REVIEW

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PEGylation – a well-proven strategy for the improvement of recombinant drugs

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Protein and peptide drugs hold great promise as therapeutic agents. But there are shortcomings: Many recombinant proteins are quickly degraded by proteolytic enzymes or are rapidly cleared by kidney filtration resulting in a short circulating half-life. Additionally they are prone to be recognized by the immune system resulting in the generation of neutralizing and non-neutralizing antibodies. PEGylation, a process by which polyethylene glycol chains are attached to protein and peptide drugs, can overcome these and other shortcomings. By increasing the molecular mass of proteins and peptides and shielding them from proteolytic enzymes, PEGylation primarily improves pharmacokinetics and helps to prevent adverse drug reactions.

1. Introduction

Proteins are not necessarily "just" amino acid polymers. The majority of proteins present in the body occur naturally in a conjugated state. Chemical reactions that modify the protein structure are phosphorylation (Hunter 1995), glycosylation (Varki 1999), methylation (Wood and Shilatifard 2004), acylation (Farazi et al. 2001) and sulfation (Hemmerich et al. 2004), just to mention the most common ones. According to the class different site-chains of a protein can be involved, for example O-linked glycans attached to the hydroxy oxygen of threonine, tyrosine, serine, hydroxylysine, and hydroxyproline side-chain. Frequently such small modifications create new entities performing different functions compared with the unmodified protein. Posttranslational modifications can affect functions like signaling, targeting, catalysis, catabolism, protein stability and immunogenicity.

Nature-inspired creation of these posttranslational modifications is now adapted and further developed in order to improve properties of recombinant drugs. The modern tools of chemistry and biology made it possible to change the rational design of chemical compounds to target specific molecules. Among these are an improved solubility, an increased stability and a decreased immunogenicity and antigenicity.

The most successful strategy is PEGylation, a strategy using polyethylene glycol (PEG) as modifying polymer. Studies in solution have shown that each ethylene glycol subunit is tightly associated with two or three molecules of water. This makes PEGylated compounds function as if they are five to ten times larger than a corresponding unmodified protein of similar molecular mass (Kozlowski and Harris 2001). The PEG polymer, along with the associated water molecules, acts like a shield to protect the attached drug from enzyme degradation, rapid renal clearance and interactions with cell surface proteins, thereby limiting adverse immunological effects.

2. First generation PEGylation

In the 1970 s Davies and Abuchowsky were the first who described the concept of PEGylation (Abuchowsky et al. 1977a, 1977b). These were key publications, because at that time it was not conceivable that enzymes could be modified to such an extent without compromising their activities. Since then, PEGylation was intensively investigated and further developed resulting in a wide range of chemical and enzymatic tools and methods for conjugation.

Amino groups were the first target for PEGylation by acylation or alkylation reactions. Now conjugation of PEG to thiol-, hydroxyl- or amide-groups is also possible, by using several specific chemical or enzymatic methods. To maximize the pharmacological benefits of PEGylation, a stable bond is formed between the PEG polymer and the recombinant drug of choice. In general, a PEG polymer is first chemically activated in order to react with a polypeptide drug (Katre 1993). A variety of chemical modifications are used to prepare an active PEG derivative with a functional group, such as active carbonate, active ester, aldehyde, or tresylate (Fig. 1). These derivatives are then covalently linked to a reactive group on the polypeptide drug, such as the α or ε amino groups of lysine or the N-terminal amino-acid groups of other amino acids (Zaplinsky and Lee 1992).

The selection of PEGylation chemistries and reaction conditions are crucial, since the physicochemical properties in a biological environment are difficult to predict. For example, PEGylation of granulocyte colony-stimulating factor (G-CSF) through an amine linkage increases the liquid-phase stability of G-CSF five times compared with the PEGylation via an amide bond (Kinstler et al. 1996).

Although amino conjugation represents so far the most common modification and is often the first approach in many new PEG-protein projects, it is not devoid of limitations, because of the high number of isomers obtained. Initially the PEG polymer was generally attached to the ε-amino groups of lysines. This resulted

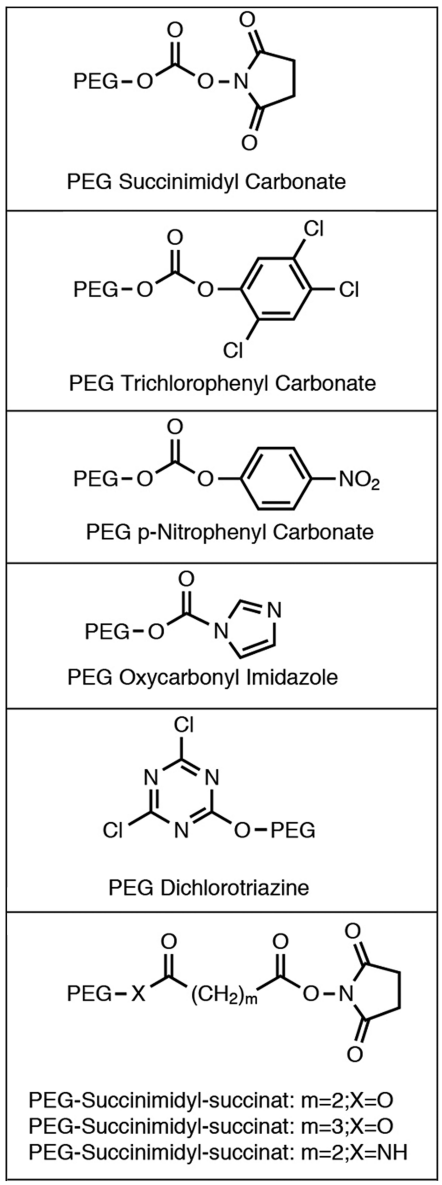

Fig. 1: Examples of PEG-derivations which can be covalently linked to a reactive group on the polypeptide drug.

mostly in the modification of multiple lysines, and gave mixtures of PEG isomers with different molecular masses (Zaplinsky 1995). The existence of such isomers makes it difficult to reproduce drug batches, and can cause problems like unpredictable antigenicity of the drug or poor clinical outcomes. However, authorities have approved isomer mixtures, if evidence for the reproducibility of the reaction was provided. FDA-approved first generation PEGylated drugs are PEG-asparaginase (Oncaspar®) (Graham 2003) or PEG adenosine deaminase (Adagen®) (Levy et al. 1988). These drugs are not approved by the EMA.

Meanwhile requirements for the approval became more stringent and a detailed characterization of each isomer is compulsory. Examples are the two α-interferon conjugates, Pegasys® (Bailon et al. 2001) and PEG-Intron® (Wang et al 2002), for which almost all the binding sites in the primary sequence were characterized.

Peginterferon alfa-2b (PEG-Intron®) is a first generation PEGylation product. The molecular mass of IFN alfa-2b is 19 kDa, whereas the molecular mass of Peg-Intron® is 31 kDa. Peginterferon alfa-2b is prepared by reacting interferon alfa-2b with activated methoxy-polyethylene glycol (mPEG) which results in covalent bonds between mPEG and amino groups of the

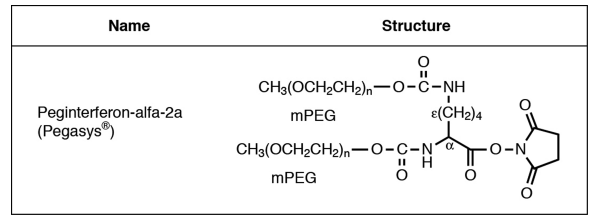

Fig. 2: Peginterferon-alfa-2a (Pegasys®) containing two mPEGs linked to IFN-alfa-2a.

interferon alfa-2b. Predominantly mono-PEGylated species are formed with small amounts of di-PEGylated species and unmodified interferon. It has been demonstrated that the substance heterogeneity is essentially consistent. Structural and biological characterization of PEGylated recombinant IFN-alfa-2b indicates that the principal PEGylation site (about 50% of PEGylation) is His^{34}, although PEGylation also occurs at lysines and at the N terminus (Grace et al. 2001).

3. Second generation PEGylation

Second-generation PEGylation chemistry strives to avoid the pitfalls associated with mixtures of isomers, diol contamination, unstable bonds and low-molecular mass mPEG. A huge array of chemistries has been developed in order to improve the production of these important conjugates. An overall goal of second-generation PEGylation methods is to create larger PEG polymers to improve the pharmacokinetic and pharmacodynamic effects seen with lower molecular mass PEGs. In some cases, the changes are dramatic, such as PEGylation of interleukin-6 (IL-6) which increases the half-life of IL-6 100-fold, which in turn results in a 500-fold rise in its thrombopoietic potency (Harris et al. 2001).

A method devised by Kinstler et al. (1996) takes advantage of the lower pKa of the N-terminal α-amino groups compared with that of the ε-amino group in lysines (Wong 1991). In this case the conjugation was performed on Filgrastim, a recombinant granulocyte colony stimulating factor (G-CSF), by attaching a 20 kDa polyethylene glycol-aldehyde (PEG-aldehyde) to the N-terminal amino acid of Filgrastim by a reductive alkylation, leading to the marketed Pegfilgrastim (Neulasta®) (Kinstler et al. 1996). Pegfilgrastim has a relative molecular mass of approximately 39 kDa.

Peginterferon-alfa-2a (Pegasys®) is a second generation PEGylation product. It contains IFN-alfa-2a connected to bis-[monomethoxy-polyethylene glykol] (mPEG) of a molecular mass of about 44.000 at a degree of substitution of one mole of polymer/mole of protein. The active PEG reagent contains two mPEG chains, each of a molecular mass of 20.000, attached to the α- and ε-amino groups of lysine via urethane bonds. The lysine carboxylic acid end group is functionalized to an N-hydroxysuccinimide ester, which reacts with lysine within IFN to form an amide bond with the protein (Fig. 2). There are potentially twelve sites for PEGylation in the polypeptide chain of IFN-alfa-2a, comprising the N-terminus plus eleven lysine residues. The process has been adjusted to ensure that no free interferon or free PEG is detectable in the product and that the product is consistent regarding the isomer pattern. The average molecular mass is approximately 60.000 of which the protein moiety constitutes approximately 20.000.

Also a PEGylated form of the human growth hormone antagonist called Pegvisomant (Somavert®) has been developed, which is indicated for the treatment of acromegaly. Patients suffering from acromegaly experience extremely high serum levels of insulin-like growth factor-1 (IGF-1) that contributes to soft-tissue enlargement. It has been shown that daily treatment with

Pegvisomant normalizes levels of IGF-1 and improves soft-tissue enlargement (Drake et al. 2001; Van der Lely et al. 2001). Pegvisomat is formed by covalent addition of polyethylene glycol with 4 and 5 PEG groups per molecule resulting in a molecular mass of 40 – 50 kDa. The molecular heterogeneity is reduced as far as possible by an appropriate downstream purification process. Pegvisomant has been approved in Europe and in the USA.

An interesting product is Mircera® with its active compound Methoxy-Polyethyleneglycol-Epoetin beta. In this case the 165 amino acid long Epoetin beta (NeoRecormon®) is PEGylated. Epoetin beta is a glycoprotein of approximately 30 kDa containing three carbohydrate trees at asparagine positions 24, 38 and 83 and a fourth carbohydrate tree at serine position 126. The PEGylation is carried out through integration of an amide bond between Methoxy-polyethyleneglycol-succinimidyl butanoic acid (PEG-SBA) and either the N-terminal amino group or the ε-amino group of lysine, predominantly either Lys^{52} or Lys^{45}. This results in a molecular weight of around 60 kDa.

4. Thiol modifications

PEGylation at thiol groups of cysteines not involved in disulphide bonds is one of the most specific methods because cysteinyl residues are rarely present in proteins nor are required for biological activity. Therefore the cysteine-modification has to be created such that it is still biologically active after PEGylation. At present no drugs are approved with PEGylated cysteines. This might change in the future when antibodies might be considered as targets for PEGylation.

But thiol chemistry not only allows targeting of thiol-groups. PEG–maleimide, for example, can also react with amines at pH > 8. This is realized in Certolizumab pegol (Cimzia®). Here the humanized Fab' antibody fragment Certolizumab pegol is covalently bound to a maleimido terminated bis-methoxypoly-(ethylene glycol) modified lysine (PEG2MAL40K) through a thioether linkage. The PEG2MAL40K moiety comprises two 20 kDa PEG chains linked to a reactive maleimide group. It is a polydisperse mixture of an average molecular weight of 40.000 Da, which is attached to the C-terminus of the molecule. The experimentally determined molecular mass of Certolizumab pegol is approximately 90.8 kDa, which is consistent with the addition of a single molecule PEG2MAL40K per molecule of the Certolizumab pegol Fab' fragment - the latter is composed of a 214 amino acid residues light chain and a 229 amino acid residues heavy chain.

5. Specific PEGylation by enzymes

Several strategies for site-specific PEGylation have been successfully exploited to conjugate PEG to pharmaceutical proteins. One of them is the conjugation of PEG to the amide group of glutamines or the hydroxyl group of serines and threonines with the help of the transglutaminase, which catalyzes the formation between a covalent bond between a hydroxyl group and the acyl group**.** These enzymes are labile molecules so the coupling reactions should utilize only mild chemical conditions. Sato (Sato 2002) discovered that one property of a glutamine residue is its ability to act as an amine acceptor in a transglutaminase-catalyzed reaction if an amino PEG is used as nucleophilic donor. The enzyme links PEG to the protein via a transglutamination reaction (Sato 2002).

In accordance with the procedure of Neose Technology (De Frees et al. 2004) the hydroxyl group of a specific serine or threonine is in the first step glycosylated. The enzyme O-GalNAc-transferase refers to an enzyme that catalyzes the addition of N-acetylgalactosamine (GalNAc). The natural occurring glycosylation site possesses a terminal sialic acid, which is crucial for the half-life period of the protein.

In the Neose process this sialic acid is conjugated to PEG and the binding to a O-GalNAc residue as the acceptor site is catalyzed by a sialyltransferase. Other enzymes have also been proposed for specific PEGylation, for example tyrosinase for conjugation to tyrosine (Chen et al. 2003; Harduin-Lepers et al. 2001).

6. GlycoPEGylation of filgrastim in order to yield lipegfilgrastim

A variation of the aforementioned method for specific GlycoPEGylation by enzymes is used to modify *E. coli* derived Filgrastim in order to yield Lipegfilgrastim. Lipegfilgrastim is a covalent conjugate of recombinant human N-methoxy granulocyte-colony stimulating factor (G-CSF, Filgrastim, company code: XM21), containing a glycine moiety, two carbohydrate moieties and a single methoxypolyethylene glycol (mPEG) molecule. mPEG is attached at the natural glycosylation site ($threonine^{134}$) of the polypeptide backbone, to create a drug with a longer half-life than unPEGylated G-CSF (Kubota et al. 1990).

The $threonine^{134}$ residue of the purified and refolded human G-CSF is modified *in vitro* in a single reaction via the sequential action of two specific glycosyltransferases, GalNAcT2 and ST6GalNAc1. These glycosyltransferases have distinct specificities: (i) acceptor specificity for a particular target molecule, i.e. an amino acid or a terminal sugar residue already linked to a polypeptide or glycan chain, (ii) donor specificity for a particular nucleotide-activated sugar, and (iii) reaction specificity which determines the position and stereochemistry (α vs β) of the glycosidic bond formed between the transferred sugar and the acceptor.

7. Assessment of the glyco-PEGylated filgrastim and conclusion

Glyco-PEGylation as applied for Lipegfilgrastim is certainly the most specific mPEGylation method available to date. The resulting product "Lipegfilgrastim or XM22" is, from a pharmaceutical point of view, the mostly developed G-CSF molecule used in a therapeutic manner or the next generation in the family of therapeutically applied G-CSF molecules. Appropriately set up glyco-PEGylation selectively adds one molecule of mPEG to a sugar-activated threonine ($threonine^{134}$) in Filgrastim. In addition to this defined glycol-PEGylation pattern the position of the methoxyPEGylation site is mentionable as well and could be the basis for hypothesis generation. Since one particular aspect of a PEG modification is shielding the protein from enzymatic degradation and protection from recognition by the immune system. A centrally positioned mPEG-chain (as in Lipegfilgrastim) might be favorable over an N-terminally positioned PEG-chain (as in Pegfilgrastim).

These hypotheses can be tested and there are clinical trials ongoing to test that at least the first assumption might be valid (Bondarenko et al. 2013).

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