Review

PEGylation of therapeutic proteins

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Since the first PEGylated product was approved by the Food and Drug Administration in 1990, PEGylation has been widely used as a post-production modification methodology for improving biomedical efficacy and physicochemical properties of therapeutic proteins. Applicability and safety of this technology have been proven by use of various PEGylated pharmaceuticals for many years. It is expected that PEGylation, as the most established technology for extension of drug residence in the body, will play an important role in the next generation therapeutics, such as peptides, protein nanobodies and scaffolds, which due to their diminished molecular size need half-life extension. This review focuses on several factors important in the production of PEGylated biopharmaceuticals enabling efficient preparation of highly purified PEG-protein conjugates that have to meet stringent regulatory criteria for their use in human therapy. Areas addressed are PEG properties, the specificity of PEGylation reactions, separation and large-scale purification, the availability and analysis of PEG reagents, analysis of PEG-protein conjugates, the consistency of products and processes and approaches used for rapid screening of pharmacokinetic properties of PEG-protein conjugates.

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1 Introduction

Protein and peptide biopharmaceuticals have been successfully used as very efficient drugs in therapy of many pathophysiological states since the first recombinant product insulin was approved in 1982. They have become widely available after the rapid development of recombinant DNA technology over the last few decades. One group of approved first-generation protein biopharmaceuticals mimics native proteins and serves as replacement therapy, while another group represents monoclonal antibodies for antagonist therapy or activating malfunctioning body proteins [1]. The main drawbacks of the first-generation biopharmaceuticals are their suboptimal physicochemical and pharmacokinetic (PK) properties. Main limitations are physicochemical instability, limited solubility, proteolytic instability, relatively short elimination half life, immunogenicity and toxicity. Consequently, protein therapeutics are mainly administrated parenterally.

Many technologies have been developed during the last decade focusing on improvement of characteristics of the first-generation protein drugs to gain the desired PK properties. Half-life extension technologies include amino acid manipulation to reduce immunogenicity and proteolytic instability, genetic fusion to immunoglobulins domains or serum proteins (albumin) and post-production modifications – conjugation with natural or synthetic polymers (polysialylation, HESylation and PEGylation).

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Abbreviations: AEX, anion-exchange chromatography; **CEX**, cation-exchange chromatography; **DBC**, dynamic binding capacity; **FDA**, Food and Drug Administration; **G-CSF,** granulocyte colony-stimulating factor; **HES**, hydroxyethyl starch; **hGH**, human growth hormone; **IFN**, interferon; **PK,** pharmacokinetic; **RPC**, reversed-phase chromatography; **SEC**, size-exclusion chromatography; **TNF,** tumor necrosis factor; **VEGF**, vascular endothelial growth factor

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In addition, new drug-delivery systems, such as microspheres, liposomes and nano- or micro-particles, are employed to optimize drug properties. It is difficult to judge which of these approaches will most benefit the patient either for short-term or long-term therapy. Amino acid engineering is the basic strategy, but other approaches, especially fusions and post-production derivatizations or conjugations bring significant elimination half-life extension, thus enabling much less frequent administration, which is undoubtedly one of the main and most important benefits for patients.

The covalent attachment of the polymer polyethylene glycol (PEG) to protein – PEGylation – is a well-established, widely employed and fast-growing technology that fulfils many of the requirements for safe and efficacious drugs. Several PEGylated products have been on the market for some time, confirming their efficacy and safety. First attempts to PEGylate proteins were undertaken in the 1970s, when Abuchowski *et al*. [2, 3] conducted first conjugations of PEGs to protein and observed improved characteristics of PEG-protein conjugates. The first Food and Drug Administration (FDA)-approved PEGylated biopharmaceutical appeared on the market in 1990: a PEGylated form of adenosine deaminase, Adagen® (Enzon Pharmaceuticals, USA), for the treatment of severe combined immunodeficiency disease (SCID) [4]. Since then, nine different PEGylated products have received FDA approval. Eight are PEGylated proteins and one [pegaptanib (Macugen® or Macuverse®)] is a PEGylated anti-vascular endothelial growth factor (VEGF) aptamer (an RNA oligonucleotide) for the treatment of ocular vascular disease [5]. It is worth mentioning that four of these eight approved PEGylated biopharmaceuticals are blockbuster drugs: PegIntron® (Schering-Plough, USA), a PEGylated form of interferon (IFN)-α2b; Pegasys® (Hoffman-La Roche, Inc., USA), a PEGylated form of IFN-α2a, both for the treatment of hepatitis C; Neulasta® (Amgen, USA), a PEGylated form of granulocyte colony-stimulating factor (G-CSF) for the treatment of chemotherapy-induced neutropenia; and Mircera® (Hoffman-La Roche, Inc., USA), a PEGylated protein (epoietin-β) approved by FDA in 2007 for the treatment of anemia associated with chronic renal failure in adults [but not approved for the treatment of anemia in patients with cancer because of an increased risk for mortality – European Medicines Evaluation Agency (EMEA) product information http://www.emea.europa.eu/humandocs/PDFs/EPAR/mircera/H-739-PI-en.pdf]. As an alternative to full monoclonal antibodies, a PEGylated antibody fragment has already been FDA approved; this belongs to the tumor necrosis factor (TNF) inhibitors drug family. Cimzia® (UCB Pharma, Belgium), a PEGylated anti-TNF-α Fab' was approved in April 2008 for the treatment of Crohn's disease and in May 2009 for rheumatoid arthritis. The next PEGylated antibody fragment originating from UCB Pharma di-Fab' anti-platelet derived growth factor (CDP860) [6] is in the advanced stages of clinical trials.

In addition to these already approved PEGylated biopharmaceuticals, many more new products can be expected in the near future and are currently in different stages of clinical trials. For example, at the beginning of July 2009, BiogenIdec Inc. received a Fast Track designation from the FDA for its PEGylated IFN-β1a for treatment of multiple sclerosis, which means that a global Phase III study evaluating the efficacy and safety of less frequent administration of PEGylated IFN-β1a will start soon (http://www.medicalnewstoday.com/articles/156976.php) [7]. In addition, an improved version of PEGylated G-CSF (Maxy G34), a site-specifically PEGylated G-CSF analog successfully completed Phase IIa clinical trials (http://www.maxygen.com/products-mye.php). A PEG conjugate of recombinant porcine-like uricase, an enzyme that substantially and persistently reduces plasma urate concentrations, successfully passed Phase III clinical trials [8].

Until now PEGylation has generated several successful therapeutics available on the market with improved PK behavior, and has also played a role in life-cycle management for several proteins, resulting in four PEGylated blockbuster drugs [9–11]. With the further development of scaffold- and nanobody-based biopharmaceuticals, an increasing number of approved PEGylated drugs can be expected, because PEGylation is the most established technology for extension of drug elimination half-life.

Protein scaffolds represent a new generation of universal binding structures for future biopharmaceutical drug design, complementing the existing monoclonal antibody-based therapeutics. Engineered protein scaffolds are generated from small, soluble, stable, monomeric proteins derived from several families, such as lipocalins (Anticalin), fibronectin III (AdNectin), protein A (Affibody), thioredoxin (Peptide aptamer), and BPTI/LACI-D1/ITI-D2 (Kunitz domain), and equipped with binding sites for the desired target [12–14]. Such engineered protein scaffolds are stable, can be overexpressed in microbial expression system and are, due to their small size, efficient in tissue penetration, possessing therapeutic potential for intracellular targets in addition to extracellular and cell

surface targeting [13]. To prolong their residence time in the body, they are often PEGylated. Several protein scaffold-based drugs are in preclinical and clinical trials, including the following PEGylated scaffolds: CT-322, an Adnectin-based antagonist of VEGF receptor-2 (VEGFR-2); and DX-1000, a Kunitz-type inhibitor for blocking breast cancer growth and metastasis [12].

Another structure for the development of new generation biopharmaceuticals are nanobodies – single-domain antibody fragments devoid of the light chain found in camelids (variable domain of camelid heavy chain antibody, VHH) and sharks (variable domain of shark new antigen receptor, VNAR), which are fully functional and capable of binding antigen without domain pairing. The nanobodies are soluble, very stable, do not tend to aggregate and are overexpressed in microbial expression systems, making them very attractive for biotechnological and biopharmaceutical applications. An example of a successful application of PEGylation to nanobodies is the PEGylated nanobody neutralizing foot and mouth disease (FMD) virus [15]. Several obstacles still have to be circumvented to allow the clinical applications of the nanobodies-based therapeutics. With further progress in this field nanobodies-based therapeutics can be expected that will target toxins, microbes, viruses, cytokines and tumor antigens [15, 16].

Conjugation of PEG to protein results in a new macromolecule with significantly changed physicochemical characteristics. These changes are typically reflected in alterations of receptor binding affinity, *in vitro* and *in vivo* biological activity, absorption rate and bioavailability, biodistribution, PK and pharmacodynamic profiles, as well as reduced immunogenicity and reduced toxicity. The main drawback of PEGylation is usually reduced biological activity *in vitro*, which is compensated *in vivo* by significantly improved PK behavior [17, 18]. Generally, the longer the PEG chain, the longer the elimination half-life of the PEG-protein conjugate (Fig. 1). In addition to PEG length, its shape greatly influences absorption and elimination half-life. Various sources have confirmed that branched PEGs extend elimination half-life more than linear PEGs of the same nominal molecular weight [19].

2 PEG reagents and their availability

PEG reagents are commercially available in different lengths, shapes and chemistries, allowing them to react with particular functional groups of proteins for their covalent attachment. There are several commercial suppliers, *e.g.*, NOF Corporation

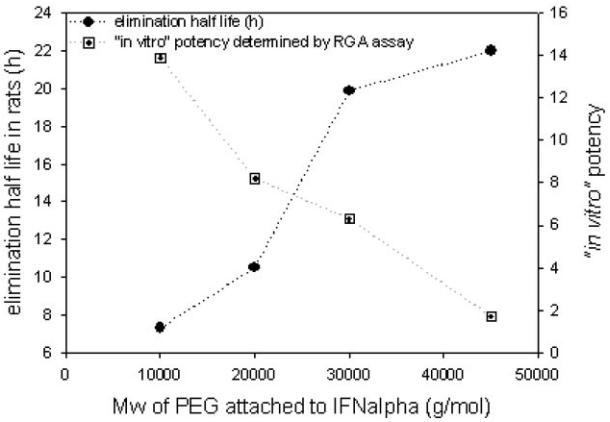

Figure 1. Influence of the molecular weight (Mw) of N-terminally PEGylated IFN-α2b conjugates (bearing linear 10-, 20-, 30- and branched 45-kDa PEGs) on their *in vitro* potency determined by reporter gene assay assay [45] and elimination half-life in rats after *i.v.* administration.

(Japan); SunBio (South Korea); Chirotech Technology Ltd. (UK), their PEG business was taken over by Dr. Reddys in 2008; JenKem (China); Creative PEGWorks (USA).

PEG is generally regarded as a non-biodegradable polymer, but some reports clearly show that it can be oxidatively degraded by various enzymes, such as alcohol and aldehyde dehydrogenases [20, 21] and cytochrome P450-dependent oxidases [22]. PEG chains shorter than 400 Da are metabolized *in vivo* by alcohol dehydrogenases to toxic metabolites. Longer PEG chains, which are used for PEGylation of proteins, are not subjected to metabolism, and the elimination mechanism depends on their molecular mass. PEG molecules and PEG-protein conjugates with PEGs of below 20 kDa are eliminated by renal filtration, while protein conjugates with larger PEG molecules are cleared from the body by other pathways, such as liver uptake, via the immune system and proteolytic digestion of the protein part of the conjugate. These are also the natural clearing mechanisms for large protein molecules with molecular masses above 70 kDa [23].

PEG has a long history as a non-toxic, nonimmunogenic, hydrophilic, uncharged and non-degradable polymer, and has been approved by the US FDA as 'generally recognized as safe' [24]. PEG is typically polydisperse. PEGs and PEG reagents with broad polydispersity were used in the past, whereas nowadays polydispersity indexes of approximately 1.05 are the accepted standard for PEG reagents of up to 30 kDa. For higher molecular weight forms, a polidispersity of 1.1 may still be acceptable; however, the general trend is directed to PEGs with narrower distributions. Polydispersity of the polymer is one of the factors that aggra-

vate final characterization of PEG-protein conjugates. The current practice employs linear and branched PEGs with molecular masses up to 40 kDa, which bring the desired improvement of PK properties. Nevertheless, new PEG formats such as forked, multi-arm and comb-shaped PEGs show great promise for the future. The macromolecular structure of the conjugating PEG polymer appears crucial for the improved properties of the conjugates. In this sense, comb-shaped PEGs bearing numerous short PEG chains attached to the polymer backbone that can be prepared by transition metal-mediated living radical polymerization, offer an additional advantage of relatively tightly controlled polymer molecular weight and architecture [11]. A promising approach is releasable PEGylation – an attachment of PEG reagent with releasable linker to the protein. This overcomes drug inactivation by conjugation and enables release of the full-potency drug, increases the solubility of poorly soluble drugs and deposits such drugs at the target, allows random PEGylation, and by appropriate selection of the linker also control of PK parameters. At the same time some major benefits of traditional PEGylation are lost, *e.g.*, long elimination half-life, reduced immunogenicity, reduced proteolysis, and easier formulation and analytics of stable PEGylated proteins [25].

Significantly improved physicochemical characteristics after coupling of PEG to a protein can be explained by the increased hydrodynamic volume of the PEG-protein conjugate, which results from the ability of PEG to coordinate water molecules and from the high flexibility of the PEG chain. Consequently, apparent molecular weight of the PEG-protein conjugate is around five- to tenfold higher in comparison to the globular protein of the same nominal molecular weight [26]. PEG chains can sweep around the protein to shield and protect it from the environment (or *vice versa*), but they also influence the interactions of the protein that are responsible for its biological function. This is considered as the basis for the certain discrepancy between the *in vitro* and *in vivo* activities of PEGylated proteins. Generally, the preserved *in vitro* biological activity after PEGylation is reduced, sometimes very significantly; nevertheless, the *in vivo* pharmacological effects are usually enhanced. Pegasys®, a PEGylated IFN-α2a, is a typical example of a very efficient PEGylated protein drug that displays an *in vitro* activity of only a few percent of the level of the unmodified IFN-α, while its efficacy justifies replacement of the first-generation IFN-α in therapy [27].

3 Immunogenicity and safety of PEGylated proteins

PEGylation normally reduces immunogenicity of proteins; there are examples of transforming immunogenic proteins into a tolerogen by PEGylation [28]. Generally, it is often not easy to predict characteristics of PEG-protein conjugates, because they strongly depend on the physicochemical properties of the protein, polymer and final conjugate. The likelihood of an immunogenic reaction of conjugates increases with the level of immunogenicity of the non-modified protein. A typical example is PEG-uricase, a recombinant enzyme capable of degrading high levels of uric acid in patients with hyperuricemia. Unlike most mammals, humans lack an uricase enzyme, therefore, for therapeutic purposes, an enzyme totally foreign to the human body (porcine-like) is used, and has to be sufficiently PEGylated to mask its immunogenicity [8]. However, during Phase I clinical trials the formation of unusual anti-PEG antibodies was detected in some patients [29]. Presumably the methoxyl group in the PEG chain at the terminus remote from the linker to the protein ([8], WO 2004/030617 A2) was identified as a source of antigenicity, which is rather surprising since methoxyl end-capped PEGs are generally used in modern marketed PEGylated biopharmaceuticals without reports on PEG immunogenicity. However, a few reports on induction of anti-PEG immune responses in the case of repeated administration of PEGylated liposomes [30, 31] or PEG-glucuronidase can be found in literature [32]. High levels of PEG used as an intravenous therapeutic agent *per se* have been shown to generate concentration and molecular mass-dependent serum complement activation [33]; however, the quantities of PEG administered in PEGylated therapeutics are 10000- to 1000-fold lower. In toxicology studies, very high doses of PEG-protein conjugates have been demonstrated to be capable of inducing renal tubular vacuolization that is not associated with functional abnormalities and that disappears after the treatment [34]. Therefore, PEG-protein conjugates remain regarded as immunologically safe and non-toxic. It has also been demonstrated that potential protein immunogenicity can be better alleviated by attachment of larger and branched PEGs than by shorter and linear PEGs. In general, low immunogenicity of PEG and relatively low dosages of PEG-conjugates reduce the risk for an immunogenic response significantly [23, 35–38].

Conjugation might sometimes lead to the formation of new epitopes as a consequence, e.g., of partial protein denaturation after conjugation or

use of an inappropriate spacer between protein and PEG chain. Hence it is important to pay attention to suitable PEGylation chemistry, solution conditions and careful selection of the PEGylation site [37].

The size and shape of the PEG-protein conjugates determines their distribution and accumulation in the liver and other organs that are rich in reticuloendothelial cells, such as the spleen, lymph nodes, lungs and kidneys. Clearance from these organs is lower for PEG-protein conjugates than for native or glycosylated proteins. Severe side effects have not been reported, but consequences of lifelong therapies with high dosages of PEG-protein conjugates containing PEG conjugates of high molecular weight are hardly predictable. Occasional warnings that significant PEG-protein accumulation in the liver may increase the risk of toxicity have appeared [23, 39, 40].

4 PEGylation reaction/chemistry

PEGylation of proteins is usually achieved by a chemical reaction between the protein and suitably activated PEGylation reagents. There are various chemical groups on the amino acid side chains that could in principle be exploited for the reaction with the PEG moiety, such as -NH_2, -NH-, -COOH, -OH, -SH groups as well as disulfide (-S-S-) bonds. However, not only the protein attachment site for the PEGylation reagent is important. When speaking about PEGylation and PEGylated proteins, especially about their altered properties, various aspects of the process have to be considered, such as the attachment site on the protein, activation type of the PEG reagent, nature (permanent or cleavable), length and shape of the linker, length, shape and structure of the PEG reagent as well as end capping of the PEG chains.

4.1 Random PEGylation

When looking back into the history of PEGylation it can be seen that, until recently, the majority of the PEGylation reagents developed targeted amino groups on the protein, most frequently the ε-amino groups on the side chains of lysine residues. Lysines are polar and relatively abundant amino acid residues usually located on the protein surface, which make them prone to chemical reactions with PEG reagents. Consequently, such reactions advance quickly and lead to complex mixtures of conjugates, differing in the number and site of the attached PEG chains. In addition, most of the PEGylation reagents employed are not strongly specific for the reaction with the amino groups of the lysine residues, but react to a minor degree also with other protein nucleophiles: *N*-terminal amino groups, the imidazolyl nitrogens of histidine residues and even with the side chains of serine, threonine, tyrosine and cysteine residues. Although the reaction can be directed to some extent by the pH of the medium (for the reaction to proceed, the nucleophile must always be in the non-protonated form), this type of conjugation reactions always lead to complex PEGylation mixtures. The historical development of such "random" PEGylation reagents began in the 1970s with PEG-chlorotriazine reagents [2, 3] and continued with succinimidyl succinate (SS-PEG) [41] and succinimidyl carbonate PEG reagents (SC-PEGs) [42, 43]. SS-PEG reagents produce stable amide bonds (-CO-NH-protein) with the protein, but due to another ester linkage in the polymer backbone the resulting PEG-protein conjugates are susceptible to hydrolysis. SC-PEG reagents form urethane linkages (-O-CO-NH-protein) and react, beside with lysine residues, also with histidine residues, resulting in hydrolytically unstable linkages. The weak linkage could be used to advantage in the preparation of controlled-release or pro-drug formulations, as in the case of PegIntron®, or it could be a severe disadvantage if the conjugate instability was not desired. Although numerous other chemistries have also been tried in the past, most of the random PEGylation reagents possess the activated carbonyl group in the form of *N*-hydroxy-succinimide esters that form stable protein-PEG conjugates via amide linkages. Depending on the reaction conditions (reaction time, temperature, pH, amount of PEG reagent and protein concentration), mono-, di-, tri- and numerous higher-PEGylated conjugates can be formed. However, due to reactions with different nucleophilic groups on the protein, even mono-PEGylation leads to positional isomers that can differ substantially in their biological and biomedical properties.

The first PEGylated pharmaceuticals Adagen® (pegademase) and Oncaspar® (PEGylated asparaginase, pegaspargase) were actually complex mixtures of various PEGylated species. Pegademase has been proven to be much more efficient than the partial exchange transfusions of red blood cells that represented the standard therapy before approval of pegademase. Pegaspargase serves for treatment of various leukemias and has addressed the problem of neutralizing antibodies associated with the use of native asparaginase.

However, also subsequently approved drugs, PegIntron® and Pegasys®, are produced by random PEGylation. Both biopharmaceutical drugs are

mixtures of mono-PEGylated positional isomers, containing linear 12-kDa PEG chains bound to different sites of IFN-α2b in the case of PegIntron® (http://www.emea.europa.eu/humandocs/PDFs/EPAR/Pegintron/024400en6.pdf) and branched 40-kDa PEG chains bound mainly to four Lys residues of IFN-α2a in the case of Pegasys® (http://www.emea.europa.eu/humandocs/PDFs/EPAR/pegasys/199602en6.pdf).

Another interesting PEGylated biopharmaceutical drug, produced by random PEGylation, is pegvisomant (Somavert®, Pfizer) that was approved in 2003 for the treatment of acromegaly. Pegvisomant has been designed to function as an antagonist of the human growth hormone (hGH) receptor (HGHR) by substitution of certain amino acids in the backbone of the protein hGH. Modifications include several mutations on the protein and conjugation with four to six 5-kDa PEG chains per molecule (http://www.emea.europa.eu/humandocs/PDFs/EPAR/somavert/486302en6.pdf).

Even Mircera®, which was FDA approved in 2007, is a mixture of mono-PEGylated conjugates of erythropoietin, with a 30-kDa PEG attached either to lysine residues (mostly Lys52 and Lys45) or the N terminus of the protein (http://www.emea.europa.eu/humandocs/PDFs/EPAR/mircera/H-739-en6.pdf).

4.2 Site-specific PEGylation

Although purification would allow homogenous product preparation, one of the obvious trends in the development of the PEGylation technology is a shift from random to site-specific PEGylation reactions, leading to better defined products. Examples of classical, well-known approaches toward site-specific PEGylation reactions are N-terminal and cysteine-specific PEGylations.

N-terminal PEGylation, performed as a reductive alkylation step with a PEG-aldehyde reagent and a reducing agent (*e.g.*, sodium cyanoborohydride) [44], was employed in the development of Neulasta® [45], which is an N-terminally mono-PEGylated G-CSF bearing a 20-kDa PEG, (EMEA product information, http://www.emea.europa.eu/humandocs/PDFs/EPAR/neulasta/296102en6.pdf). The improved PK behavior enables administration only once per chemotherapy cycle compared to the first generation, Neupogen®, which is administered daily (up to 2 weeks in each chemotherapy cycle).

PEGylation of thiol groups in natural or genetically introduced unpaired cysteines is another well-known approach to site-specific PEGylation. A variety of thiol-specific reagents are available, such as maleimide, pyridyl disulfide, vinyl sulfone, thiol reagents, *etc*. Due to the stability of the formed linkages, maleimide-PEG reagents have become very popular. In native proteins, cysteine residues are usually involved in disulfide bridges or responsible for interaction with metals or other proteins, but a good example of single cysteine PEGylation of G-CSF has been described. To achieve site-specific PEGylation of the unpaired Cys18, which is only partially exposed, the PEGylation was performed under transient denaturing conditions [46]. Genetically introduced cysteines represent an opportunity to direct the PEG moiety to an exactly determined site in the molecule. In the case of IFN-α2a, several cysteine analogues with high preserved *in vitro* activity were identified and used for specific PEGylations [47, 48]. Another very promising group of proteins for cysteine-specific PEGylations are Fab' fragments. PEGylation appears an ideal method to reduce their antigenicity and prolong the *in vivo* circulation times. However, the main benefit of using PEGylated Fab's instead of full antibodies can be elimination of undesired side effects originating from the Fc region. Cysteine residues in the hinge region of Fab' fragments that are far from the antigen-binding region, offer the possibility of specific conjugation leading to a well-defined product. The most prominent example of this strategy is Cimzia®, a PEGylated Fab' fragment of a humanized anti-TNF-α monoclonal antibody bearing a 40-kDa branched PEG site-specifically attached to a hinge cysteine. Recently, it has also been shown that the PEGylation efficiency of Fab' fragments can be substantially increased by exploitation of interchain disulfide bond after its reduction for PEGylation. The final Fab'–PEG product does not retain the interchain disulfide bond. Such molecules, although without covalent linkage between both antibody chains, retain very high levels of chemical and thermal stability and normal performance in PK and efficacy models [49].

A novel approach to the PEGylation of protein disulfide bonds, TheraPEG™, is a PEGylation technology of PolyTherics using special PEG monosulfone reagents. The site-specific bisalkylation of both sulfur atoms in the natural disulfide bond that is sufficiently exposed on the protein surface results into the insertion of the PEG linker into the disulfide bond and formation of a three-carbon PEGylated bridge [50, 51]. The strategy is appropriate for specific PEGylation of Fab' fragments and will presumably be used for the production of a novel type of PEGylated IFN-α (http://www.chime.plc.uk/press-releases/de-facto-appointed-by-polytherics).

A new approach to PEGylation using histidine affinity tags as targets for PEG attachment has been recently published by PolyTherics (WO 2009/047500 A1). Taking into account that histidine affinity tags are among the most frequently used tools for easy and rapid purification of recombinant proteins the strategy appears to offer a certain potential.

PEGylation via non-natural amino acids requires the genetic manipulation of the protein and of the host organism to allow incorporation of non-natural amino acids (so-called Amber technology), which can be specifically conjugated with appropriate PEG reagents.

Following triazole formation by the (3+2) cycloaddition of an alkyne and an azide for selective conjugation, azido and ethynyl derivatives of serine are interesting as they are capable of reacting specifically with matching ethynyl-PEG or azido-PEG reagents [52].

Through the Amber technology approach, the Ambrx company generated site specific mono-PEGylated hGH molecules with improved pharmacological properties. Phase I/II clinical trial data demonstrated that the long-acting hGH analog developed in collaboration between Ambrx and Merck Serono, normalized insulin-like growth factor I (IGF-I) levels and showed an acceptable safety and tolerability profile in adults with growth hormone deficiency (http://www.ambrx.com/wt/page/pr_1226513229). More long-acting therapeutics can be found in the Ambrx pipeline (*e.g.*, IFN-β, FGF21, leptin), all still in preclinical trials.

Instead of traditional conjugation reactions performed by chemical procedures, enzymes can also be employed to achieve specific PEGylation. Thus, transglutaminase is capable of catalyzing the incorporation of PEG-alkylamine reagents into the protein glutamine residues, which can be either natural or genetically introduced [53]. The reaction offers a high degree of specificity since only those glutamine residues that are encompassed in a flexible or unfolded region are modified [54]. Even more promising appears to be a two-enzyme step GlycoPEGylation™ technology developed by Neose Technologies Inc, which allows the introduction of PEG chains at natural *O*-glycosylation sites [55]. Starting materials are non-glycosylated recombinant polypeptides obtained by *Escherichia coli* production systems. Proteins must contain a single *O*-glycosylation site in which a serine or threonine residue functions as an acceptor for selective addition of *N*-acetylgalactosamine (GalNAc) by the recombinant enzyme *O*-GalNAc-transferase. In the next step the glycosylated protein is PEGylated on *O*-GalNAC by a cytidine monophosphate derivative of sialic acid-PEG using another recombinant enzyme sialyltransferase. The technology has been tested so far on various pharmaceutically relevant proteins, including G-CSF. GlycoPEG-G-CSF is currently in the most advanced phase of clinical trials (http://www.medicalnewstoday.com/articles/112612.php).

A short overview of PEGylation technologies used today is summarized in Table 1.

5 Purification of PEGylated proteins

Purification of PEGylated proteins is required to obtain the final product from the complexity of PEGylation mixtures. The target PEG-protein conjugate has to be separated from unreacted protein, over-PEGylated proteins, unreacted PEG reagent and from other reagents eventually added to the PEGylation mixture. For isolation of the target PEG-protein, differences in charge, hydrodynamic radius, hydrophobicity and in some cases also affinity are exploited [56]. Efficiency of the purification process that results in desired product homogeniecity typically depends on the complexity of the PEGylation mixture.

Historically, size-exclusion chromatography (SEC) has been widely used for separation of PEG conjugates as increase of molecular weight is one of the most evident changes caused by PEGylation. SEC is very efficient in removing low molecular weight impurities (by-products formed by hydrolysis of functionalized PEG and other low molecular weight reagents) as well as unreacted protein. SEC has several limitations, *i.e.*, the inability to separate positional isomers of the same molecular weight, poor resolution for PEG-protein conjugates, low throughput and high costs. Using only SEC, even unreacted PEG cannot always be efficiently removed. The removal depends on the molecular size difference between the PEG reagent and the protein. PEG is very well hydrated and exhibits larger hydrodynamic radius than proteins of the same molecular weight (Fig. 2).

In SEC, the prediction of separation efficiency can be made by calculating the hydrodynamic radius of the PEG using Eqs. (1) or (2) and PEG-protein conjugate using Eqs. (3) and (4). ($M_{r,PEG}$ molecular mass of PEG in Da; R_h - hydrodynamic radius).

$$R_{h,PEG} = 0.0201 M_{r,PEG}^{0.556} \quad (1)\ [57]$$

$$R_{h,PEG} = 0.0191 M_{r,PEG}^{0.559} \quad (2)\ [58]$$

Table 1. Overview of PEGylation technologies currently used

Attachment site	PEG reagent	Applicable for	Development status				Marketed drug (active substance)
			Research	Preclinic	Clinic	Market	
Random PEGylations							
Predominantly ε amino groups of lysine residues and N-terminal amino groups	PEG NHS-esters, PEG NHS-carbonates, PEG-p-nitrophenyl carbonates, PEG-triazine reagents, etc.	All proteins	√	√	√	√	Adagen® (Adenosine deaminase) Oncaspar® (Asparaginase) PegIntron® (Interferon-α2b) Pegasys® (Interferon-α2a) Somavert® (Human growth hormone mutein) Mircera® (Erythropoietin)
Site-specific PEGylations (chemical)							
N-terminal amino group	PEG-aldehydes and reducing agent	All proteins	√	√	√	√	Neulasta® (G-CSF)
-SH group of unpaired cysteine residues	PEG-maleimides Pyridyl disulfides Vinyl sulfones Thiol reagents	Proteins with natural or engineered surface Cys residues	√	√	√	√	Cimzia® (Anti-TNF-α Fab')
Non-natural amino acids (azido and ethynyl derivatives of serine)	PEG-ethynyl reagents PEG-azido reagents	Proteins with non-natural amino acids – (genetic manipulation of the host organism needed)	√	√	√		
Disulfide bond	PEG monosulfones	Proteins with surface -S-S- bonds	√	√	?		
Histidine affinity tag	PEG sulfones	Engineered His-tagged proteins	√				
Site-specific PEGylations (enzymatic)							
Serine or threonine	*N*-Actylgalactosamine and PEG-sialic acid derivatives (*O*-GalNAc-transferase and sialyltransferase mediated)	Non-glycosylated proteins with exposed Ser or Thr residues	√	√	√		
Glutamine residues	PEG-alkylamines (transglutaminase mediated)	Proteins with natural or engineered Gln residues in flexible regions	√				

NHS, N-hydroxy-succinimide ester

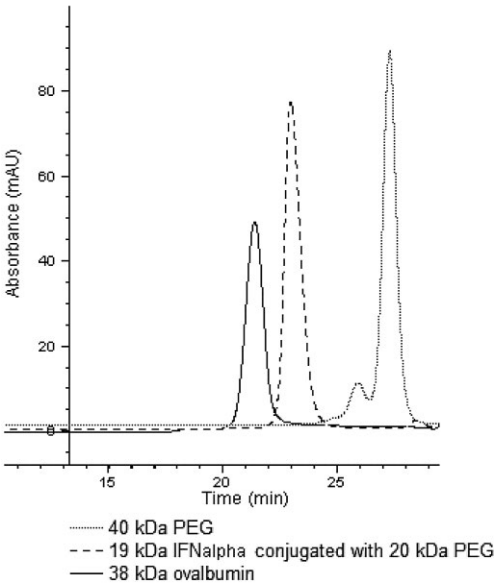

Figure 2. SEC analysis of various types of molecules having a similar molecular mass of approximately 40 kDa: 40-kDa branched PEG-CHO reagent (derivatized with *p*-aminobenzoic acid to enable UV detection), IFN-α of 19 kDa conjugated with 20-kDa PEG-CHO reagent and ovalbumin of 38 kDa.

$$R_{h,PEGprot} = \frac{A}{6} + \frac{2}{3A}R_{h,PEG}^2 + \frac{1}{3}R_{h,PEG} \quad (3)\ [58]$$

$$A = \left(\begin{array}{l} 108R_{h,prot}^3 + 8R_{h,PEG}^3 \\ +12\left(81R_{h,prot}^6 + 12R_{h,prot}^3 R_{h,PEG}^3\right)^{1/2} \end{array}\right)^{1/3} \quad (4)\ [58]$$

A difference in hydrodynamic radius larger than 1.26 enables efficient separation [56]. Applying only one SEC step in large-scale production would not result in a product with sufficient purity; therefore SEC is usually used in combination with hydrophobic interaction chromatography (HIC), or with cation-exchange chromatography (CEX) [56].

HIC can also be applied for purification and isolation of PEGylated proteins, although it is not widely used. The main reasons are poor resolution of PEGylated species and binding of unreacted PEG reagent to the HIC columns. The elution of PEGylation mixture components depends on the degree of protein modification. Unmodified protein is eluted first, followed by mono-PEGylated and higher PEGylated conjugates [56]. However, higher PEGylated species are usually not well resolved. The removal of unreacted PEG from the target PEG-protein conjugate is not always predictable and depends on the size difference and hydrophobicity of the protein.

Generally, the method of choice for isolation of PEGylated proteins is CEX. CEX enables a single-step purification of the target PEG-protein conjugate from un-PEGylated protein, higher PEGylated molecules and unreacted PEG. Due to charge differences, CEX also possesses the ability to separate positional isomers of the same molecular weight. The elution order of PEG-protein conjugates is determined by the PEG to protein mass ratio [59, 60]. Higher-PEGylated molecules elute first, followed by mono-PEGylated and unreacted protein. Figure 3 shows efficient CEX separation of higher-, mono-PEGylated and un-PEGylated protein. Additionally, retention times on CEX also depend on the molecular weight of PEG attached to the protein. This is also illustrated in Fig. 3, where separations of PEGylation mixtures prepared with PEGs of different sizes have been performed under the same conditions. It is seen that conjugates with PEGs of higher Mw exhibit lower retention times. The same elution order as in CEX is also obtained with anion-exchange chromatography (AEX). PEGylated proteins posses a lower average surface charge, either positive or negative, due to PEG shielding of the protein surface. Reduced interactions between the PEG-protein and the chromatographic resin cause elution of PEG-protein conjugates before un-PEGylated protein in CEX as well as in AEX. The PEG shielding effect is so pronounced that CEX separation can also be applied in the case where there is no charge difference between PEGylated and un-PEGylated protein [56, 57, 60–62].

PEGylation reactions are usually performed with an excess of PEG reagent and at high protein concentrations. All these factors combined with large hydrodynamic radius of PEGs make PEGylation mixtures very viscous. High viscosity and the tendency of PEG to absorb nonspecifically onto the surfaces are two major reasons that cause problems during CEX purification. High back-pressure and column fouling can be avoided by dilution of PEGylation mixtures before loading onto the CEX column. Unreacted PEG reagent does not bind to the CEX resin and elutes in the flow through. The presence of PEG reagent in the load reduces CEX resolution; therefore, it is recommended to remove unreacted PEG as soon as possible in the purification process [56]. To achieve efficient removal of PEG reagent and efficient separation two consecutive ion exchange separations can be performed, the first for removal of unreacted PEG and the second for fractionation of PEG-protein conjugates [63, 64]. In the first separation step, resins with high porosity and larger particles can be used allowing higher flow rates and higher viscosity of the loaded

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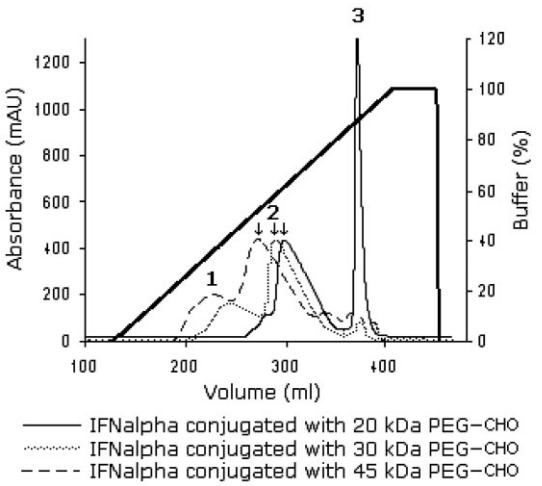

Figure 3. Comparison of preparative CEX separations of IFN-α pegylation mixtures prepared with various mPEG-CHO reagents of different lengths and shapes (20 kDa linear, 30 kDa linear and 45 kDa branched) on TSK-gel SP-5PW column (Tosoh Bioscience, Japan). Peak 1, higher-PEGylated IFN-α forms; 2, mono-PEGylated IFN-α forms; 3, un-PEGylated IFN-α.

sample, while for the second step resins with good resolution are recommended.

Increased hydrodynamic radius and masking of the protein surface by PEG are two characteristics that tend to denote CEX purification. PEGylated proteins are associated with lower equilibrium and dynamic binding capacities (DBCs). Equilibrium binding capacity is reduced as the consequence of weaker interactions between the protein and the resin, caused by PEG shielding, while DBC is reduced due to slower mass transfer and hindered diffusion effect associated with molecules of large hydrodynamic radius. Compared to un-PEGylated proteins, the DBC for PEGylated proteins is on average ten times lower [65]. Some producers offer resins specially designed for separation of PEGylated proteins (http://www6.gelifesciences.com/aptrix/upp00919.nsf/Content/5E17BDCA59B77FC3C125718600812910/$file/28409465AA.pdf).

For a proper choice of the ion exchange resin, special attention has to be paid to the appropriate particle pore size enabling penetration of the PEGylated proteins. In one interesting study, a couple of industrial scale AEX resins were compared. Some resins maintained some DBC after PEGylation, while for few a complete loss of DBC was observed. The loss of DBC after PEGylation was explained by PEG-protein inability to penetrate into the particle pores [65].

An important non-chromatographic step in the production of pharmaceutical proteins is also the ultrafiltration/diafiltration (UF/DF) unit operation. The UF/DF process step enables buffer exchange in between the chromatographic steps or into the final drug substance buffer, allowing concentration of the drug substance to the desired final concentration. Since PEGylated proteins are frequently administrated at relatively high concentrations, usually around 10 mg/mL, UF/DF is a well-suited final processing step. As the PEG attachment significantly increases the hydrodynamic radius, it would seem possible that membranes with larger cut-offs could be employed. Surprisingly, membranes with cut-offs similar to those used for un-PEGylated protein should be employed after PEGylation with linear PEG reagents to avoid the so-called "snake effect" of PEGs, whereby PEGylated product can escape through larger pore membranes causing substantial losses [58, 66, 67]. In the case of branched PEG, and for multi-PEGylated conjugates membranes with larger cut-offs can be successfully used. However, it should be noted that the difference in sieving coefficients is not proportional to the difference in molecular weight of conjugated and unconjugated protein.

6 Analytics of PEG reagents and PEGylated proteins

The development of an efficient PEGylation process and safe PEGylated therapeutic require analytical methods for PEG-protein conjugates and PEG reagent at various stages. PEG reagents represent a crucial raw material, therefore the ability to characterize them fully is essential for successful development of PEGylated therapeutics. The quality of PEG reagents may vary substantially with regards to the molecular mass, polydispersity index, presence of activated and non-activated impurities and degree of activation.

Terminal activity or degree of activation, with typical values of 70–90%, is a very important characteristic of PEG reagents, and directly influences the efficiency of the PEGylation reaction process; depending on its value a different PEG excess may be needed for the same conversion yield. As PEG reagents contribute a substantial part to the manufacturing costs of PEGylated proteins, a high degree of reagent activation, as well as the ability to control the activation efficiency, play a very important role in the production process of PEGylated proteins. NMR is frequently used for a qualitative and quantitative determination of the functional groups, and is used by all manufacturers as the release method for terminal activity determination for activated PEGs. Alternatively, HPLC methods combined with derivatization of the terminal group can be employed effectively. PEG itself, and a ma-

jority of PEG reagents, are UV transparent and nonfluorescent, therefore a derivatization method is needed to produce UV absorbance. For example, methoxy-PEG aldehyde can be derivatized with 4-aminobenzoic acid and analyzed using reversed phase (RP)-HPLC [60]. This alternative method is very powerful for detecting activated impurities in the PEG reagent, which should be kept at a very low level to avoid formation of undesired by-products.

Without derivatization, PEG reagents can be detected by evaporative light scattering or corona discharge charged aerosol detectors that detect particulate matter in the gas phase [68]. Reversed-phase chromatography (RPC) and SEC in combination with corona detection mode can be employed for determining the difference in molecular weight of PEG chains and thus enable detection of impurities in final PEG reagent; however, it does not have a power to distinguish between activated and non-activated PEG species.

Another characteristic of PEG that has to be well controlled is molecular weight as it determines the final half-life of the PEGylated protein and directly influences the bioavailability of the PEGylated therapeutics. The average molecular weight of the PEG reagent is usually established by SEC. The same method is used for polydispersity determination and determination of the main peak fraction in PEGs. However, a more precise determination of the molecular weight of PEGs can be obtained using the MALDI-TOF technique. This technique is more complicated and expensive, but, although not in routine use yet [69], it will most probably soon become a standard technique in the production of modern biopharmaceuticals.

In the production process of PEGylated therapeutics, full characterization of the PEG-protein conjugates represents a very challenging task. It starts with analysis of PEGylation reaction mixtures, analysis of individual fractions during purification and complete characterization of the final product. The characterization of PEGylated proteins is influenced by the fact that the PEG molecule attached to the protein changes the characteristics of the protein substantially. As previously mentioned, the most evident changes caused by PEGylation are a larger moleculer size and a larger hydrodynamic volume. The molecular weight of proteins and PEG-protein conjugates can be determined by several methods, such as SEC, electrophoretic methods, light scattering and mass spectrometry. Most comprehensive studies on PEG-protein conjugate sizes are based on SEC data [58, 70]. SEC is a simple and the low-cost method of choice enabling molecular weight determination of proteins and polymers on the basis of calibration curves. For analytical purposes addition of organic modifiers into the mobile phase can improve separation of PEG-protein conjugates and reduce peak broadening caused by the polydispersity of PEG as well as peak tailing caused as consequence of non-specific adhesion of PEGs to the stationary phase [63, 71, 72].

Comparison of behavior of PEG reagents, PEG-protein conjugates and proteins of approximately the same nominal molecular weight on SEC and SDS-PAGE show distinctly different retention times (Fig. 2) and mobility in the gel; 40-kDa PEG reagent behaves as the biggest molecule and protein without conjugation as the smallest. For PEGs and PEG-protein conjugates there is no correlation with protein molecular weight standards [57]. PEG standards seem to be more adequate for a rough estimation of the apparent molecular weight of PEG-protein conjugates than protein standards [70, 73].

Dynamic light scattering (DLS) can also be applied for molecular size evaluation of the conjugates. In contrast to SEC, it seems to be able to distinguish between protein conjugates with branched and linear PEGs, PEG-protein conjugates with branched PEGs appearing smaller than conjugates with linear PEGs of the same nominal molecular weight [57, 61]. None of the methods mentioned above is able to resolve and detect PEG positional isomers; however, CEX is efficient in their separation. By employing CEX it was demonstrated that the marketed product PegIntron®, randomly conjugated with a 12-kDa linear PEG, contains 15 different PEG positional isoforms [74], while Pegasys®, randomly conjugated with a 40-kDa branched PEG, contains 4 main isoforms [27]. CEX is also capable of separating different molecular weights in a series of well-defined N-terminally mono-PEGylated proteins conjugated with PEGs of different length. The PEG size affects the retention time, which is another indication for the strong shielding and/or steric effect of PEG, which results in weakening of the interaction between the protein and the chromatographic matrix [57].

Theoretically, efficient separation of positional isomers could be expected using RPC, as the method exploits the differences in hydrophobicity. However, positional isomers are not resolved in practice, and the PEG-to-protein ratio appears to be the predominant factor that determines the resolution [60]. Based on the fact that PEG is usually regarded as a hydrophilic molecule, PEGylated proteins should exhibit shorter retention times on RPC than their un-PEGylated counterparts; however, the opposite is observed. PEGylated proteins

show distinctly longer retention times on RPC columns, which increase with increasing PEG length exhibiting to some extent the hydrophobic nature of PEG. This is also the reason why PEGs themselves are retained and can be separated on RP matrices. RPC is an excellent and robust method for determination of purity and content of PEGylated proteins, including the amount of higher-PEGylated and un-PEGylated species, protein oxidation, deamidation and cleavage of the protein backbone [72] as well as for RP-HPLC peptide mapping [44, 63].

Various modes of mass spectroscopy (MS) nowadays represent valuable and generally applied tools for protein characterization [75]. However, the high intensity of PEG-related signals hinders the detection of PEGylated peptides and their fragmentation analysis, which can lead to ambiguous identification of PEGylated proteins because the signals and their intensities in MS spectra depend on the intrinsic properties of the peptide. Nevertheless, peptide mapping and MS are used for identification and quantification of PEGylation sites by comparing PEGylated and un-PEGylated counterparts [61, 76], and for characterization of impurities that are sometimes not resolved and detected by simpler techniques. However, in the case of monodisperse PEG reagents, a direct identification of the PEGylation site(s) is possible using electrospray ionization (ESI) MS [77].

7 Determination of PK profiles of PEG-protein conjugates

The modulation of protein PK characteristics focused on elimination half-life extension is the main driver for protein modification, including PEGylation. A first screen of PK properties is usually performed in rodents, most frequently rats, which are large enough to allow time course sampling required for PK profile determination. To estimate the concentration during time course of the PEG-protein conjugate in the blood sera with satisfactory sensitivity, specificity, reproducibility and accuracy, an universal analytical method for detecting the conjugate in complex biological samples is necessary (http://www.emea.europa.eu/pdfs/human/ewp/8924904enfin.pdf).

Immunoassays, bioassays and radioassays are frequently used [78], while a more universal anti-PEG ELISA test has been developed and offered recently by Epitomics (USA) (http://www.epitomics.com/Kits/ELISA_PEG.php). Although it seems to be a universal and very elegant solution, its applicability in practice is limited due to the relatively high detection limit. Its sensitivity is usually satisfactory for multi-PEGylated conjugates, but it is often not sensitive enough for determination of lower amounts of mono-PEGylated conjugates in blood sera (our unpublished results). Most of the marketed PEGylated therapeutics are mono-PEGylated, and it is not expected that this will change in the future.

Hence the method of choice is still a protein-specific ELISA, which are usually available commercially, with antibodies directed to the protein that is conjugated. The main drawbacks of such ELISAs are their ability to recognize only the protein part, and therefore, lower sensitivity for the PEGylated protein compared to the un-PEGylated counterpart. Lower sensitivity is a consequence of the shielding effect of PEGs that frequently masks amino acid sites important for receptor binding, resulting in weaker interaction with receptor and with the target antibodies. Generally, larger PEGs and multi-PEGs attached to protein reduce the affinity for the antibody, resulting in the reduced steepness of the dose-response curves. To avoid inaccuracy of the determined concentration of PEG-protein conjugates, it is important to use the same purified PEG-conjugate for the standard curve. The final concentrations of PEG-conjugates in blood sera should thus be calculated using the standard curve derived from purified PEG-conjugate, and should not be compared to the un-PEGylated protein.

However, in the production of PEGylated therapeutics highly sensitive and specific ELISA methods using anti-PEG capture antibodies and detection antibodies for the respective protein part of the conjugate are now routinely used in PK studies, and in the determination of drug concentrations in body fluids and in tissue extracts ([71], and unpublished results). Most of these methods are proprietary and not available in the public domain.

8 Conclusions

In this review numerous aspects of PEGylation technologies have been covered. These include PEG reagents, their development and novel trends, as well as their analysis, PEGylation reactions, large-scale purifications, and the analysis of the PEG-protein conjugates. PEGylation is a mature and tested technology, which has already resulted in nine FDA-approved therapeutics, testifying to the safety and applicability of the methodology (Table 2). Since its introduction, PEGylation has been focused mostly on existing therapeutic proteins and their life-cycle management. However,

Table 2. Marketed PEGylated biopharmaceuticals

Name	Company	Original protein	Therapeutic indication	Engineering rationale	Year to market
Adagen (Pegadamase)	Enzon	Bovine Adenosine Deamidase	Severe combined immunodeficiency (SCID)	Increased serum half-life	1990
Oncaspar® (Pegaspargase)	Enzon	Asparaginase	Acute lymphoblastic leukemia	Increased serum half-life, less allergic reactions	1994
PEG-Intron® (PEGylated IFN-α2b)	Schering-Plough / Enzon	IFN-α2b	Hepatitis C	Increased serum half-life	2001
Pegasys® (PEGylated IFN-α2a)	Hoffmann-La Roche	IFN-α2a	Hepatitis C	Increased serum half-life	2002
Neulasta® (pegfilgrastim)	Amgen / Nektar	G-CSF	Neutropenia	Increased serum half-life	2002
Somavert® (Pegvisomant)	Pfizer / Nektar	hGH mutein	Acromegaly	hGH–receptor antagonist	2003
MIRCERA® PEGylated epoetin-β	Hoffmann-La Roche	Epoetin-β	Anemia associated with chronic renal failure	Increased serum half-life	2007
Certolizumab pegol (Cimzia)	UCB	Anti TNF Fab	Rheumatoid arthritis and Crohn's disease	Increased serum half-life	2008
Macugen® or Macuverse® (pegaptanib)	Pfizer	Anti-VEGF aptamer (an RNA oligo-nucleotide)	Treatment of ocular vascular disease		2004

with the development of protein nanobodies [79] and scaffolds [12], which are believed to represent the next generation therapeutics but require half-life extension to exert a clinically meaningful effect, even wider medical use can be expected. A very complex intellectual property (IP) situation exists covering site-specific PEGylation and branched PEG reagents, hindering the wider use of modern PEGylation technologies for new products. Expiry of these patents will give modern PEGylation technology freedom to operate in the near future and may result in the expansion of its use in the production of new therapeutics.

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