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PEG-modified biopharmaceuticals

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PEGylation is a process in which one or more units of chemically activated polyethylene glycol reacts with a biomolecule, usually a protein, peptide, small molecule or oligonucleotide, creating a putative new molecular entity possessing physicochemical and physiological characteristics that are distinct from its predecessor molecules. In recent years, PEGylation has been used not only as a drug delivery technology but used also as a drug modification technology to transform existing biopharmaceuticals clinically more efficacious than before their PEGylation. PEGylation bestows several useful properties upon the native molecule, resulting in improved pharmacokinetic and pharmacodynamic properties, which in turn enable the native molecule to achieve maximum clinical potency. In addition, PEGylation results in sustained clinical response with minimal dose and less frequency of dosing, leading to improved quality of life via increased patient compliance and reduced cost. During the course of development of various pegylated protein therapeutics, several new insights have been gained. This review article focuses on the approaches, strategies and the utilization of modern PEGylation concepts in the design and development of well-characterized pegylated protein therapeutics.

Keywords: biopharmaceuticals, biosimilar, EPR, NME, PEG, PEG-biosimilar, PEG-linkers, PEGylation, pharmacokinetics renal clearance, poly(ethylene glycol), putative, tumor-targeting

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1. Introduction

Nearly three decades ago, PEGylation was a fledgling technology seeking universal acceptance as a legitimate drug delivery technology for protein therapeutics. Today, PEGylation is a well-established technology used extensively to transform proteins, peptides, small molecules and oligonucleotides into more potent drugs than their corresponding unmodified native molecules [1-6]. Pegylated biomolecules distinguish themselves from their predecessor molecules by possessing more useful clinical properties, such as increased stability and solubility, reduced renal clearance, longer circulating half-life, reduced immunogenicity and antigenicity, as well as protection from proteolytic degradation; all of which contribute to the increased potency compared to the unmodified native molecule. The pioneering PEGylation research conducted by Davies and Abuchowsky [7,8] resulted in the Food and Drug Administration (FDA) approval of Adagen® (Enzon Pharmaceuticals, USA), a pegylated form of adenosine deaminase for the treatment of severe combined immunodeficiency (SCID), closely followed by the approval of Oncaspar® (Enzon Pharmaceuticals, USA), a pegylated form of L-asparaginase for the treatment of pediatric acute lymphoblastic leukemia (ALL). These two pegylated enzymes were the first FDA-approved agents for enzyme replacement therapy. The other major pegylated therapeutics approved by FDA are Pegintron® (Schering-Plough, USA), a pegylated form of interferon α-2b, and Pegasys® (Hoffman-La Roche, Inc., USA), a pegylated form of interferon α-2a, both for the treatment of hepatitis C; Neulasta® (Amgen, USA), a pegylated form of G-CSF for the treatment of chemotherapy induced neutropenia; Pegvisomant® (Pfizer Pharmaceuticals, USA), a pegylated growth hormone antagonist for the treatment of acromegaly or hyperplasia

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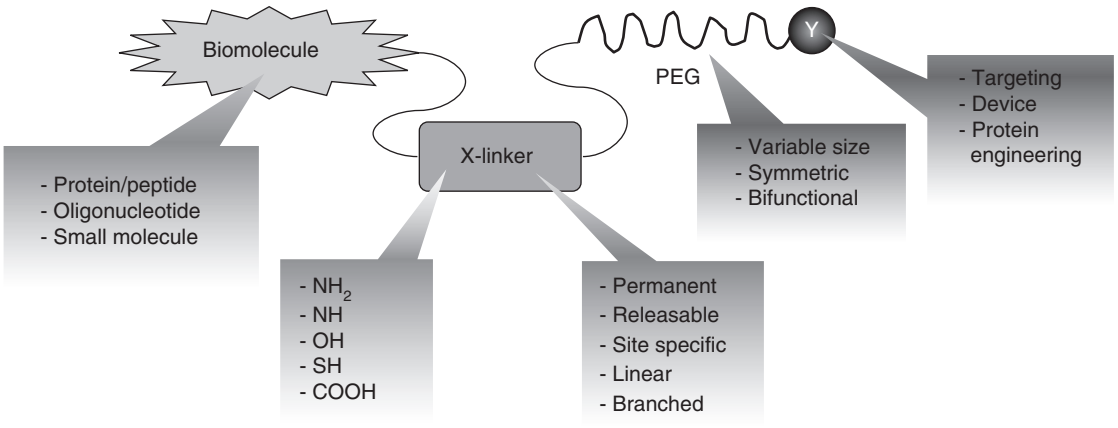

Figure 1. Versatility of PEG modification.

of the extremities; and Macugen® (OSI Pharmaceuticals, USA), a pegylated form of anti-VEGF aptamer for the treatment of wet AMD or age-related macular degeneration. Mircera® (Hoffman-La Roche, Inc., USA), or continuous erythropoiesis receptor activator, is the next major pegylated protein (epoietin-β) approved by FDA in 2007 for the treatment of anemia associated with chronic renal failure (CRF) in adults, including patients on dialysis and not on dialysis. Pegylated antibodies, antibody fragments and single chain antibodies are also in various stages of clinical development. Examples of pegylated antibody fragments are Cimzia® (UCB Pharma, Belgium), an anti-TNF-α Fab′ approved in 2008 for the treatment of Crohn's disease and a pegylated di-Fab′ anti-PDGF (CDP860), which is in the advanced stages of clinical trials for the treatment of coronary restenosis [9].

2. What is PEGylation?

PEGylation is a process by which one or more molecules of activated polyethylene glycols chemically reacts with a biomolecule, usually a protein, a peptide, a small molecule or an oligonucleotide, resulting in the production of a putative new molecular entity (NME) with improved pharmacokinetic and pharmacodynamic properties, thus enabling it to attain maximum clinical potency. The NME concept has gained credence due to the fact that all approved PEG conjugates have undergone FDA-mandated clinical trials, even though their corresponding predecessor molecules like IFN, G-CSF and EPO have been previously clinically tested and FDA approved. In addition, the US Patent and Trademark Office often issues patent to PEG conjugates, provided that NME criteria (e.g., new and surprising properties) are met.

PEGylation is a versatile drug modification and drug delivery technology, whose main features are illustrated in **Figure 1**.

The available functional group X in the biomolecule is used to link the polyethylene glycol (PEG) moiety via a linker. For proteins and peptides the most commonly used functional group for PEGylation is the primary amino group -NH_2, which could be from the N-terminus or from the lysine residues. Another functional group commonly used for PEGylation of proteins is sulfhydryl (-SH), especially used for site-specific PEGylation of antibodies and antibody fragments. Oligonucleotides contain a secondary amine (-NH-) for PEGylation, whereas small molecules may contain functional groups such as carboxylic acid (-COOH) or hydroxyl (-OH) or sulfhydryl (-SH) or amine (-NH_2) suitable for PEGylation.

The PEG linker could be permanent or releasable, randomly or site-specifically linked, linear or branched. The PEG could be of variable size or shape, or could be mono-functional, bi-functional or heterobifunctional or polyfunctional. The PEG may contain a biomolecule like a cell-killing toxin on one end and a targeting moiety like an antibody on the other end. A bioengineered fusion protein exhibiting both targeting as well as cell-killing functionalities (e.g., scFv-toxin or single chain Fv-toxin) could be used instead.

3. Historical perspective

Since its conceptualization in the 1970s by Davies and Abuchowsky [7,8], it took over 30 years for PEGylation to grow into a fully fledged technology for the modification of efficacious protein therapeutics. The events that led to the evolution of PEGylation into a critically important drug modification and delivery technology are summarized in **Table 1**.

During the last three decades, PEGylation has grown from a single linking chemistry (SS-PEG or succinimidyl succinate-PEG) to numerous innovative linking chemistries providing permanent linkages: i) SC-PEG or succinimidyl carbonate-PEG; ii) NHS-PEG or *N*-hydroxy succinimide-PEG; iii) T-PEG or 2-mercaptothiozoline-PEG; iv) UPEG or branched PEG; and v) ALD-PEG or aldehyde-PEG, as well as releasable or hydrolysable linkages, for example r-PEG, aromatic, aliphatic, or disulfide (see the PEGylation chemistry

Table 1. The 30-year history of PEGylation technology – main linkers, attributes, limitations, indications and utilities.

	1970s	1980s	1990s	2000s
Main linkers*	SS	SC	NHS, TPEG, UPEG	rPEG
Attributes	Inert, reduced immunogenicity, long half-life	Inert, reduced immunogenicity, long half-life, convenience	Inert, long half-life, convenience, increased efficacy, site selectivity, patent protection	Inert, tailored half-life, convenience, prodrugs, branching, increased efficacy, site specificity, EPR effect, targeting, patent protection
Limitations	PEG reagent hydrolysis, reactivity of precursor, lack of site selectivity, parenteral	PEG reagent hydrolysis, reactivity of precursor, lack of site selectivity, parenteral	Lack of site selectivity, full NDA/BLA, cost of goods, parenteral	Full NDA/BLA, cost of goods, parenteral
Indications	Enzyme replacement	Enzyme replacement	Anti-cancer Anti-infective	Full spectrum
Utility	Research	Orphan drugs	'Blockbuster' drugs	'Blockbuster' drugs

*See text for description of linking chemistries.

section, below, for details). PEGylation could take place randomly or site-specifically. Many of the aforementioned PEG reagents are commercially available from companies like NOF Corporation, Japan, SunBio Company, Korea, JenKem, China, and Laysan Bio, USA, among others. The specialized PEG reagents like rPEG are proprietary to the inventors and may not be available commercially.

The historical development and use of the major PEG reagents in each of the last four decades, starting with SS-PEG (1970s), SC-PEG (1980s), NHS-PEG, T-PEG and UPEG (1990s) and rPEG (2000s) are listed in **Table 1**. Also listed in the Table are the attributes (e.g., improved pK/pD, selectivity, EPR effect and patent protection), along with the limitations (e.g., hydrolytic instability of PEG reagent or PEG conjugate, lack of site-specificity, need for parenteral administration, stringent regulatory requirements and the like). Pegylated protein therapeutics now covers the full spectrum of disease areas (see the introduction, above). Since 1990, PEGylation or PEG modification has produced orphan drugs to blockbuster drugs.

4. Modern PEGylation insights

During the course of development of various pegylated biomolecules (e.g., G-CSF, Interferon α, insulin, epoietin β and leptin, among others), several new insights have been gained on PEGylation. Awareness and utilization of these modern PEGylation concepts are essential in the design and development of potent, long-acting forms of protein therapeutics, especially for the class of proteins including cytokines, hormones and growth factors. One common property of these proteins is that their biological activity is dependent upon receptor interactions. The major insights on PEGylation are described in the following paragraphs.

4.1 Renal clearance versus PEG mass

It is now well established that a minimum of 20 kDa PEG is needed for the significant retardation of renal clearance via glomerular filtration [10]. This is illustrated in **Figure 2**. The renal clearance of PEG is compared with that of inulin, which is a naturally occurring polysaccharide clinically used as a reference to accurately measure renal clearance of flexible polymers and other substances. A 0.15 M NaCl solution containing 1% inulin or 1% PEGs (nominal molecular weights ranging from 1000 – 10,000 Da) were administered to male rats (300 – 350 g) by infusion. Sixty minutes after infusion urine samples were collected, subjected to gel permeation chromatography in Sephadex G-200 and column fractions were analyzed by spectrometry for inulin and turbidimetry for PEG, and the respective renal clearances were determined. The relative clearance is the ratio (%) of renal clearance of PEG to that of inulin.

It could be extrapolated that a total PEG mass of 20 kDa is the lower limit to obtain the clinically useful circulation half-life. Additional factors that may influence renal clearance are the added mass due to the molecular weight of the protein and protein/protein interactions (e.g., receptor/ligand interactions).

4.2 Renal clearance and hepatic uptake

Using interferon modified with various sizes of polyethylene glycol (PEG), it is shown that as the renal clearance decreases due to increased PEG mass, the hepatic uptake of the pegylated interferon increases [11-14]. A similar pattern is also seen with PEG by itself [15].

4.3 *In vitro* activity versus *in vivo* biological activity

The next major discovery is that the *in vitro* activity determined by cell-based bioassays is not predictive of

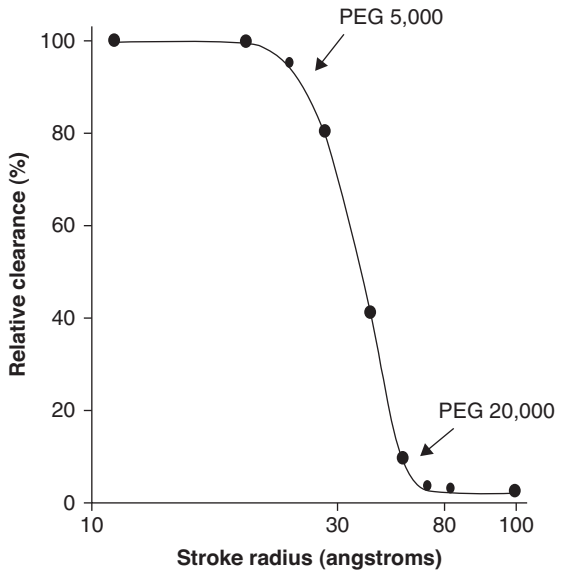

Figure 2. Renal clearance as a function of PEG Stokes radius. Relative renal clearance rates of various MW. PEGs are expressed as a ratio (%) in relation to the clearance rate of inulin, the glomerular filtration reference standard.

the *in vivo* therapeutic effect. As shown in the following paragraphs, this phenomenon is observed with several pegylated cytokines, hormones and growth factors, whose pharmacological effect is dependent upon complex receptor-ligand interactions.

In **Figure 3A**, the antiviral activities of unmodified and PEG modified interferon α-2a are expressed as a function of branched PEG mass. As the attached PEG mass has increased, there is a corresponding loss in the *in vitro* antiviral activity. The *in vitro* antiviral activities are determined in a microtiter bioassay based on reduction of cytopathic effect employing Marvin–Darby bovine kidney (MDBK) cells challenged with vesicular stomatitis virus (VSV) [16]. The antiviral activity corresponding to the 40 kDa sample is that of Pegasys, which exhibited only 7% of the original antiviral activity of interferon α-2a [17] as described.

It is remarkable that PEG-IFN used in clinical trials had only 5 – 10% of the original *in vitro* antiviral activity, yet it was significantly more effective in treating hepatitis C infection in humans than the standard interferon treatment (180 µg monotherapy for PEG-IFN and 3 – 6 MIU thrice-weekly for interferon [18,19].

In **Figure 3B**, the *in vitro* and *in vivo* activities of PEG-GCSF are expressed as a function of PEG mass [4]. The *in vitro* activity is expressed as the ^{3}H-thymidine uptake by IL-3 dependent murine myelogenous G-NFS-60 cells incubated with various preparations of PEG-GCSF [20]. The *in vivo* activity is determined as the increased absolute neutrophil counts (ANC) in female C57BL/6 mice (6 – 8 weeks old) after subcutaneous (s.c.) injection of various MW types of PEG-GCSF [20]. While there is an inverse relationship between PEG mass and *in vitro* activity, a direct relationship between *in vivo* activity and PEG mass is observed with a practical threshold of around 70 kDa. Similar observations are observed with other pegylated molecules (e.g., interferon-α, OB protein or leptin and epoietin-β [21,22].

4.4 Site of PEGylation versus biological activity

Another myth about PEGylation of biomolecules, which requires receptor interactions for their biological activity, is that PEGylation at or near the binding domain results in loss of biological activity. This is not really the case, as demonstrated by examples cited in **Table 2**. However, PEGylation may affect the retention of biological activity when it takes place at the level of protein binding region rather than at the level of protein binding sequence. The *in vivo* biological activities shown in **Table 2** are determined based upon various combinations of indicators like drug-induced biomarkers for IFN (e.g., 2′-5′ OAS), absolute neutrophil counts (ANC) for G-CSF, reticulocytes production for erythropoietin (EPO), and determination of the lowering effect of blood glucose concentration for insulin. PEGylation at the heart of the receptor-binding domain of interferon α-2a does not result in loss of its biological activity. A similar phenomenon is observed with a consensus PEG-GCSF, in which PEGylation takes place near Glu^{19} of G-CSF, whose interaction with Arg^{288} of G-CSF receptor is essential for its biological activity [23]. Erythropoietin is a biomolecule, whose receptor binding involves sugars [24,25]. Yet PEGylating specifically at the sugars of erythropoietin does not result in loss of biological activity [21]. Caliceti and Veronese [26] had shown that PEGylating at the biologically important Gly^{1} of insulin once again does not affect its biological function.

An explanation for this phenomenon is that the major effect of PEGylation is steric hindrance and not conformational changes. Steric hindrance does not completely shut down on/off receptor–ligand interactions. Though steric hindrance lowers the binding affinity to the receptor, due to the increased circulating half-life, there are plenty of opportunities for the receptor–ligand interactions to occur, thus producing the signal transduction events that lead to the pharmacological effect. It is also conceivable that the flexibility of PEG may play a role here.

4.5 Overlapping activities

A protein conjugate mixture (e.g., PEG-GCSF) comprised of zero, mono-, di-, tri-, etc PEG conjugates exhibits various individual absorption and clearance rates. The higher the degree of PEGylation, the slower the rates of absorption and the rates of clearance. The unPEGylated biomolecule is absorbed and cleared first, followed by mono-, di-, etc, while each component leaves behind some residual activity

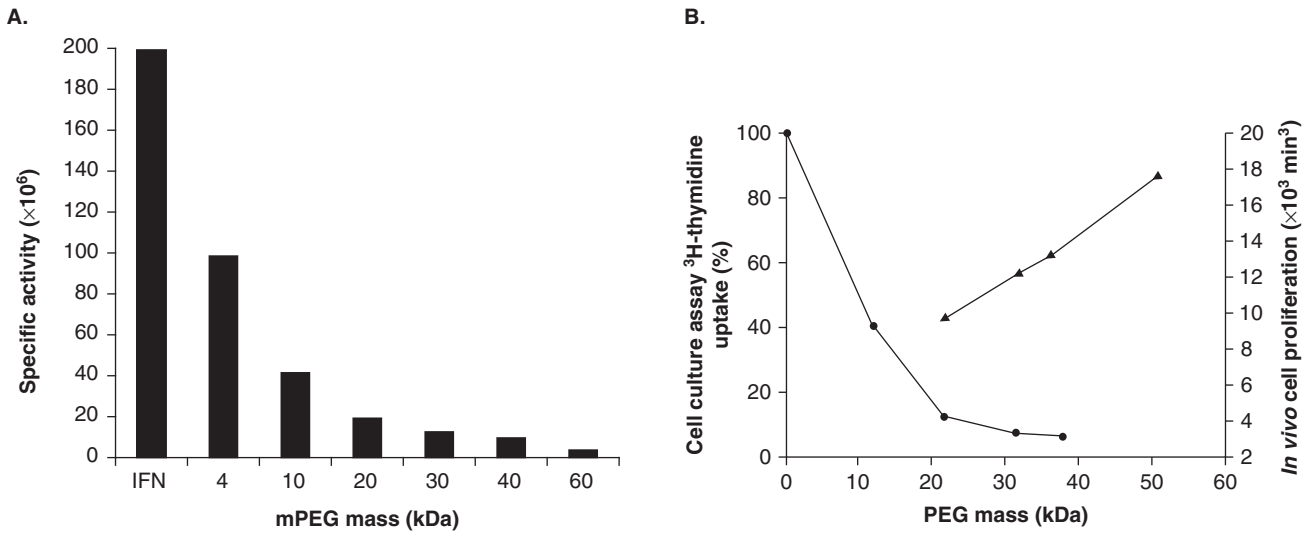

Figure 3. *In vitro* versus *in vivo* activities of PEG-interferon and PEG-GCSF. A. *In vitro* antiviral activities of interferon and branched PEG-interferons are determined in a microtiter cell-culture bioassay based on the 50% reduction of cytopathic effect (end-point) employing Marvin–Darby bovine kidney (MDBK) cells challenged with vesicular stomatitis virus (VSV) [16]. A laboratory standard 640 U/ml interferon is used to determine the absolute titer. **B.** PEG-GCSF, as a function of PEG mass. *In vitro* activity is expressed as ^{3}H-thymidine uptake by G-NFS-60 cells after treatment with PEG-GCSF, whereas the *in vivo* activity is expressed as cell proliferation (increase in neutrophil counts) after the s.c. injection of PEG-GCSF in female C57BL/6J mice. See text and Ref. 20 for details.

Table 2. Effect of PEGylation site on biological activity of PEG conjugates.

PEG protein	Binding domain	PEG site	Biological activity*
IFN	Cys^{29}-Asp^{35} Phe^{123}-Trp^{140}	Lys^{31} $Lys^{121,131,134}$	+
G-CSF	Glu^{19}	$Lys^{16,23,34,40}$	+
Epoietin-β	Sugars	Sugars	+
Insulin*	Gly^{1}	Gly^{1}	+

Bailon, Ehrlich. Innovative Drug Delivery Systems Symposium; 2002.
Caliceti, Veronese. Proc Intl Symp Control Rel Bioact; 2000.
*See the text for the type of biological activities determined.

that results in overlapping activities (see **Figure 4**) [4]. However, in order to clinically exploit the overlapping bioactivities of a PEG conjugate mixture containing various pegylated components, it is imperative that the manufacturing process has to be consistent and reproducible.

5. Other PEGylation insights

5.1 Binding affinity of biomolecules

Biomolecules like cytokines, hormones and growth factors may possess greater binding affinities than needed for cellular activity [27]. Using site-directed mutagenesis, Pearce *et al.* [27] reduced the receptor binding affinity of human growth hormone up to 500-fold. Their results indicate that only when binding affinity was reduced 30-fold (~ 3% of original binding affinity); there is any effect on cellular (biological) activity. These results suggest that one should not be overly concerned when a pegylated cytokine exhibits significantly reduced binding affinity from *in vitro* measurements (e.g., Biacore).

5.2 pK/pD modeling

pK/pD modeling is an important tool in the design of optimally pegylated biomolecules. A mathematical model that predicts efficacy based upon pK/pD analyses can be elucidated [11,28]. The model can predict efficacy based upon the trough levels of drug induced surrogate biomarkers (e.g., 2′-5′ OAS induction in PEG-IFN-α. For example, in the case of PEG-IFN, the model predicts that based upon the trough levels of 2′-5′ OAS induction, an eightfold increase over the half-life of interferon α-2a is necessary for achieving the target profile of once-a-week dosing [11].

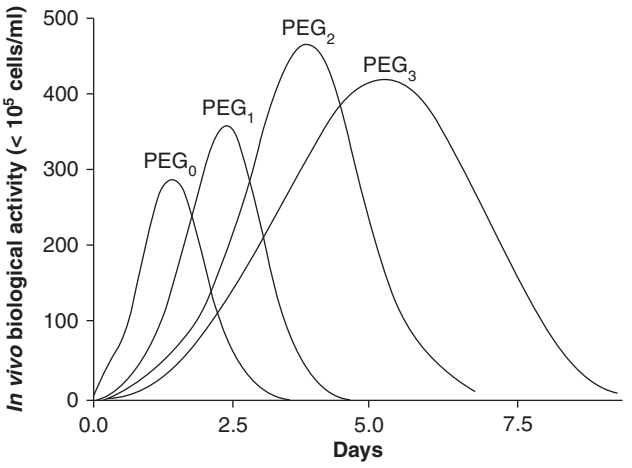

Figure 4. Simulation of overlapping bioactivities of individual components of a typical PEG-GCSF conjugate mixture. Components of the mixture are: (**1**) free G-CSF (PEG_0); (**2**) monopegylated G-CSF (PEG_1); (**3**) dipegylated G-CSF (PEG_2); and (**4**) tripegylated G-CSF (PEG_3). The simulated overlapping curves are constructed based upon the previously determined duration of individual *in vivo* activities (absolute neutrophil counts or ANC) in mice.

5.3 Altered biological properties

In rare instances, PEGylation may result in altered biological properties. After PEGylation, GM-CSF exhibits differentiation of neutrophil priming activity and colony stimulating activity [29]. Pegylated cholesterol oxidase and cholesterol esterase exchanges specificity from total cholesterol to high-density lipoprotein cholesterol (HDL cholesterol) [30]. This phenomenon has been taken advantage of in developing an assay kit for HDL cholesterol. Upon PEGylation, IL-15 becomes an antagonist and competes for receptor binding [31]. Self-regulating pharmacokinetics exhibited by Pegfilgrastim is yet another such example of altered biological properties after PEGylation [32].

5.4 Self-regulating pharmacokinetics

In chemotherapy-induced neutropenia patients, Pegfilgrastim undergoes a very slow clearance from the body. Pegfilgrastim clearance accelerates with neutrophil recovery. It undergoes neutrophil-mediated clearance involving internalization via G-CSF receptors present on the neutrophils. This is a unique example where a cytokine is removed from the body by its own biological effect; a novel drug clearance mechanism. This phenomenon is called 'self-regulation' or 'self-regulating pharmacokinetics' [33].

5.5 Steric hindrance, not conformational change

High Resolution Nuclear Magnetic Resonance (NMR) studies have shown that the predominant effect of PEGylation is steric hindrance and not conformational changes. This phenomenon has been observed with hGRF [34], IFN α-2b [35,36], insulin [37] and IFN-α-2a [38]. Non-NMR methods such as light scattering, differential scanning calorimetry, circular dichroism and fluorescence spectroscopy have shown that there is very little structural difference between unmodified and modified biomolecules [38].

5.6 Physicochemical changes

Biomolecules undergo several physicochemical modifications due to PEGylation. PEG is comprised of ethylene oxide subunits, each of which takes up three molecules of water leading to the observed increased hydrodynamic volume and the resulting increase in Stokes's radius. There is also the possibility of charge modification (e.g., acylation) leading to aggregation, which is more evident when evaluated after incubation at higher temperatures. Preservation of charge on α-amino group after PEGylation leads to less aggregation [39]. PEGylation may also mask charge and glycosylation functions, thus causing reduced phagocytosis by RES and liver cells [40]. Epitope shielding produced by the presence of PEGs on proteins could reduce proteolytic degradation and immunogenicity; other routesof elimination.

5.7 Blood concentrations versus routes of administration

Molecular weight and the injection site are the two most important factors that determine the concentration profile of PEG-modified drugs in blood circulation [41]. The elimination rates from the injection sites are characterized by intraperitoneal (i.p.) > *s.c.* > *i.m.* The intramuscular (i.m.) administered drug initially form a depo at the injection site and slowly diffuses into the bloodstream, and to a lesser extent this is also true for the s.c. administered drug. For s.c. and i.m., the elimination rate decreases with increasing MW of PEG [41]. The administration route plays a major role in the pharmacokinetic profile of PEG conjugates [42].

5.8 Biodistribution and tissue uptake

Biodistribution according to a two-compartment model [15] indicates the following: i) higher MW PEGs circulate in blood longer, while urinary clearance is decreased; ii) smaller PEGs translocate freely from circulation to extravascular tissues and return to circulation again by diffusion, whereas larger PEGs translocate much slower [40]; iii) PEG accumulates in tissues/organ (muscle, skin, bone and to a higher extent in the liver), irrespective of MW; iv) time dependence of tissue accumulation is dependent upon vascular permeability; v) PEG uptake by the Küpffer cells increases when the MW approaches ≥ 50 kDa; and vi) smaller linear PEGs are distributed throughout the body with a larger distribution volume, while branched PEGs are distributed in a smaller distribution volume; and (g) early on, branched PEGs preferentially accumulate in the liver and spleen [43].

5.9 Enhanced permeability retention effect (EPR)

Solid tumors are characterized by extensive angiogenesis and the resulting hypervasculature, defective vascular architecture

and impaired lymphatic drainage. Hence blood-borne macromolecules are preferentially distributed in the tumor. This phenomenon was first discovered by Maeda's group in 1985 and described as the enhanced permeability and retention (EPR) effect [44]. The aforementioned characteristics of tumor tissues could be taken advantage of in delivering macromolecular drugs to the tumor site. PEG promotes accumulation of PEG-modified drugs (e.g., anticancer agents) into permeable hypervasculature of tumors via EPR effect [45]. Maeda also observed that accumulation of polymer-based drug is > 1000× in tumor than in plasma [44].

5.10 Tumor-selective targeting

The enhanced vascular permeability of solid tumors facilitates the delivery of macromolecular drugs to tumor sites [46-48]. The EPR concept is now widely used for the selective delivery of macromolecular anti-cancer agents to tumors, resulting in improved efficacy and better safety at lower doses. Relative to normal tissues, macromolecular drugs penetrate and accumulate preferentially in tumors, leading to prolonged pharmacological effects relative to normal tissues. Polymeric anticancer drugs exhibit two types of targeting modalities; passive and active [49]. The accumulation of PEG modified drugs into permeable tumors by EPR is an example of passive targeting. Active targeting augments passive targeting by EPR and improves selectivity. Attaching targetingmoieties to the polymer backbone can further differentiate between normal and cancerous cells through receptor-mediated endocytosis.

5.11 Anti-PEG IgM

The discovery and availability of a high affinity IgM monoclonal antibody AGP3 against PEG facilitates the development of sensitive and highly specific enzyme-linked immunoassay (ELISA) for the quantitative determination of intact PEG-molecules in body fluids. The anti-PEG antibody enables the histochemical detection of PEG and PEG metabolites in tissues. It should be noted that PEG by itself is not antigenic and does not induce antibody production. PEG antibodies are induced when PEG is linked to proteins involving linking chemistries that leave behind bulky aromatic or heterocyclic group or groups in the PEG conjugate. There is no evidence that the presence of PEG antibodies affects the biological activities of pegylated biomolecules. However, PEG antibodies accelerate the clearance of pegylated molecules [50].

6. PEGylation of antibody and antibody fragments

At the present, there are at least 10 FDA approved antibody products on the market [51,52]. They are used in the treatment of cancer and chronic diseases. Pharmaceutical Research and Manufacturers of America (PhRMA) reported in August 2006 that of the 400 biotechnology products in various stages of clinical trials or awaiting FDA approval, 160 are different monoclonal antibodies. By 2000, monoclonal antibodies had become the second largest biopharmaceuticals, second only to vaccines [53].

As early as 1984, murine antibodies were pegylated to reduce immunogenicity [54]. Advances made in antibody technology (e.g., humanized antibody) since then have made this practice unnecessary today. Since the MW of IgG (~ 150 kDa) is above the glomerular filtration limit, no additional benefits are gained by PEGylation in retarding renal clearance of antibody. However, PEGylation could improve the *in vivo* circulation of IgG by: i) interfering in the interaction of carbohydrate with specific receptors; ii) masking specific sequences from cellular receptors; and iii) reducing proteolysis and reducing antigenicity. PEGylation of whole length antibody is of still interest in immunotherapy because of the bivalency of IgG and the resulting high affinity in antigen binding, as well as PEG facilitating antibody localization to tumors.

Alternative to pegylated full-length antibody is pegylated antibody fragments (e.g., Fab′, diFab′ and scFv), which improve serum half-life and facilitate tumor-targeting [55]. The interest in antibody fragments is due to their reduced size compared to IgG, enabling them to penetrate tissues and tumors more easily than the whole antibody. Lack of an Fc region in these fragments could be useful in circumventing unwanted side effects associated with Fc. Commercial scale production of antibody fragments is now carried out using recombinant DNA technologies. Using antibody engineering techniques, one or more cysteine residues could be engineered into the hinge region of Fab′, which enables site-specific PEGylation. This type of PEGylation alleviates loss of antigen binding usually associated with random PEGylation [56]. In order for the minimum antigen binding scFv proteins to be clinically useful, it is imperative that their circulation half-life have to be prolonged and made adjustable. This requires the synthesis of tailor-made mono-PEG-scFv. Engineered free cysteine residues at the C-terminus or within the linker at the both scFv orientations are used to site-specifically attach various MW PE Gs to scFv [57-59].

7. Random versus site-specific PEGylation

Examples of the effects of random or site-specific PEGylation on the antigen binding properties of antibody and antibody fragments are listed in **Table 3** [51,54,60-62]. Random PEGylation results in the substantial loss of antigen binding in all samples tested. Antigen binding affinity is also adversely affected (data not shown). In contrast, site-specific PEGylation results in full retention of antigen binding, as well as retention of binding affinity [51]. PEGylation at or near the antigen-binding domain may cause steric hindrance. Overall, PEGylation of antibody fragments is potentially a very useful strategy for the development of new therapeutic entities in oncology and autoimmune diseases.

Table 3. Random and site-specific PEGylation of antibody and antibody fragments; effect on antigen binding.

Antibody	PEGylation		Ag binding effect	Ref.
	Random	Site-specific		
Hu anti-hepatitis B IgG	4 × PEG 1.7 kDa	–	30% loss	Suzuki [54]
	15 × PEG 1.7 kDa	–	> 80% loss	
Mu A5B7 F(ab')$_2$	2 × PEG 5 kDa	–	12% loss	Pedley [60]
Mu A5B7 DFM (diFab')	1 – 2 × PEG 25 kDa	–	40% loss	Casey [61]
Hu diFab'	1 × PEG 40 kDa	–	78% loss	Chapman [51]
Mu A5B7 Fab'	2 × PEG 5 kDa	–	20% loss	Pedley [60]
Anti-Tag-72 scFv	1 × PEG 20 kDa	–	40% loss	Lee [62]
Anti-TNF-a-SCA	–	1 × PEG 40 kDa	87% loss	Yang [59]
Hu Fab'	1 × PEG 40 kDa	–	47% loss	Chapman [49]
	–	1 × PEG 25 kDa	107% retention	
Hu Fab'	–	2 × PEG 25 kDa	100% retention	Chapman [49]
	–	1 × PEG 40 kDa	102% retention	

8. Polyethylene glycols (PEG)

Polyethylene glycols are amphiphilic polymers comprised of repeating ethylene oxide subunits, each of which has a MW of 44 Da and whose number is indicated by the whole integer n (**Figure 5**) [1,3]. The molecular weight of PEG is equal to n × 44 Da. Pharmaceutical grade PEGs are inert, non-toxic and contain two terminal hydroxyl groups that can be chemically activated. However, it is common practice to convert one of the two hydroxyl groups to methoxy or other alkoxy groups, in order to make the PEG unifunctional and thus avoid crosslinking during conjugation. In addition to linear PEG chains, there are branched PEG chains in which two or more PEG units are linked together via linkers like lysine [63] and propanol diamine [64], among others. Other types of PEG are the forked and multi-arm PEGs. They are useful for conjugating small molecules to increase drug loading capacity and the preparation of PEG-based hydrogels. Some representative PEG molecules are illustrated in **Figure 2**.

9. PEGylation chemistries

9.1 Permanent PEG linkers

A variety of PEG linking chemistries is now readily available [65-67]. They differ in their relative chemical reactivity and specificity. The most commonly used PEGylation reaction involves an electrophilically activated PEG and ε-amino group of lysine residues or the protein's N-terminal amino group. Examples of such reagents include:

- PEG-Succinimidyl succinate, amide bond [68].
- PEG-Succinimidyl propionate or succinimidyl butyrate, amide bond [3,69].
- PEG-2-Mercapto-thiazoline (T-PEG), amide bond [70].
- PEG-Epoxide, alkyl bond [71].
- PEG-Carbonyl imidazole, alkyl bond [72].
- PEG-Tresilate, alkyl bond [73].
- PEG-Succinimidyl carbonate, carbamate bond [74].
- PEG-*p*-Nitrophenyl carbonate, carbamate bond [75].
- PEG-Benzotriazole, carbamate bond [76].
- PEG-Aldehyde and PEG-Aldehyde hydrates, Schiff's base, N-terminus [77,78].
- PEG-Hydrazide, hydrazone bond, via oligosaccharides or serines [79].
- PEG-Isocyanate, urea or carbamate bond via amino and hydroxyl groups, respectively [80].

PEGylation involving the primary amino groups of lysine residues in the polypeptide chain of proteins often produces heterogeneous PEG conjugates (positional isomers) due to random PEGylation at multiple sites. Site-specific PEGylation achieved using other functional groups (e.g., N-terminal amine, free cysteines, oligosaccharides and hydroxyl groups) on the protein surface, avoids the random PEGylation and the resulting heterogeneity. The most commonly used PEG reagents for site-specific PEGylation are:

- PEG-Maleimide, via free cysteine [81].
- PEG-Vinyl sulphone, via free cysteine [82].
- PEG-Iodoacetamide, via free cysteine [83].
- PEG-Orthopyridyl disulfide, via free cysteine [84].
- PEG-Aldehyde, via N-terminal amine [77,78].

9.2 Releasable PEG-linkers (RPEG)

Releasable PEG linkers are designed for the controlled release of amino drugs [85]. This approach is most suited for peptides, small molecules and oligonucleotides. It is not usually used for protein drugs. In order to be effective, prodrugs derived

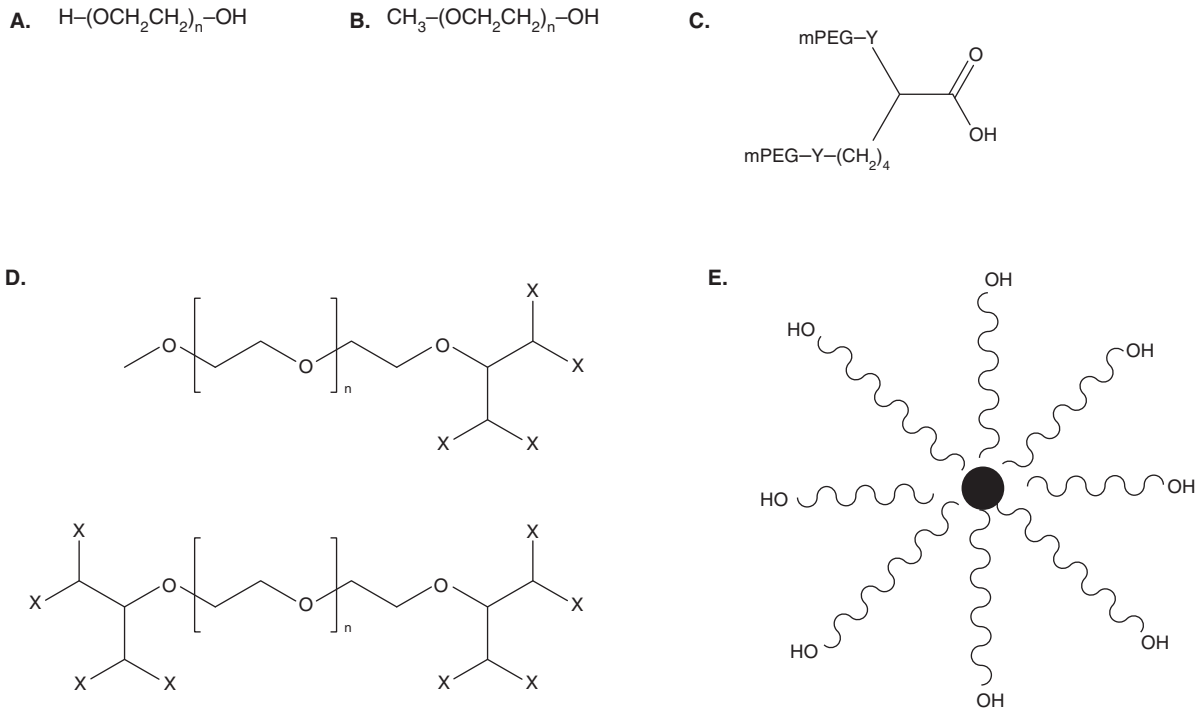

Figure 5. Representative chemical structures of PEG. (**A**) Linear PEG diols with two free end hydroxyl groups. (**B**) Linear monomethoxy-PEG with one of the end hydroxyl group is converted to a methoxy group. (**C**) Branched PEG (PEG2); two linear mPEGs are linked to amino groups of lysine, where Y represents the linker. (**D**) Fork-shaped PEG reagents; providing multi-proximal reactive groups at one PEG chain end or both chain ends, where X represents functional groups. (**E**) Multi-arm PEG; carrying multi-hydroxyl or functional groups, eight-arm PEG is prepared by hexaglycerine as a core.

from releasable PEGylation must be chemically or enzymatically transformed into their active form after administration to patients. With these goals in mind, Zalipsky *et al.* [86] and Greenwald *et al.* [87,88] have fashioned rPEGs that are releasable *in vivo*. They differ in their release mechanisms. Releasable PEGs designed by Zalipsky and co-workers used a para- or ortho-disulfide benzyl urethane, which undergoes reduction in the endosomal compartment of a cell under mild conditions. The rPEGs synthesized by Greenwald and co-workers involve a tripartite system consisting of a spacer containing a trigger or specifier, and an enzymatically cleavable linker attached to the drug moiety. The rate at which the drug is cleaved from the linker is dependent upon the steric hindrance introduced by the chemical structure of the linker. The linker could be substituted or non-substituted aromatic structures or entirely aliphatic.

A slow rate spontaneously hydrolysable insulin prodrug denoted PEG (40)-Fmoc-insulin was synthesized by Shecter *et al.* [89]. They reported that this reversibly pegylated insulin undergoes hydrolysis at physiological conditions and maintains low levels of biologically and immunologically active insulin. Another example of reversible PEGylation involves the novel interferon α-2 conjugate reported by Peleg-Shulman *et al.* [90] and designated as PEG (40)-FMS-IFN α-2.

The clinical utility of rPEG is based upon its ability to release continuously and maintain clinically significant levels of the drug moiety in circulation, thus enabling sustained exposure. Although promising, drugs modified by releasable or reversible PEGylation have yet to be proven effective in the clinic and are devoid of the potential side effects (e.g., immunogenicity, antigenicity, toxicity etc) often associated with the free drug, whereas conventional PEGylation of proteins involving permanent linkage is well-established and proven to be effective.

10. PEGylation reaction and purification

The factors that affect PEGylation reactions involve: i) reaction pH; ii) protein to PEG reagent molar ratio; iii) protein concentration; iv) time of reaction; and v) reaction temperature. Controlling one or more of these parameters, the reaction could be directed towards producing predominantly mono-, di- or tri-PEG conjugates, as desired. PEGylation reactions could be performed in solution-phase [4] or solid-phase [4,91]. Isolation and purification of various PEG conjugates (mono-, di- and oligo-) from the reaction mixture (solution-phase) free of unmodified protein, excess reagents, reaction by-products, salts, etc are performed on an ion exchange column as described [4]. First, the reaction mixture is diluted and

applied on an ion exchange column equilibrated with buffer. The unabsorbed materials are washed away and the adsorbed oligo-, di- and mono-pegylated proteins, and the unmodified protein still remaining on the column are eluted in that order using increasing concentrations of salt. The desired mono-pegylated protein is then exchanged into storage buffer via diafiltration, concentrated and stored at 2 – 8°C until further use.

11. Formulation

Biopharmaceutical formulation has evolved from 'empirical trial and error' to 'science-based' development based on a better understanding of protein stability, protein degradation mechanisms and the application of state-of-the-art analytical techniques [92,93]. PEGylation allows for simple formulations involving buffers, salts, sugars/sugar alcohols and surfactants, among others. The major formulation advantages of PEG-biopharmaceuticals over unmodified proteins are concentrations can be > 10 mg/ml and no need for human serum albumin-based stabilization.

Specific formulation problems associated with PEG-modified proteins include: i) viscosity of formulation and potential phase separation; ii) effect of PEG and solution conditions on protein stability; and iii) cleavage of PEG caused by peroxides in solution. The most common chemical degradation pathways of PEG proteins include deamidation and oxidation. Deamidation of glutamine and asparagines can be prevented by maintaining the pH 3 – 5. Oxidation of methionine can be prevented by non-exposure to oxygen.

Using the formulation of Neulasta as an example, Piedmont and Treuheit [94] recommend the following general considerations for the formulation of PEG proteins: i) effect of pH; ii) effect of buffer; iii) choosing the right tonicity modifier; iv) optimal buffer and excipient concentrations; v) effects of PEG protein concentration (e.g., viscosity and phase separation); vi) effects of surfactants; vii) effects of freeze/thaw; viii) effects of vortex or agitation; and ix) effects of transportation and thermal quiescent stability. The formulation components to be considered are buffer type and strength, ionic compounds, sugars, polyols, certain amino acids, surfactants, antioxidants, chelating agents and other suitable substances.

In order to choose the right excipients, a better understanding of protein stability and the application of sophisticated analytical methods are absolutely necessary. Analytical techniques include: i) UV spectrometry for the determination of concentration (280 nm region); ii) Fourier transform infrared spectrometry for the quantitative determination of secondary structure as well as for the determination of α-helices and β-sheets; iii) circular dichroism for the determination of secondary and tertiary structure; iv) fluorescence spectroscopy for the detection of changes in tertiary structure; v) differential scanning calorimetry for the determination of melting point (T_m) and equilibrium point between native and first stage of denaturation; the higher the T_m, the more stable the protein; vi) isothermal titration calorimetry, for the determination of thermodynamic parameters (ΔH, K_a, ΔS and n binding stoichiometry); vii) dynamic and static light scattering for the determination of protein size and soluble aggregates; viii) analytical ultra-centrifugation for the determination of sedimentation coefficients, MW and quantification of soluble aggregates; ix) size exclusion chromatography for MW and size determination; x) isoelectric focusing non-reducing for the determination of charged amino acids arising from PEGylation, hydrolysis and deamidation; and xi) non-reducing and reducing SDS-PAGE for the determination of MW of protein subunits and reducing disulfide bonds to complete protein unfolding, among others.

12. Biochemical and biological characterization

'Conjugated proteins are considered well-characterized (well-identified) if the starting materials (protein and reagent) are well-characterized and certain parameters are met' [95].

Major points to consider when characterizing pegylated protein therapeutics are:

- They cannot be characterized solely based upon the protein's therapeutic use or biological activity.
- PEG proteins are produced from various therapeutic proteins using a variety of manufacturing processes, resulting in a number of different modifications.
- PEG proteins are heterogeneous; they are neither protein nor polymer but are a hybrid of the two.
- Identification and characterization methods have to be developed on an individual basis.
- Identity, stoichiometry, purity, consistency, short- and long-term stability and potency must be determined.
- Drug product must be tested for the effect of conjugation.
- Validated and reproducible derivatization and manufacturing processes are required.
- Determination of the extent of the heterogeneity in the drug product has to be performed.

13. PEGylation sites and positional isomers

PEGylation sites and the corresponding positional isomers are determined using a combination of techniques [96-99] (**Figure 6**) that include HIEC, SEC, RP-HPLC, peptide mapping, Edman sequencing, amino acid analysis and MALDI-TOF MS. The potential number of positional isomers in a pegylated biomolecule can be calculated from the factorial equation [4,100]. For N PEGylation sites, taken k at a time,

$$\frac{N!}{(N-k)!\cdot k!} = \text{Possible number of positional isomers.}$$

For example, in pegylated interferon α-2a, n = 12 (11 lysines plus N-terminus) and k = 1 – 12. The potential number of positional isomers in mono-PEG-IFn = 12, Di-PEG-IFn = 66 and so on.

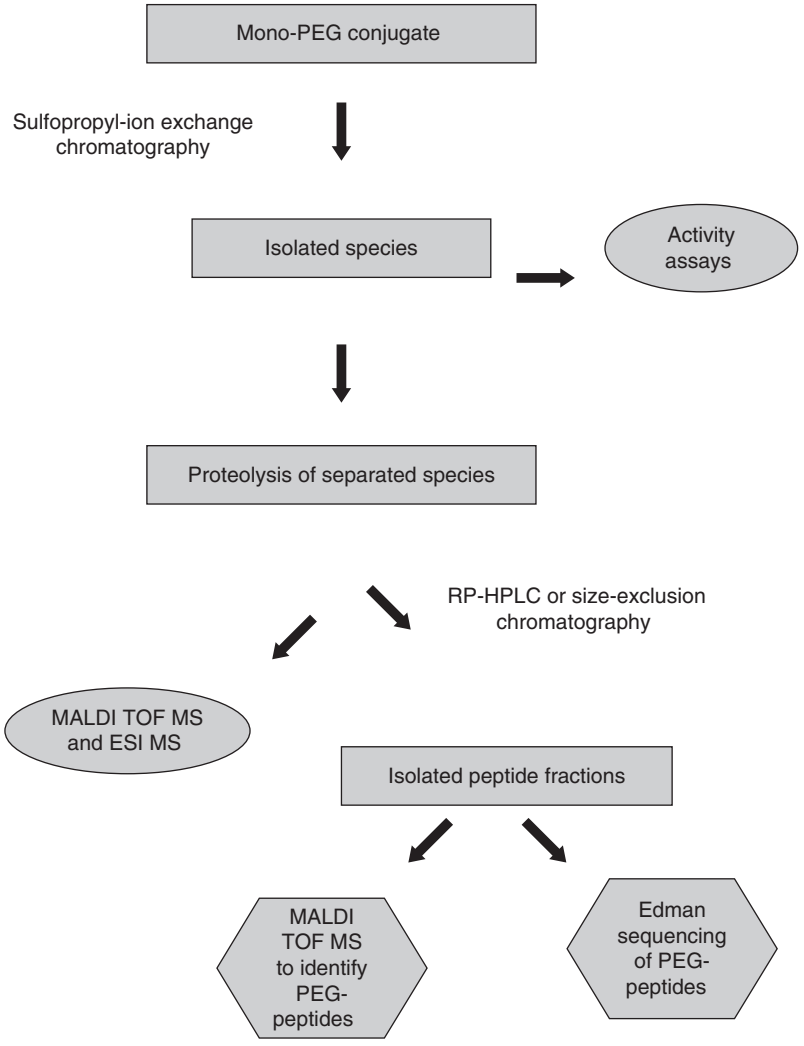

Figure 6. Strategy for the determination of PEGylation sites and positional isomers (see text and references for details).

14. Clinical development and the FDA'S approval process

Regulatory practices related to pegylated biopharmaceuticals are still an evolving process and remain in flux. However, based upon the knowledge gained with the previously FDA-approved PEG-biopharmaceuticals, the guidelines and the steps to be undertaken for the clinical development and approval process are deduced and listed in the following subsections. It is natural to expect that modifications have to be made in the guidelines and steps based upon the biomolecules involved (e.g., protein, peptide and oligonucleotide, among others).

14.1 Pre-clinical studies (regulatory requirements)

- pK/Proof of principle in relative species (~ 3 months).
- Single dose toxicity studies in two species (~ 3 months).
- 2-week non-GLP toxicology/toxicokinetic range-finding study in two species (~ 4 months).
- 4-week GLP toxicology/toxicokinetic study in two species (~ 6 months).
- Mouse micronucleus assay (genotoxicity) to evaluate linker (~ 3 months).
- 13-week GLP toxicology/toxicokinetic study in two species to support 8-week dosing in man (~ 9 months).
- IND (Investigational New Drug) application filing.

14.2 Three-part clinical trials

Phase I

- Testing of drug in healthy volunteers.

Phase II

- Several hundred patients, establishment of efficacy to a particular disease and common side effects.

Phase III

- Testing of several 100s to several 1000s of people.

14.3 NDA (New Drug Application) filing

Manufacturer submits application to FDA describing chemistry manufacturing, patent information and statistics.

- FDA accepts or rejects application.
- FDA's CDER (Center for Drug Evaluation and Research), including outside experts, hold meetings with the company.
- FDA inspects manufacturing site and engages in labeling discussions.
- FDA issues its final decision.

The FDA regulations are intended to assure the long-term safety and efficacy of the yet-to-be-marketed investigational drug, as well as the marketed medical product.

15. Conclusion

Over the last 30 years, PEGylation has grown into a fully fledged protein drug modification and delivery technology that substantially improves the pharmacokinetic and pharmacodynamic properties of therapeutically important biomolecules, thus enabling them to attain maximum clinical potency. So far, nine pegylated drug products are FDA approved and marketed and many more are presently undergoing clinical trials (see introduction, above). All but one of the nine marketed products involve proteins, the sole exception being Macugen, which is a pegylated aptamer. Until now PEGylation has been considered best suited for high value and potent proteins like cytokines, hormones and growth factors. However, the use of this technology has now expanded from proteins to peptides to oligonucleotides and small molecules and covers a full spectrum of therapeutic areas.

16. Expert opinion

In recent years, PEGylation technology has scored some spectacular successes in producing several blockbuster drugs of the likes of Pegintron, Pegasys, Neulasta and Mircera for the treatment of life-threatening diseases (e.g., hepatitis C, cancer, chemotherapy-induced neutropenia and renal anemia, among others). It is now common practice to use PEG-modification to improve the therapeutic value of biopharmaceuticals. Advances made in PEGylation concepts and in linking chemistries have greatly expanded the role of PEGylation as a drug modification and drug delivery platform for protein and peptide therapeutics.

No one questions the usefulness of PEG-modification of protein therapeutics to achieve enhanced clinical potency with equivalent safety, as well as patient-friendly dosing regimens, which were unattainable for protein therapeutics before the maturation of PEGylation technology. However, there is a controversy swirling about the definition of PEG-biopharmaceutical. Is it simply a PEG attached biomolecule or a new molecular entity (NME) produced by the reaction between a biomolecule and a chemically reactive PEG? Arguments in favor of the NME concept are presented elsewhere in this article. All reasonable people will agree that PEG-modified biopharmaceutical has no parts or components, or cannot be taken apart physically. Further support to the NME concept is the physicochemical and physiological changes that occur with the biomolecule after the PEGylation reaction. It should also be pointed out that early on, all manufacturers, in order to avoid conducting expensive three-phase clinical trials, suggested that the PEG-biopharmaceuticals are similar to the corresponding already-tested unmodified biomolecules and need not have to undergo rigorous clinical trials. At the same time, for intellectual property protection purposes, they argued in support of the NME concept. Obviously, you cannot have it both ways. The issue of the NME classification for PEG-biopharmaceuticals is yet to be resolved.

So far, all the marketed PEG-modified biopharmaceuticals utilize 'permanent' linking chemistries. Permanent linkages between the polymer and the drug moiety often lead to inactivation; especially in the case of peptides and small molecules. A well-designed prodrug or (releasable) type of conjugation could possibly alleviate the potential pitfalls of permanent linkage, especially associated with peptides and small molecules. In addition to increased solubility and improved pK/pD characteristics, the releasable prodrugs could act as a 'circulating depo' as well as providing means for tumor targeting via the EPR effect.

PEG-modified biopharmaceuticals require only a simple formulation involving common excipients, devoid of the use of human serum albumin. High drug concentrations are achieved with no additional efforts. However, releasable PEG-modification may require non-aqueous storage formulation (e.g., lyophilization) to maintain linker stability. In order to fully characterize the PEG-modified biotherapeutics, sophisticated analytical methods and instrumentation are needed.

All marketed PEG-modified therapeutics are administered by injection via parenteral routes that are not very patient-friendly and result in less patient compliance. Much work is presently being undertaken to develop advanced delivery technologies (e.g., parenteral, pulmonary and nanospheres) for pegylated biotherapeutics. It is only a matter of time before innovative drug delivery technologies become available for the delivery of PEG-modified biopharmaceuticals.

The majority of pegylated biopharmaceuticals are either cytokines or hormones or growth factors. However, PEG modification among biomolecules has been now extended to antibodies and antibody fragments to take advantage of the long circulating half-life and the tumor targeting properties of PEG conjugates. Several PEG-modified antibody fragments are presently in development for the treatment of life-threatening diseases like cancer and autoimmune diseases.

PEGylation has a potential role to play in the area of biosimilars. Several FDA-approved biopharmaceuticals are already off patent or coming off patent in the next five years. This opens up new avenues for the use of PEGylation technology to improve the therapeutic value of biosimilars [101]. In order

to be acceptable to the medical community and to be competitive in the marketplace, PEG-biosimilars must be safe, more potent, longer acting and importantly cost-effective compared to their corresponding unmodified biosimilars.

Declaration of interest

The authors declare no conflict of interest and have received no payment in preparation of this manuscript.

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