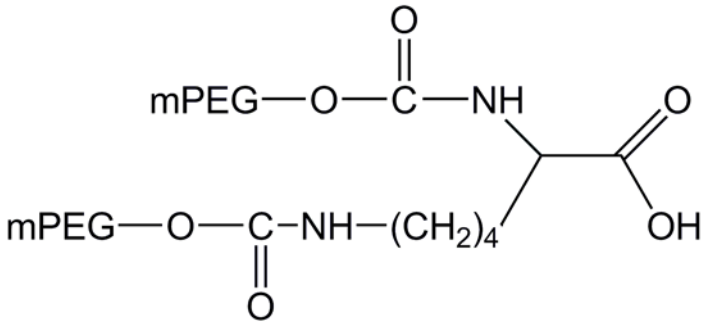


Figure 1. Structural formulae of different PEG molecules: linear PEG, linear mono-methoxy-PEG (mPEG) and branched mono-methoxy-PEG (mPEG2).

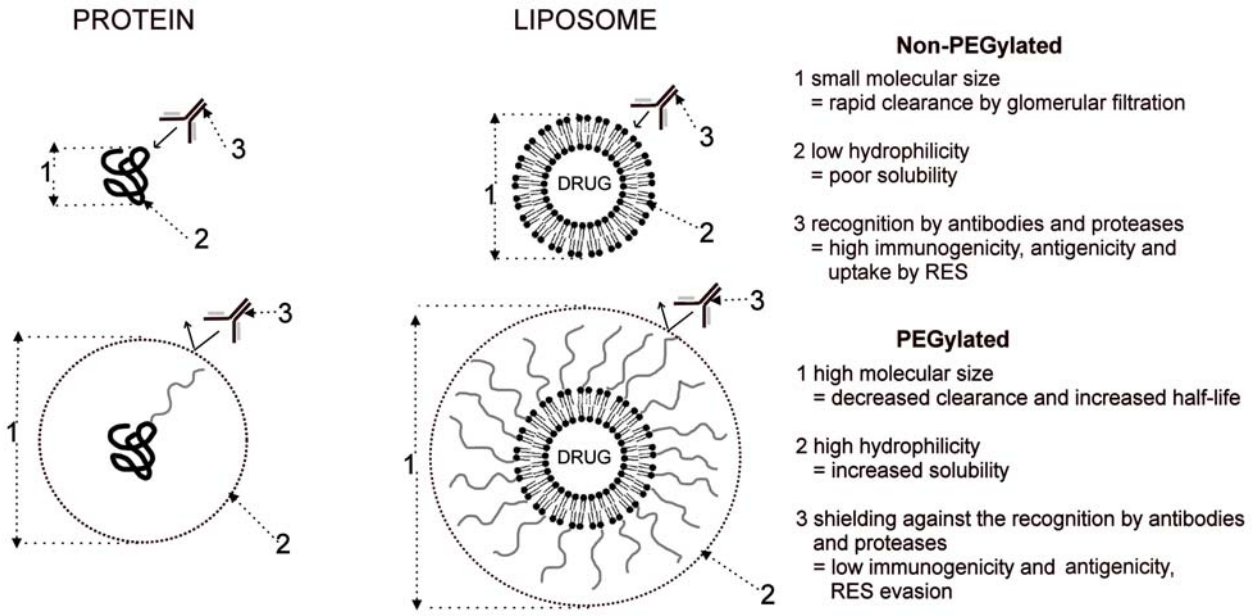


Figure 2. Main advantages for PEGylation of proteins and liposomes.

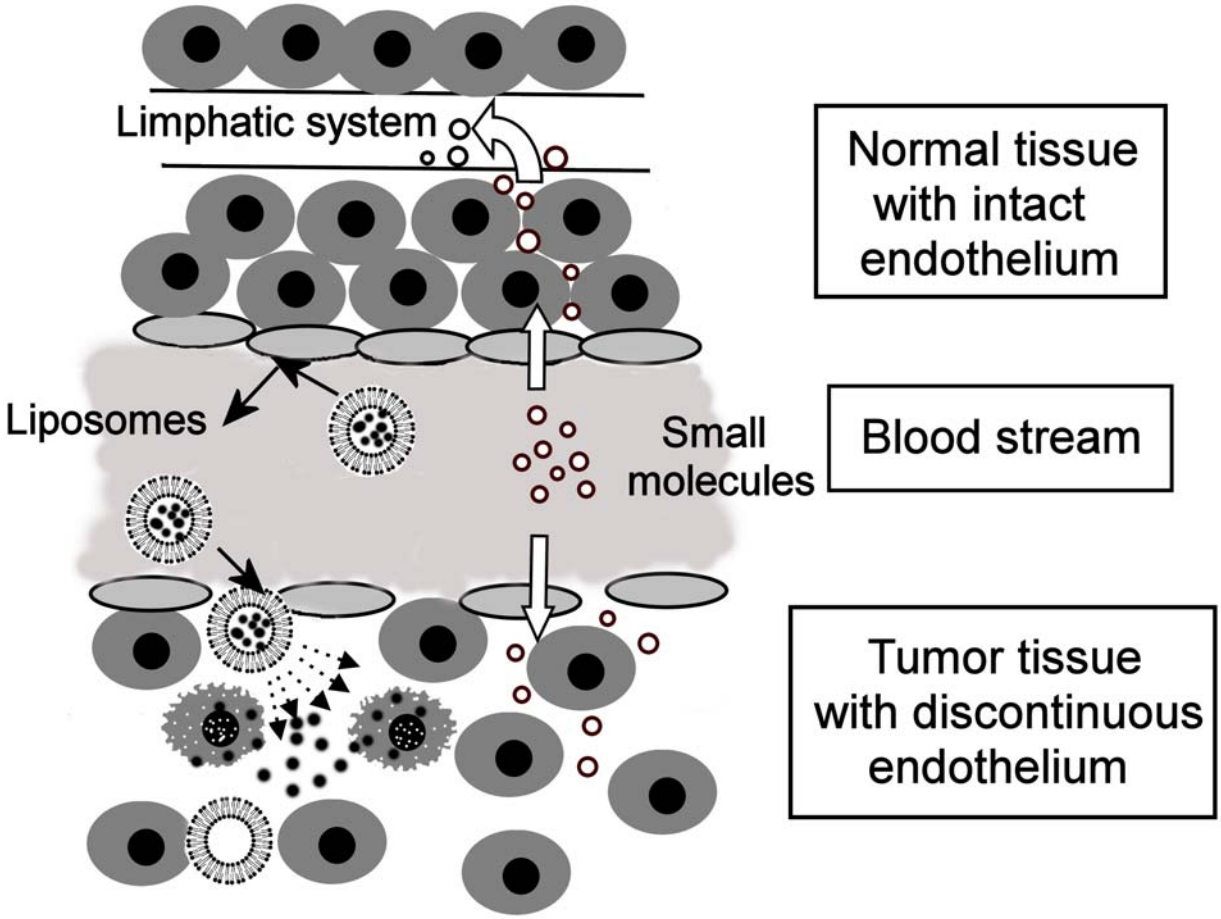


Figure 3. EPR (Enhanced Permeation Retention) effect [14, 15].

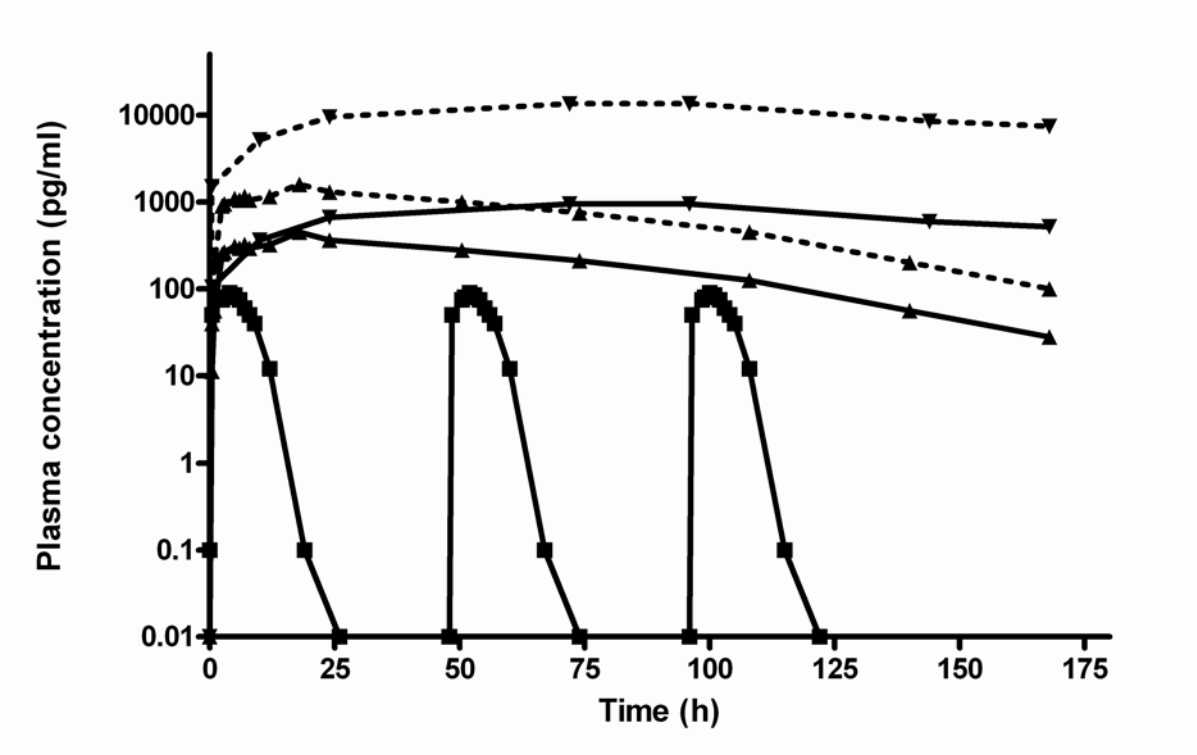


Figure 4. Pharmacokinetics of non-PEGylated IFN-α2b (Intron® A) 3 million units three times a week (-■-), PEG-INF-α2b (PegIntron®) 1.5 μg/kg once-weekly (-▲-), and PEG-INF-α2a (Pegasys®) 180 μg once-weekly (-▼-). Dotted lines represent the protein total concentration and solid lines represent the protein active concentration (about 28% for PEG-INF-α2b and 7% for PEG-INF-α2a) [49, 58].

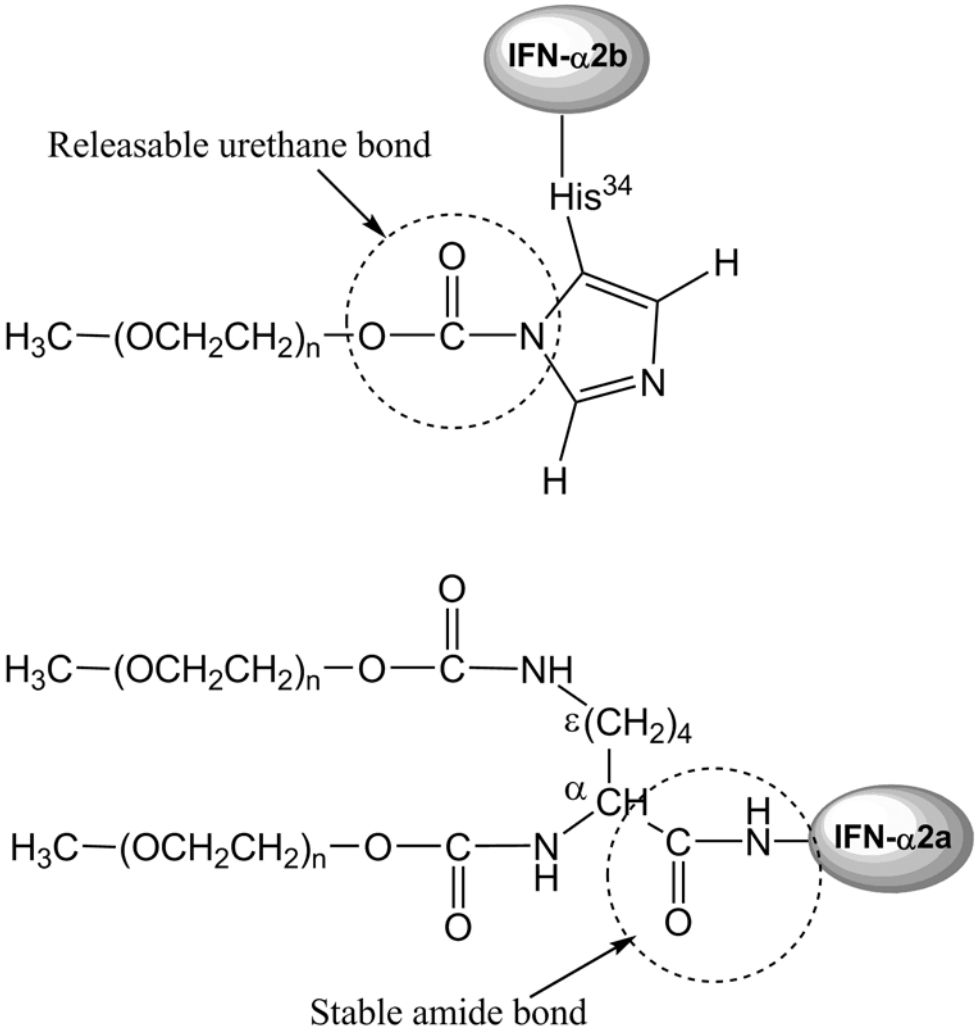


Figure 5. Differences on the PEGylation strategy for PegIntron® (PEG-IFN-α2b) and Pegasys® (PEG-IFN-α2a).

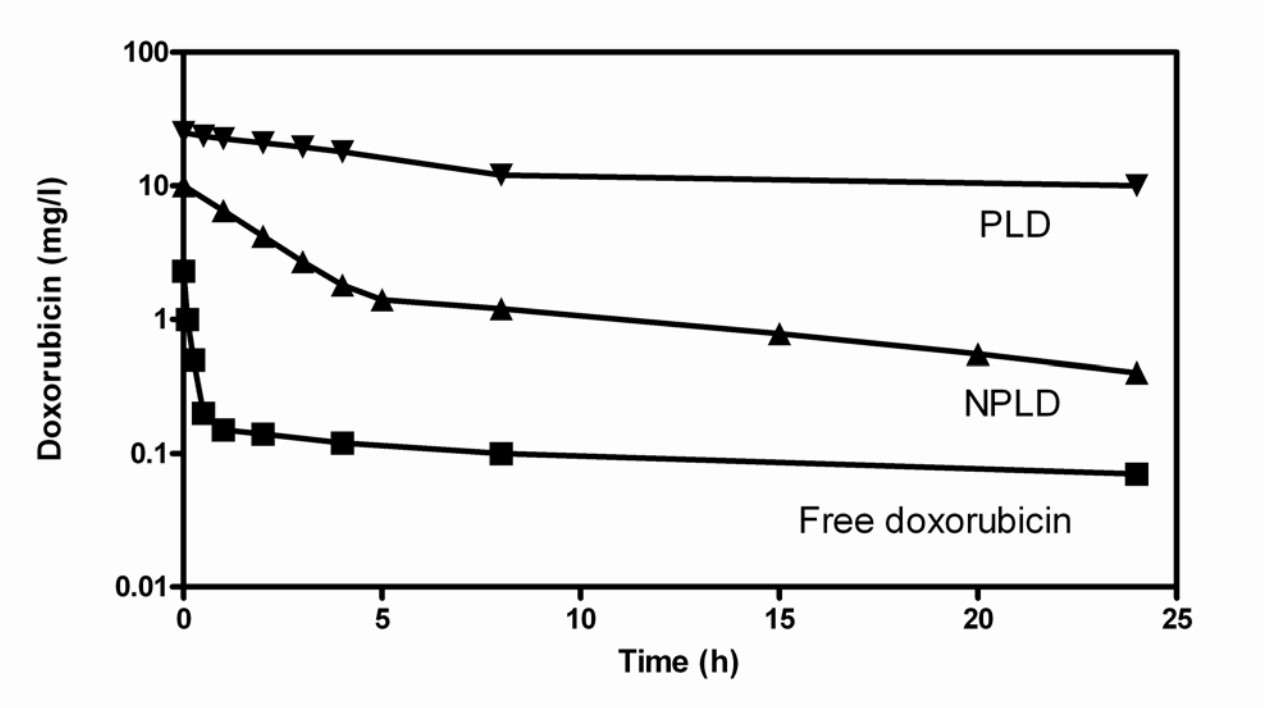


Figure 6. Plasma mean concentrations of doxorubicin in patients receiving a single intravenous dose of PLD 50 mg/m^2 (14 patients), NPLD 60 mg/m^2 (10 patients), or free doxorubicin 50 mg/m^2 (4 patients) [66, 67].

Tables

PEG conjugates (original protein)	Brand name (company)	PEGylation	Therapeutic indication	Approved year	Engineering rationale
Pegademase (bovine adenosine deaminase)	Adagen® (Enzon Inc.)	Random, amine PEGylation, multiple linear 5kDa PEGs	Severe combined immunodeficiency disease (SCID)	1990	Increased half-life
Pegaspargase (L-asparaginase)	Oncaspar®, (Enzon Inc.)	Random, amine PEGylation, multiple linear 5kDa PEGs	Acute lymphoblastic leukemia	1994	Increased half-life, decreased immunogenicity
Peginterferon-α2b (IFN-α2b)	PegIntron® (Schering-Plough Corp.)	Random, amine PEGylation, linear 12kDa PEG	Hepatitis C	2000-2001	Increased half-life, decreased immunogenicity
Peginterferon-α2a (IFN-α2a)	Pegasys® (Hoffmann La Roche Inc.)	Random, amine PEGylation, branched 40kDa (20kDa + 20kDa) PEG	Hepatitis C	2002	Increased half-life, decreased immunogenicity
Pegfilgrastim (G-CSF)	Neulasta® (Amgen)	Site-specific, N-terminal PEGylation, linear 20kDa PEG	Treating of neutropenia during chemotherapy	2002-2003	Increased half-life
Pegvisomant (genetically engineered analogue of hGH)	Somavert® (Pfizer Inc.)	Random, amine PEGylation, multiple (4-6) linear 5kDa PEGs	Acromegaly	2002-2003	hGH-receptor antagonist, increased half-life
CERA (epoetin-β)	Mircera® (Hoffman-La Roche Ltd.)	Random, amine PEGylation, linear 30kDa PEG	Anemia associated with chronic renal failure	2007	Increased half-life
Certolizumab pegol (anti-TNF-αFab')	Cimzia® (UCB S.A.)	Site-specific, thiol PEGylation, branched 40kDa PEG	Rheumatoid arthritis and Crohn's disease	2008	Increased half-life, decreased immunogenicity

Table 1. Marketed PEGylated proteins.

	Non-PEGylated IFN-α2b (Intron® A)	IFN-α2b (PegIntron®)	IFN-α2a (Pegasys®)
Chemical/biochemical parameters			
Molecular weight (kDa)	19	31	60
PEG structure	-	Monoconjugate, 12 kDa, linear (mPEG)	Monoconjugate, 40 kDa, branched (mPEG2)
Type of PEG-protein bond	-	amine PEGylation, releasable urethane bond	amine PEGylation, stable amide bond
PEGylation site	-	His 34 (50%), lysine residues (35%) and other residues (15%)	lysine residues 31, 121, 131 or 134 (94%) and other residues (6%)
Specific activity (%)	100	28	7
Pharmacokinetic parameters			
Elimination half-life (hours)	6	40	80
Vd (l)	31-73	69	8-12
Clearance (l/h)	6.6-29.2, renal	0.725-1.50, renal	0.06-0.10, hepatic
Posology, administration and formulation			
Dose	3 million units three times a week	1.5 μg/kg once-weekly	180 μg once-weekly
Route of administration	Subcutaneous	Subcutaneous	Subcutaneous
Formulation	ready-to-use solution	lyophilized powder that should be administered immediately after the reconstitution	ready-to-use solution

Table 2. Comparison between non-PEGylated IFN-α2b and the two PEGylated IFN-α approved by FDA [34, 47-59, 61].

Formulation	Dose (mg/m^2)	C_{max} (mg/l)	AUC (mg·h/l)	Cl (l/h)	V_d (l)	$t_{1/2}\beta$ (h)
Free doxorubicin	50	5.9	3.5	25.3	365	10.4
NPLD (conventional liposomes)	75	7.8	20.6	9.49	139	52.6
PLD (PEGylated liposomes)	60	33.7	4082	0.023	3.0	83.7

Table 3. Pharmacokinetic properties in human of marketed preparation of doxorubicin (free or encapsulated in conventional or PEGylated liposomes) [8, 68-70].