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Optimizing Pharmacological and Immunological Properties of Therapeutic Proteins Through PEGylation: Investigating Key Parameters and Their Impact

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Abstract: Protein PEGylation represents a significant technological advancement in the development of protein-based therapeutics and is widely used to reduce immunogenicity, enhance pharmacokinetics, and/or improve stability. The improved pharmacokinetic profile of PEGylated proteins compared with the native protein results in sustained versus fluctuating plasma concentrations and carries the potential of less frequent administration. However, attachment of PEG to therapeutic proteins can alter their structural conformation, which exposes new epitopes to the immune system. The design of PEGylated proteins thus needs to balance the intended benefits with the potential risks associated with the immunogenicity of the PEG moiety itself or resulting from alterations in the conformation of the therapeutic protein. In recent years, advancements in protein PEGylation chemistry have offered the capability to target PEG attachment to specific amino acids to create more stable and bioactive therapies. The biophysical and biopharmaceutical features of PEGylated proteins can vary based on polymer size, shape, density, and conjugation site, and the immunogenicity of the conjugate can be further impacted by the properties of the therapeutic protein itself and the characteristics of the patient. It is important to note that not all patients will develop an immune response toward the PEG moiety, and not all immune responses are clinically meaningful. A comprehensive understanding of the factors that influence immunogenic responses to PEGylated proteins is important to optimize their therapeutic benefits. This article reviews the design and optimization of PEGylation strategies to enhance the clinical performance of protein-based therapeutics while minimizing immunogenic responses to the PEG moiety or PEGylated proteins.

Keywords: PEGylation, protein-based therapeutics, immunogenic response

Introduction

In recent years, advancements have been made in the development of protein- and peptide-based therapeutics that offer superior binding selectivity and specificity, along with an improved safety profile, compared with small-molecule medicines.[1] However, strategies are needed to overcome the inherent limitations of these treatments, including poor in-vivo stability, short half-life, low bioavailability, immunogenicity, and an increase in aggregation during large-scale storage.[1]

Protein PEGylation is the conjugation of polyethylene glycol (PEG) to functional amino acid groups on the protein surface. This technique was first developed in the 1970s by Abuchowski and Davis at Rutgers University (New Jersey, USA) to yield new chemical entities with different biological behaviors with respect to the native protein.[2–5] PEGylation has been increasingly used to address some of the challenges faced by native proteins by modifying their biophysical, biopharmaceutical, pharmacokinetic, and immunological features, thus enhancing their therapeutic performance.[1] PEGylated proteins vary substantially in their PEG architecture, molecular weight, and degree of conjugation.[6] They

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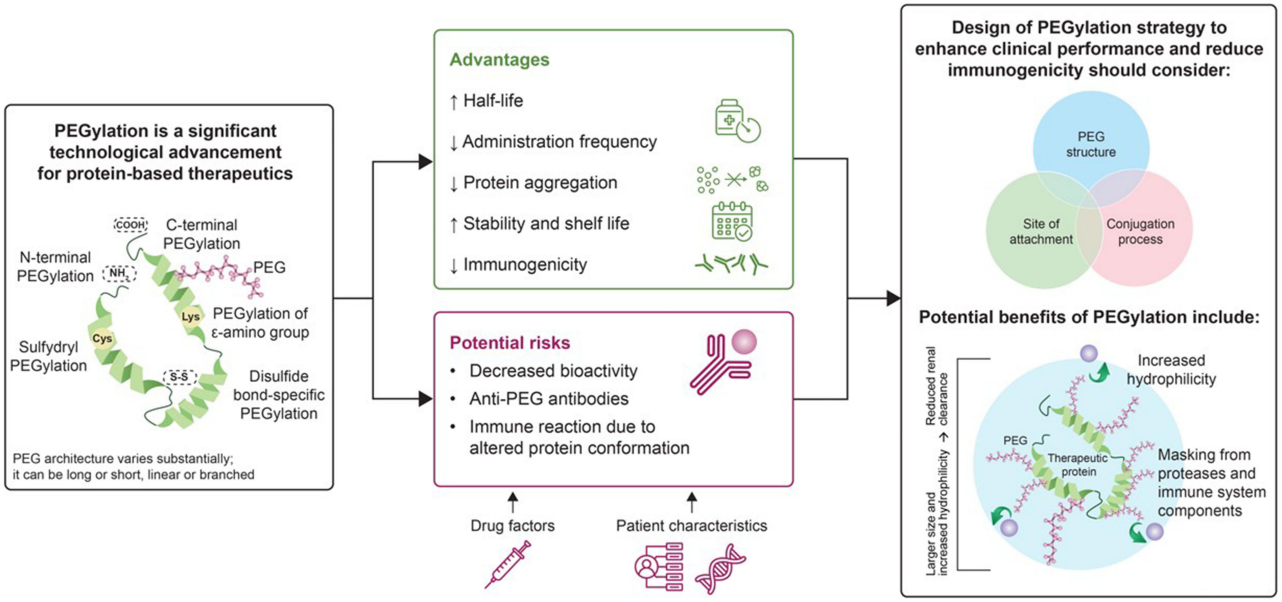
Graphical Abstract



are already widely used in some therapeutic fields (eg oncology, chronic kidney diseases, hemophilia) and are now being approved in evolving fields, such as rare diseases and ocular conditions (eg pegunigalsidase alfa [Elfabrio] for Fabry disease, avacincaptad pegol [Izervay] for geographic atrophy of the eye, and pegcetacoplan [Empaveli] for paroxysmal nocturnal hemoglobinuria).[7] Thus, it is important for healthcare professionals to understand the complexities and limitations involved when comparing the properties of PEGylated drug analogs (PEGylated proteins obtained by modification of a specific protein through different PEGylation strategies), given that each of them has its own unique structure and composition.

In 1990, the first therapeutic PEGylated protein was approved by the US Food and Drug Administration (FDA) – adenosine deaminase (pegademase bovine, Adagen), extracted from bovine intestine, for severe combined immunodeficiency syndrome.[8] In 1994, pegaspargase (Oncaspar), derived from *Escherichia coli* (*E. coli*), was approved for use in acute lymphoblastic leukemia.[9] These extensively PEGylated proteins dramatically decreased the immunogenicity of the native xenogeneic proteins.[8]

More recently, the large-scale production of pharmaceutical-grade proteins and the translation of these products into therapeutics has further expanded the use of PEGylation technology. PEGylation has been exploited to overcome limitations associated with novel protein therapeutics, such as instability, short circulation half-life, and immunogenicity.[10] However, PEGylation has certain drawbacks, including eliciting an immune response to PEG in some individuals[11] or triggering hypersensitivity reactions, which may limit the effectiveness of therapy.[12,13] It is important to note that not all individuals develop an immune response to PEGylated proteins,[11] and the presence of anti-PEG antibodies can have varying effects on PEGylated protein clearance[11] and treatment response[14] among individuals. The immunogenicity and potential adverse events associated with PEGylated proteins can vary widely based on the specific characteristics of the PEG moiety, native protein, and host.[8,15,16] These effects should be assessed in clinical trials and monitored via post-marketing surveillance.

This review provides a comprehensive overview of the biochemical, biological, and immunological properties of PEGylated therapeutic proteins. We discuss how the clinical profile of PEGylated proteins can be enhanced by optimization of PEGylation strategies and conjugation chemistry. We then consider the properties of PEGylated proteins that may influence the risk of triggering an immune response, together with strategies for overcoming such reactions.

Sources of Therapeutic Proteins

Over a third of all approved recombinant therapeutic proteins are produced using *E. coli*.[1] However, prokaryotic expression systems are limited because they lack post-translational machinery, and thus lack protein glycosylation pathways, disulfide bond formation, phosphorylation, and proteolytic processing, leading to low expression, low solubility, potentially poor functionality, and increased immunogenic potential.[1,17,18] PEGylation has the advantages of improving solubility, reducing aggregation and proteolysis of proteins, extending the stability, and decreasing immunogenicity.[5] PEGylation of proteins produced using *E. coli* has been successfully used to develop pegaspargase and calaspargase pegol (Asparlas) for the treatment of leukemia.[19] However, several proteins cannot be generated in microbial systems because of poor product quality, especially if post-translational modifications are required for protein stability and activity.[1] Chinese Hamster Ovary (CHO) cell lines are the most frequently used mammalian hosts for the biosynthesis of recombinant glycosylated proteins to produce high-quality proteins, such as monoclonal antibodies.[20]

Plant cells have also been developed for the industrial production of glycosylated recombinant therapeutic proteins, which can be further enhanced by PEGylation. Plant proteins contain both beta-(1,2)-xylose and alpha-(1,3)-fucose residues, which are absent in animal proteins. Although the immunogenicity of plant-specific N-glycans has been a concern in humans, recent research on proteins derived from plant cells, such as taliglucerase alfa (Elelyso), indicates no increase in immunogenicity compared with mammalian enzyme replacement treatment.[21,22]

Taliglucerase alfa is a recombinant active form of the lysosomal enzyme beta-glucocerebrosidase that catalyzes the hydrolysis of glycolipid glucocerebroside to glucose and ceramide.[23] This lysosomal glycoprotein is obtained from a genetically modified carrot plant root and has been approved by the FDA for long-term enzyme replacement therapy for type 1 Gaucher disease.[23] Tobacco cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) are used to synthesize alfa-galactosidase A (alfa-GAL-A), which, in its PEGylated form (pegunigalsidase alfa), is used as an enzyme replacement treatment for Fabry disease.[12] The advantage of Bright Yellow 2-cell production over other cell lines is that it yields mannose-terminated glycosylation that promotes the binding and uptake of alfa-GAL-A by disease-relevant tissues.[24,25] However, there are important differences in the properties of these two plant-manufactured therapeutics, which likely stem from differences in the dominant glycans on their surface. The dominant glycans of pegunigalsidase alfa are mannose-rich, reflecting the targeting of its alfa-GAL-A precursor to the endoplasmic reticulum, whereas taliglucerase alfa is targeted to the vacuole, resulting in ≥90% of its glycans being paucimannose structures.[24,26] The glycosylation pattern of the enzyme can play a critical role in its efficacy. Enzymes with mannose-terminated glycosylation, such as pegunigalsidase alfa produced using Bright Yellow 2 cells, are likely to be more effective in targeting disease-relevant tissues due to enhanced uptake by lysosomes.[24,25,27] In contrast, enzymes with paucimannose structures, such as taliglucerase alfa,[26] might have reduced activity due to less efficient cellular uptake, potentially impacting their overall therapeutic effectiveness.

Biopharmaceutical and Immunological Properties of PEGylated Proteins

The covalent attachment of PEG chains to the protein surface produces bioconjugates with remarkably different biophysical features compared with those of the native protein (Figure 1). These differences may include effects on drug half-life in the circulation, biodistribution and elimination, susceptibility to proteolysis, and anti-drug immunogenicity.

Permanence of PEGylated Proteins in the Circulation

PEG is an amphiphilic polymer composed of repeated ethylene oxide subunits, each of which coordinates 2–3 water molecules,[16,28] which bestows the polymer with a high hydrophilic character and hydrodynamic volume. Accordingly, PEGylation produces a hydrated cloud on the protein surface, yielding a bioconjugate with an overall hydrodynamic size that is much larger than that of the naked globular protein.[8] Consequently, the increased molecular size of the bioconjugate leads to a slower renal glomerular filtration rate and prolonged half-life in the systemic circulation compared with the non-PEGylated protein.[4] This is particularly important for small proteins below the molecular weight of albumin (67 kDa), which is considered as the kidney ultrafiltration cutoff.[8] Low-molecular-weight proteins (<40 kDa), including cytokines and

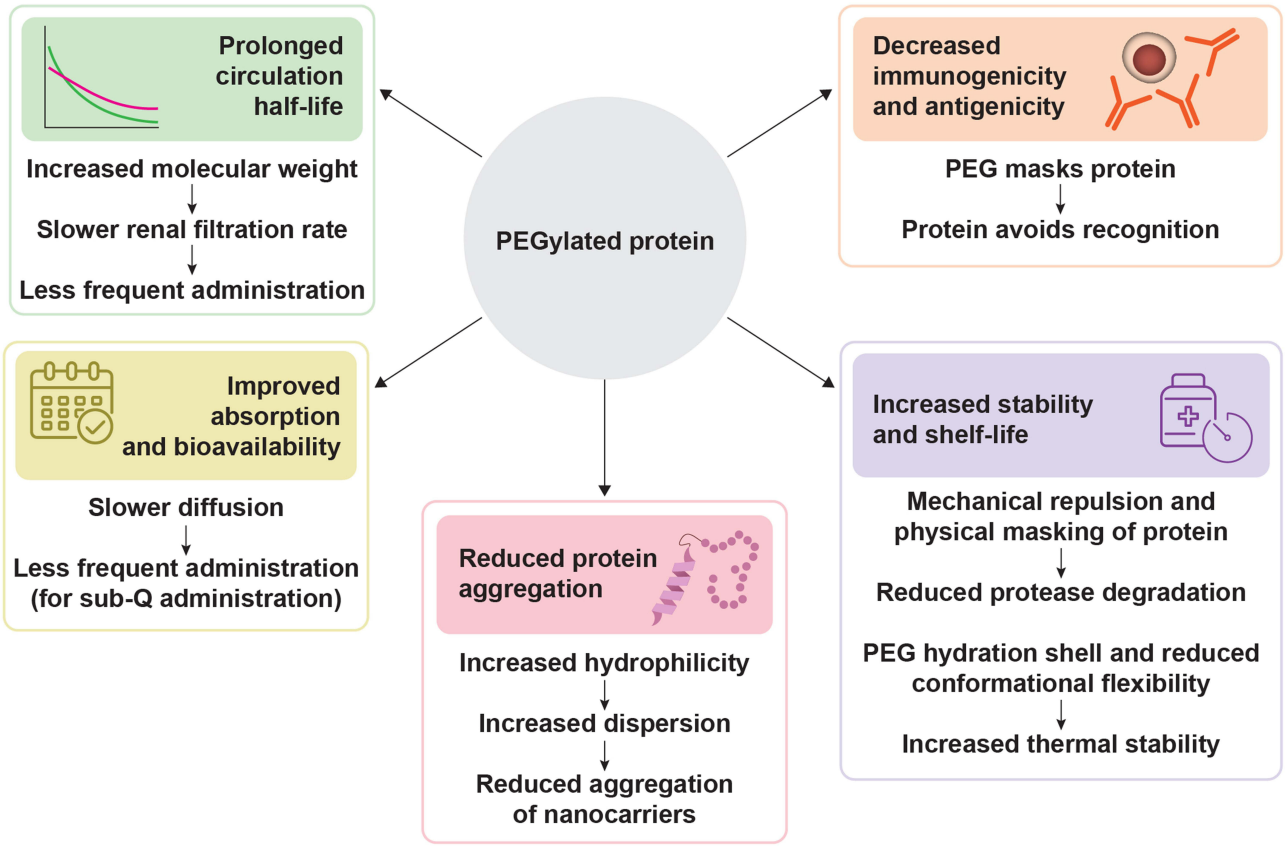


Figure 1 Enhanced biophysical properties of PEGylated proteins.
Abbreviations: PEG, polyethylene glycol; sub-Q, subcutaneous.

hormones, such as interferons (~19–33 kDa), growth hormone (GH) (~22 kDa), granulocyte colony-stimulating factor (G-CSF) (~19 kDa), and erythropoietin (30–37 kDa), are rapidly cleared from the bloodstream by kidney ultrafiltration.[8,29–32] PEG moieties may be added to therapeutic proteins to increase the molecular weight above the ultrafiltration limit along with conferring a larger hydrodynamic radius than the parent molecule, which together prolong the circulatory half-life. For example, in the case of alfa-GAL-A, an enzyme that is deficient in Fabry disease, covalent crosslinking of two PEGylated alfa-GAL-A subunits to form a recombinant homodimer (pegunigalsidase alfa, approximate molecular weight 116 kDa)[25] has extended the plasma half-life of this enzyme replacement therapy to 53−121 hours (mean 80 hours) in humans,[33] compared with a mean of ≤3 hours for the non-PEGylated alfa-GAL-A enzyme replacement therapies agalsidase alfa (Replagal, theoretical molecular weight 51 kDa)[34] and agalsidase beta (Fabrazyme, molecular weight approximately 100 kDa).[35] It should be noted that renal clearance is a complex process and is not solely dependent on size-based ultrafiltration. Involvement of several other passive and active mechanisms can make elimination of PEGylated proteins unpredictable. In addition, PEG does not have a globular structure[36] and thus has a non-linear correlation between its molecular weight and hydrodynamic size, resulting in further unpredictability.

The large size and hydrophilicity of PEGylated proteins also slow their diffusion through biological tissues and affect their absorption and bioavailability.[4] For example, subcutaneously administered PEGylated G-CSF (Neulasta) has a greater area under the serum concentration-time curve and a longer circulating half-life than the non-PEGylated derivative.[37] Similarly, PEGylation of interferon alfa-2a (peginterferon alfa-2a [Pegasys]) results in sustained absorption and a longer terminal half-life than that of non-PEGylated interferon.[38,39] The improved pharmacokinetic profile of PEGylated proteins results in more constant and sustained plasma concentrations with less frequent administration, while the native proteins may require multiple injections per day or week that are associated with fluctuating plasma concentrations and systemic side effects.[8,38]

PEG Biodistribution and Elimination

PEG is generally considered safe and biologically inert and has a favorable toxicity profile.[15] Nevertheless, theoretical concerns remain about the accumulation of large-molecular-weight PEG molecules (>20 kDa) in tissues because of their lower clearance with larger molecular size, especially in patients who require life-long administration of PEGylated products.[40,41]

The biodistribution and elimination of PEG from PEGylated drugs can vary depending on several factors, including PEG size and molecular weight, PEGylation method, and pharmacological properties of the PEGylated protein (Figure 2).[41] The predominant clearance route for smaller PEG molecules is rapid elimination by the kidney, whereas larger PEG molecules (>30 kDa) tend to have slower renal clearance.[41,42]

For large PEG sizes, other elimination pathways, such as biliary excretion, are also dependent on molecular weight.[41] For example, in mice, hepatobiliary clearance is at a minimum for PEGs with molecular weight of approximately 50 kDa, whereas PEGs with lower or higher molecular weights have increased hepatobiliary clearance.[42] Evidence suggests that the uptake of PEG by Kupffer cells in mice increases when PEG molecular weight exceeds 50 kDa.[42] While both the liver and kidney play a significant role in the excretion of PEG, urinary excretion of PEG is still likely to be the major route of elimination for molecular weights up to 190 kDa, while hepatobiliary clearance is a minor pathway.[43]

Cellular vacuolation has been reported more frequently in large-molecular-weight PEGs (≥30 kDa) as another pathway of elimination.[15] Vacuolization refers to the formation of vacuoles, which are small membrane-bound compartments within cells that can contain various substances or organelles, and is frequently observed in mammalian cells. Vacuolization can occur in response to various cellular processes, including cellular stress, injury, and pathological conditions. Vacuolization disrupts cellular homeostasis and affects important cellular functions. However, in the absence of pathological effects, such as cellular distortion, changes in surrounding tissue, or inflammation, cellular vacuolation observed with high doses of PEG is considered an adaptive rather than an adverse response.

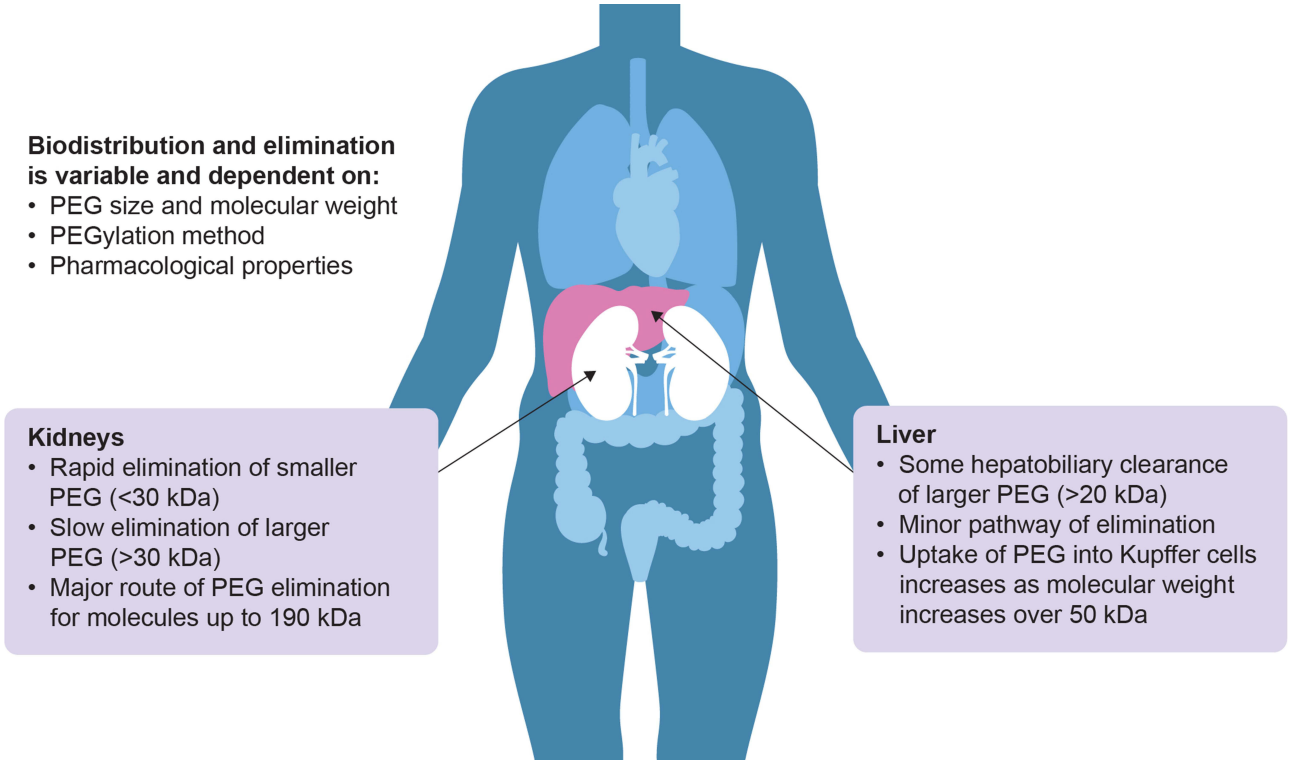


Figure 2 Biodistribution and elimination of PEGylated proteins.
Abbreviations: kDa, kilodalton; PEG, polyethylene glycol.

Non-clinical toxicology studies have suggested an increase in vacuolation in the tissues of animals administered PEGylated proteins. In a study on Sprague-Dawley rats, Bendele et al (1998) found that a recombinant dimeric tumor necrosis factor binding protein, linked by a 20-kDa PEG molecule, caused vacuole formation in the renal tubular epithelium.[44] These histological findings appear to be dependent on the PEG molecular size, with less severe renal tubular vacuolation observed with tumor necrosis factor binding protein conjugated to a larger PEG (50 kDa) than to a smaller PEG (20 kDa).[44] Consistent with this finding, Rudmann et al (2013) also reported that vacuolation observed with unconjugated high-molecular-weight PEGs was influenced by the molecular weight of PEG.[45] The clinical significance of these histological findings remains unclear.

In toxicology studies with certolizumab pegol (Cimzia, a 40-kDa branched PEG), which is approved for chronic administration to control Crohn's disease, vacuolation in hemolymphoreticular tissues and foamy macrophages was observed in several tissues of cynomolgus monkeys and rats.[15,46] In a few cases, when vacuolation was observed with lower-molecular-weight PEGs, the products were conjugated to multiple PEG molecules, resulting in a large overall PEG dose. For example, pegvisomant (Somavert), with a 5-kDa PEG (4–6 PEGs per drug molecule), was associated with vacuoles in the lymph nodes and spleen in rats.[15,47] Similarly, pegloticase (Krystexxa), with a 10-kDa PEG (8–10 PEGs per monomeric subunit), was associated with the development of vacuoles in the adrenal gland and aortic endothelia in studies in dogs.[15,48]

In phagocytic cells, vacuolation is considered a normal physiological response to the removal of foreign materials (including PEG molecules).[15,49] Vacuolation is seen mostly in phagocytes but sometimes also in non-phagocytic cells and has not been linked with adverse effects on organ function in non-clinical studies.[15,49] A recent review of non-clinical safety information for approved PEGylated drugs reported that that there is no evidence from toxicology studies of any functional impact of vacuolation, and that no indication of PEG-related effects has been reported from clinical trials or post-marketing surveillance.[50]

Protein Stability to Proteases

PEG polymers provide a hydration shell that increases the hydrodynamic volume of proteins and masks their surface, improving stability and reducing protease degradation.[4,51] Indeed, the hydrated polymer physically and chemically hinders the approach of proteases by mechanical repulsion and by chemical derivatization of amino acid sequences that are sensitive to proteolytic cleavage. Protection from proteolysis has been observed in vitro with PEGylated versions of antimicrobial peptide Onc72, cocaine esterase, taspoglutide, amylin, and pramlintide.[52–54]

Anti-Drug Immunogenicity

Many first-generation therapeutic proteins were obtained by extraction from animal organs and tissues, plants, fungi, and bacteria, which yielded intrinsically immunogenic products. Over the years, these immunogenic hurdles have been partially alleviated by the development of human recombinant proteins or humanized proteins (ie humanized antibodies) with lower immunogenicity compared with the first-generation xenogeneic proteins.[55] However, human proteins obtained by recombinant technology may also elicit an immune response owing to structural alterations that occur during the biosynthesis and purification processes.[4,56,57] The immune response elicited by immunogenic proteins can result in their inactivation by neutralization and elimination, and/or may promote severe immune reactions.[4] PEGylation can have a remarkable effect on the immunological profile of such proteins by masking surface proteins from recognition by the immune system, antibodies, and immune cells.[4] Thus, PEGylated proteins have largely been regarded as having low immunogenicity and antigenicity.[58] However, several studies have identified an increase in anti-PEG antibodies that has been shown, in some cases, to have a deleterious effect on therapeutic efficacy and an association with hypersensitivity reactions.[16,59]

Impact of PEG Structure on the Properties of PEGylated Proteins

The PEG structure of PEGylated proteins includes the PEG molecular weight and shape, and the number of PEG chains attached to the protein. Different PEG structures have different behaviors depending on their characteristics and the nature of their interactions (examples are shown in Table 1). As such, different PEGylation designs for the same protein

Table 1 Examples of the Impact of PEG Structure on the Properties of PEGylated Proteins

PEGylated Protein	PEG Modification	Effects
Pegunigalsidase alfa[24,25,33,51]	Crosslinking by a bifunctional PEG polymer of 2 kDa	Improved pharmacokinetic properties Partial masking of immune epitopes
Pegloticase[60]	Modified with a 10-kDa PEG	Extended half-life
Peginterferon alfa-2a[61]	Modified with one branched 40-kDa PEG	In-vitro antiviral activity decreased by 93%
Peginterferon alfa-2b[36] Pegfilgrastim (G-CSF)[36] Erythropoietin[36] Certolizumab pegol[36] Peginterferon alfa-2a[36]	Modified with one linear 12-kDa PEG Modified with one linear 20-kDa PEG Modified with one linear 30-kDA PEG Modified with one branched 40-kDa PEG Modified with one branched 40-kDa PEG	Conjugation with high-molecular-weight PEGs prolonged in-vivo stabilization without compromising bioactivity

Abbreviations: G-CSF, granulocyte colony-stimulating factor; kDa, kilodalton; PEG, polyethylene glycol.

produce bioconjugates with different properties. PEG size and shape are selected according to the protein structure, activity, and intended purpose of PEGylation. In general, longer chain molecules are used to reduce clearance by the kidney, whereas a higher number of short-chain molecules attached to the protein surface can increase its stability toward proteases and shield it from immune attack.[19] In some cases, PEGylation is designed to achieve both improved stability and immunogenicity (eg for pegunigalsidase alfa, PEGylation is aimed at creating a stable covalently linked homodimer while some additional PEG moieties are bound to one monomer only).[24,25,51]

Either linear or branched PEG molecules are used to PEGylate proteins (Figure 3);[59] they range in size from 2 to 80 kDa but typically are most often 5, 20, or 40 kDa. The 5-kDa linear PEG was used to PEGylate the first generation of xenogeneic proteins. Conjugation of several 5-kDa PEG molecules efficiently decreased the immunoreactivity of asparaginase and adenosine deaminase, making their PEGylated derivatives suitable for sustained delivery. Similarly, uricase was PEGylated with a 10-kDa PEG (with multiple PEG strands per uricase molecule) to produce low immunogenic pegloticase, although immunogenicity remains a major limitation of pegloticase.[60] In the case of pegunigalsidase alfa, the effect of covalent crosslinking between two alfa-GAL-A subunits by PEGs of various lengths was assessed.[51] Preclinical studies demonstrated that protein dimerization with a bifunctional PEG polymer of 2 kDa produced the most stable conjugate with improved pharmacokinetic and biodistribution profiles and low immunogenicity due to partial masking of the immune epitopes.[24,51]

The conjugation of several PEG chains does not significantly compromise protein bioactivity when the PEGylated proteins are enzymes that metabolize small substrates, which can freely diffuse through the polymer cloud to reach the enzyme active site.[4] On the other hand, the conjugation of several polymer chains can dramatically decrease the bioactivity of proteins that interact with large substrates, such as proteolytic enzymes (ie plasminogen and urokinase) or proteins that display their activity by interaction with receptors located on the cell surface, such as hormones, coagulation factors, and cytokines.[4] However, it is worth noting that the conjugation of even one PEG chain can strongly impact protein bioactivity. In the case of interferon alfa-2a, for example, the conjugation of one branched 40-kDa PEG resulted in an overall bioactivity decrease of 93%.[61] Therefore, chemists need to strike a balance between the advantages of PEGylation and the potential loss of bioactivity to produce bioconjugates with enhanced clinical performance compared with non-PEGylated proteins.

Several studies showed that, in many cases, conjugation of high-molecular-weight PEGs has proven to be successful in prolonging the in-vivo performance of a number of therapeutic proteins without compromising their bioactivity. Accordingly, early 5-kDa PEG was superseded by linear and branched 12-, 20-, and 40-kDa PEGs.[36] Examples of monoPEGylated proteins with high-molecular-weight PEG include peginterferon alfa-2b (PegIntron, Sylatron) – modified with one 12-kDa linear PEG; pegfilgrastim (G-CSF) – modified with one 20-kDa linear PEG; erythropoietin (mPEG-epoetin beta, Mircera) – modified with one 30-kDa linear PEG; and anti-tumor necrosis factor (certolizumab pegol) and interferon (peginterferon alfa-2a) – both modified with one branched 40-kDa PEG.[7,36] The number of PEG chains and their molecular weight dictate the surface PEG density and, consequently, the level of macromolecular

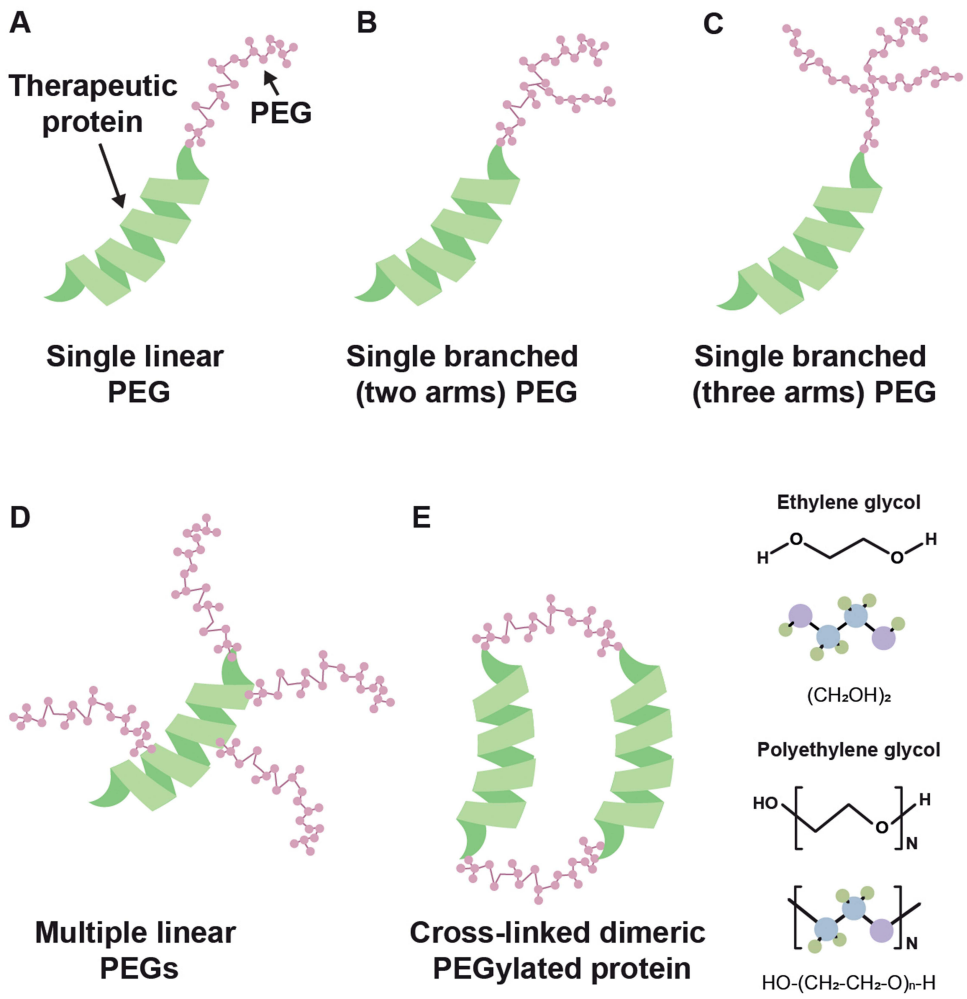


Figure 3 Representative PEG structures. (**A**) Single linear PEG. (**B**) Single branched (two arms) PEG. (**C**) Single branched (three arms) PEG. (**D**) Multiple linear PEGs. (**E**) Cross-linked dimeric PEGylated protein.
Notes: Adapted from Gao Y, Joshi M, Zhao Z, Mitragotri S. PEGylated therapeutics in the clinic. *Bioeng Transl Med*. 2024;9(1):e10600. Creative Commons.[62]
Abbreviation: PEG, polyethylene glycol.

shielding.[63] Some evidence suggests that the high surface PEG densities provided by branched PEGs can enhance the protective efficacy of PEG more than the same number of linear PEG chains with similar or higher molecular weights.[63] Comparative studies have shown that some proteins modified with branched PEG may display superior in-vitro and in-vivo therapeutic efficacy compared with their linear PEG counterparts with the same molecular weight.[64] Differences in clearance rates by glomerular filtration observed between linear and branched PEGylated proteins have been ascribed to differences in the flexibility of these polymers.[65] The use of high-molecular-weight linear and branched PEGs in the PEGylation of therapeutic proteins has a profound clinical impact by extending drug half-life, improving stability, reducing immunogenicity, and enhancing therapeutic efficacy.[1,16] Branched PEGs, in particular, offer superior protection and prolonged action,[28] making them especially valuable in the development of long-acting biological drugs. These advances translate into significant benefits for patients, including less frequent dosing, improved drug effectiveness, and better overall tolerability,[8,36] which are critical for managing chronic and complex diseases.

Impact of Conjugation Chemistry on Properties of PEGylated Proteins

Over the past few decades, several chemical strategies and protocols have been developed to conjugate PEG with proteins (Figure 4). Conjugation chemistry plays a critical role in determining the physicochemical and biopharmaceutical features of bioconjugates. Depending on the protocols and linkers, PEG can be conjugated to selected functional

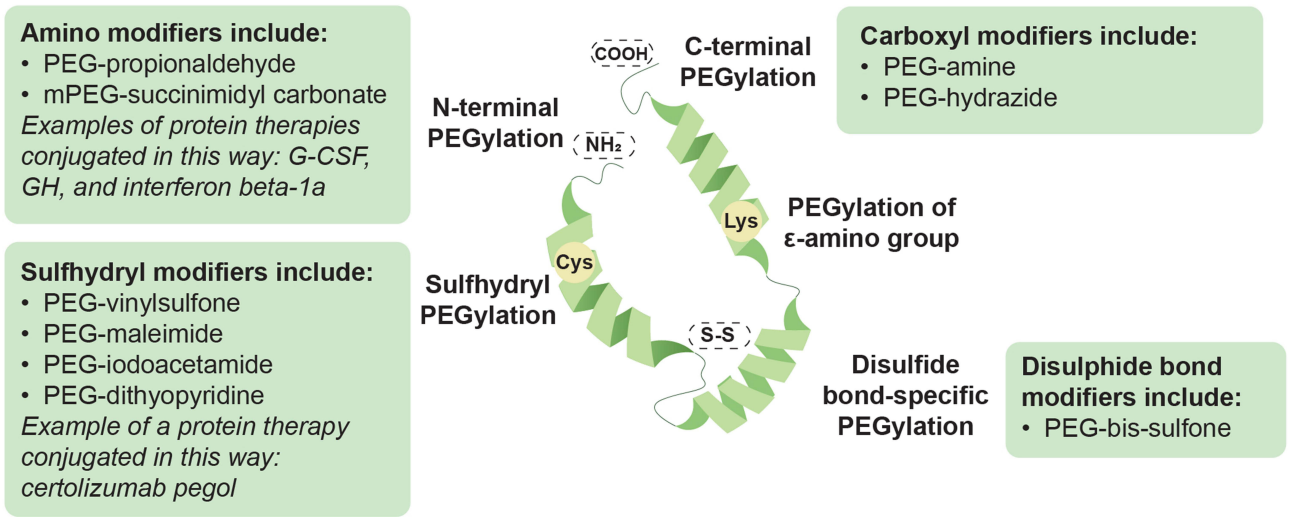


Figure 4 PEG modifiers used at various PEGylation sites in proteins.
Notes: Adapted from Li C, Li T, Tian X, et al. Research progress on the PEGylation of therapeutic proteins and peptides (TPPs). *Front Pharmacol*. 2024;15:1353626. Creative Commons.[36]
Abbreviations: Cys, cysteine; G-CSF, granulocyte colony-stimulating factor; GH, growth hormone; Lys, lysine; mPEG, monomethoxy-PEG; PEG, polyethylene glycol.

groups on protein surfaces to yield derivatives with stable or cleavable PEG-protein bonds, which determine the in-vivo behavior of the bioconjugate.[4] The chemistry of protein PEGylation has moved from procedures that allowed for unspecific and random polymer conjugation to controlled PEG attachment to specific amino acids with minimal-to-no impact on protein function. Conjugation chemistry plays a pivotal role in shaping the clinical properties of PEGylated proteins. By influencing factors such as stability, bioactivity, immunogenicity, pharmacokinetics, and production, the choice of conjugation method can directly impact the therapeutic efficacy and safety of these biologics.[36,66] Advances in selective and controlled PEGylation techniques offer significant clinical benefits, including extended drug half-life, reduced immunogenicity, improved specificity, and more predictable patient outcomes. These improvements can lead to better therapeutic options for patients, enhancing the overall effectiveness and safety of PEGylated therapeutic proteins.

PEGylation Sites on Proteins

The main amino acid side function exploited as a tag for polymer conjugation is the ε-amino group of lysine (Lys).[36,66] However, strategies have also been developed to conjugate PEG to terminal amino groups, thiol groups of cysteine (Cys), hydroxyl function of threonine and serine, carboxyl groups of aspartic acid and glutamic acid, and Cys-Cys disulfide groups.[4,36]

Chemical Activation of the PEG Molecule

Usually, PEGylation includes chemical activation of the terminal hydroxyl group of monofunctional PEG to make it reactive with protein functions exposed on the protein surface under mild conditions.[36] The use of monofunctional PEG, typically monomethoxy-PEG (mPEG), avoids crosslinking reactions that can yield heterogeneous high-molecular-weight products.[36] Nevertheless, bifunctional PEGs have been used to produce a few relevant therapeutic bioconjugates. For example, pegunigalsidase alfa was obtained by conjugating bis-activated linear 2-kDa PEG to recombinant human alfa-GAL-A.[51] In this case, the HO-PEG-OH chain terminally activated with two N-hydroxyl succinimide reactive groups (NHS-PEG-NHS) reacts prevalently with the two monomers forming the homodimer enzyme. Upon this reaction, a few PEG chains are singularly attached to one protein functional amino group, thereby behaving as a monofunctional activated PEG. The resulting product is a combination of crosslinked PEGylated alfa-GAL-A monomers with a few PEG chains bound either to crosslinked homodimers or un-crosslinked proteins, which results in an average of 8–9 PEG chains per protein monomer.[25,51] All PEGylated homodimers and monomers are biologically active. An advantage of this

approach is the reduced immunogenicity relative to the traditional PEGylation technique, where PEG moieties are usually terminated with a methoxy group, which has been associated with an immunogenic response owing to the hydrophobicity conveyed by the terminal methoxy group.[51]

The first generation of activated PEGs was directed to hyper-derivatization of the primary amino groups of proteins (εNH_2-Lys and terminal NH_2), which are the most accessible and reactive conjugation sites in the protein structure.[36] Usually, in the primary protein structure, there are several Lys residues, and the εNH_2 groups are mainly exposed on the surface where they can be easily derivatized. The selected chemical methods developed to activate the terminal hydroxyl function of PEG for Lys conjugation are summarized in Table 2. These activated PEGs exhibit different reactivities and produce conjugates with different stabilities.

These PEG activations have been found to yield efficient protein conjugation, and a few PEGylated proteins have been developed and approved for clinical applications. The 5-kDa PEG-succinimidyl succinate was used to produce hyper-PEGylated adenosine deaminase (pegademase bovine), asparaginase (pegaspargase), and a GH antagonist (pegvisomant), while 10-kDa PEG-p-nitrophenylcarbonate was used to produce hyper-PEGylated uricase (pegloticase).[4,36] However, the disadvantages of these activated PEGs include the formation of multiple reactions and degradation products, resulting in poor homogeneity, difficulties in purification, poor stability, and toxicity.[36,68] Furthermore, random PEGylation can make it difficult to predict the performance of each isomer of the product as required by regulatory authorities.[4]

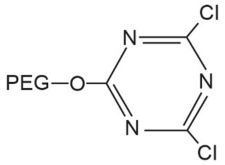
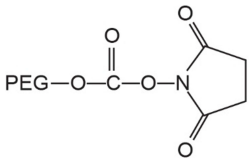
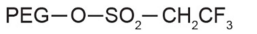
Partially controlled PEGylation yielding a limited number of derivatized amino groups was obtained using PEG-succinimide under controlled conditions (pH, protein/polymer molar ratio, etc). Interferons alpha-2a and alpha-2b were monoPEGylated with one 40-kDa branched PEG-succinimidyl succinate (2×20-kDa PEG chains) and one linear 12-kDa PEG-succinimidyl carbonate to obtain peginterferon alfa-2a[61] and peginterferon alfa-2b,[69] respectively. However, these PEG activations generated a cluster of isomers.[70] In the case of peginterferon alfa-2a, PEG was randomly conjugated to interferon alpha-2a amino groups through stable amidic bonds potentially forming up to 12 isomers.[70] In peginterferon alfa-2b, PEG was conjugated to the protein through a urethane bond forming 14 isomers; the most abundant was the derivative to the His^{34} residue through reversible linkage.[71] mPEG-epoetin beta is a monoPEGylated version of erythropoietin conjugated with 30-kDa PEG-succinimidyl butanoic acid.[72]

Selective Conjugation Procedures

Advances in conjugation strategies have made it possible to modify proteins using site-selective and controlled reactions, enabling rational design of PEGylated proteins for clinical applications.[73]

Selective conjugation of the terminal amino group of proteins is one of the most successful strategies for generating bioconjugates with well-defined structures and has been widely applied. This PEGylation method, which exploits the

Table 2 Properties of Selected First-Generation Activated PEGs

PEG Derivative	Site of Conjugation	Structure	Properties
PEG-dichlorotriazine[4,66,67]	Multiple nucleophilic functional groups, eg Lys, Ser, Tyr, Cys, His		Unspecific; potential crosslinking; toxicological concerns; maintenance of protein charge
PEG-tresylate[4,66,67]	Multiple amino groups		Heterogeneous mixture of products; maintenance of protein charge; degradable linkages
PEG-succinimidyl carbonate[4,36,66]	Preferentially Lys; can also react with His, Tyr		Selective for Lys; conjugation to His results in unstable bond; abolition of protein charge

Abbreviations: Cys, cysteine; His, histidine; Lys, lysine; PEG, polyethylene glycol; Ser, serine; Tyr, tyrosine.

Table 3 Examples of PEGylated Proteins Generated by Selective Conjugation Processes

Name	Parent Drug	Indication	Conjugation
Pegfilgrastim[29,77]	G-CSF	Neutropenia in non-myeloid malignancies	End monoPEGylation with linear 20-kDa PEG-aldehyde derivative
Long-acting PEGylated rhGH[78,79]	rhGH	GH deficiency	End-derivatization with branched 40-kDa PEG
Peginterferon beta-1a[80]	Interferon beta-1a	Multiple sclerosis	MonoPEGylation with 20-kDa PEG-methylpropionaldehyde

Abbreviations: G-CSF, granulocyte colony-stimulating factor; GH, growth hormone; kDa, kilodalton; PEG, polyethylene glycol; rhGH, recombinant human growth hormone.

lower pKa of the αNH_2 group with respect to εNH_2-Lys, is rather simple because the PEGylation selectivity is controlled by the reaction pH.[74] Accordingly, aldehyde-terminating PEGs, typically propionaldehyde-PEG, can form a Schiff base adduct with the terminal amino group of proteins, which can then be reduced to an amino group.[66,74] Examples of therapeutic proteins PEGylated with PEG-aldehyde are listed in Table 3.[75,76]

A more selective PEGylation procedure involves conjugation of the polymer to the thiol groups of Cys residues.[58] In most proteins, Cys is engaged in disulfide bonds (Cys-Cys) that stabilize the structural conformation, whereas, in a few cases, free Cys plays a biological role.[81] When available in the reduced form, Cys is usually located inside grooves of the folded protein to avoid crosslinking and it is not available for PEGylation.[82,83] The strategy to PEGylate Cys includes both the development of activated PEG that preferentially reacts with thiol groups and the introduction of an artificial Cys in the protein sequence to obtain muteins that can be PEGylated by -SH conjugation.[84] In muteins, Cys is introduced into a part of the protein sequence that is not involved in its bioactivity so that its PEGylation does not hamper protein function.[4] PEG-vinylsulfone, PEG-iodoacetamide, PEG-dithyopyridine, and PEG-maleimide react preferentially with Cys, forming polymer-protein bonds with different stabilities.[4] Certolizumab pegol is the PEGylated TNF-alpha inhibitor Fab' fragment obtained by free Cys PEGylation with 40 kDa (2×20-kDa PEG chains) of branched PEG-maleimide.[8,85] PEG-orthopyridyl disulfide specifically reacts with the sulfhydryl groups.[66]

Chemical PEGylation has also been performed on the glycosylic groups of proteins. Glucosyls are oxidized with periodate, and the resulting aldehydes are conjugated to PEG-hydrazine to form reversible hydrazone bonds that can eventually be stabilized by reduction.[4]

Enzymatic PEGylation

Enzymatic PEGylation has been proposed as an alternative to chemical procedures. The use of enzymes enables selective PEGylation of protein tags that are not easily derivatized by chemical protocols and/or result in the production of isomers with defined structures and compositions.

Transglutaminase, an enzyme that catalyzes crosslinking between side chains of Lys and glutamine, has been successfully used to PEGylate glutamine in the protein sequence.[86] For example, transglutaminase was found to selectively conjugate amino-terminating PEG to a glutamine moiety of interferon alfa-2b and GH, generating monoPEGylated isomers.[87,88]

Another enzymatic PEGylation process occurs via a double step that exploits the polypeptide N-acetylgalactosamine (GalNAc) transferase and sialyltransferase. GalNAc post-transcriptionally glycosylates threonine and serine.[4] Subsequently, sialyltransferase conjugates sialic-PEG to the newly introduced GalNAc residue.[4] This strategy was adopted to PEGylate non-glycosylated proteins, including recombinant human G-CSF (lipegfilgrastim, Lonquex), interferon alfa-2b (peginterferon alfa-2b),[4,89] and recombinant coagulation factor IX (nonacog beta pegol, Rebinyn).[8]

Finally, glucose oxidase was used to PEGylate glycosylated proteins. In such cases, the glycosyl moieties are enzymatically oxidized to yield aldehyde groups, which are then modified with PEG-hydrazine, similar to the oxidized products obtained by periodate treatment.[66]

Mechanism of Immunogenicity Against PEGylated Proteins

Although PEGylation may mask epitopes and decrease the immunogenicity of a protein, an immune response to the PEG moiety itself is also possible.[90–92] In general, an immune response toward a PEGylated protein tends to predominantly

consist of antibody formation; however, a T-cell response may be important in recognizing and responding to the protein after it has been taken up and processed by antigen-presenting cells.[19,93]

Humoral Responses

The clinical impact of antibody generation on PEG or PEGylated proteins can vary and is not well known. For example, the consequences, if any, of circulating low levels of antibodies toward PEG found in consumer products are not well understood.[93,94] However, both humoral and cellular responses can potentially lead to adverse events, such as infusion reactions or accelerated blood clearance, resulting in an impact on efficacy. Infusion responses can manifest as mild or, alternatively, as hypersensitivity, cytokine release syndrome, or anaphylaxis in severe cases[11,16,59,95–97] and could also result in immune complex formation[8] leading to inflammation and tissue damage. The frequency and severity of such reactions can be highly variable and depend on the characteristics of the PEG structure and design, the immunogenicity of the conjugated protein, and host factors.

In general, the formation of anti-drug antibodies (ADAs) toward PEG or PEGylated proteins may impact both infusion reactions and efficacy of biologic drugs as an underlying mechanism; however, it is important to note that changes in tolerability and efficacy can also occur independently of each other and may be driven by unrelated mechanisms.[98] The occurrence of infusion-related reactions is relatively common with biologic drugs and does not necessarily indicate a problem with the overall effectiveness of the drug or the formation of ADAs in all cases.[98,99] These reactions are often managed by adjusting the infusion rate, administering premedications, or implementing other supportive measures.[99] In many cases, patients can continue receiving the drug with proper management of their reactions. However, the formation of neutralizing antibodies, especially when targeting clinically relevant epitopes on the therapeutic protein, can lead to reduced efficacy.[98]

Cellular Responses

T-cell-mediated immune responses can occur against therapeutic proteins,[100] including PEGylated proteins. T-cell-dependent immunogenicity can contribute to the initiation of humoral responses, along with T-cell-independent antibody development,[101] although it has also been reported that, in some cases, PEGylation may reduce uptake of therapeutic proteins by dendritic cells and subsequent epitope presentation to T cells.[102]

Overall, the development of cellular immune responses to PEGylated proteins is a complex process that may involve multiple mechanisms. Further research is needed to fully understand the impact of the PEG moiety on the induction or modulation of such responses.

Induction of Immunogenic Memory

Memory T cells, a subset of long-lived T cells, play a crucial role in the rapid response to specific antigens upon re-exposure.[103] Memory T cells can form in response to therapeutic proteins, including PEGylated proteins, with potential clinical implications.[1] For example, researchers have found that individuals who had previously responded to PEGylated interferon alpha for the treatment of hepatitis B had memory T cells that recognized and responded more vigorously to the hepatitis B antigen upon re-exposure, compared with T cells from patients who did not respond.[104,105] This can be particularly problematic for protein-based drugs with a narrow therapeutic window. However, more research is needed to understand the specific role of the PEG moiety in induction of immunogenic memory. It is important to note that, in general, the development of immunogenicity and the subsequent impact on pharmacokinetics can vary between different protein drugs and patient populations.[106] Clinical monitoring, including the assessment of immune responses and pharmacokinetic parameters, can help inform treatment strategies and potentially mitigate the effects of immunogenicity on drug clearance and efficacy.

Factors Which Can Influence the Development of Immunogenic Responses to PEG or PEGylated Proteins

It is important to note that the immunogenicity of PEG is highly variable and depends on the interplay between drug- and host-related factors, including the design of the PEG moiety, immunogenicity of the protein itself, stability, dose, frequency of exposure, route of administration, and genetic, demographic, and medical characteristics of the treated patient.[19,58,97,107] Figure 5 provides an overview of the factors discussed in this section.

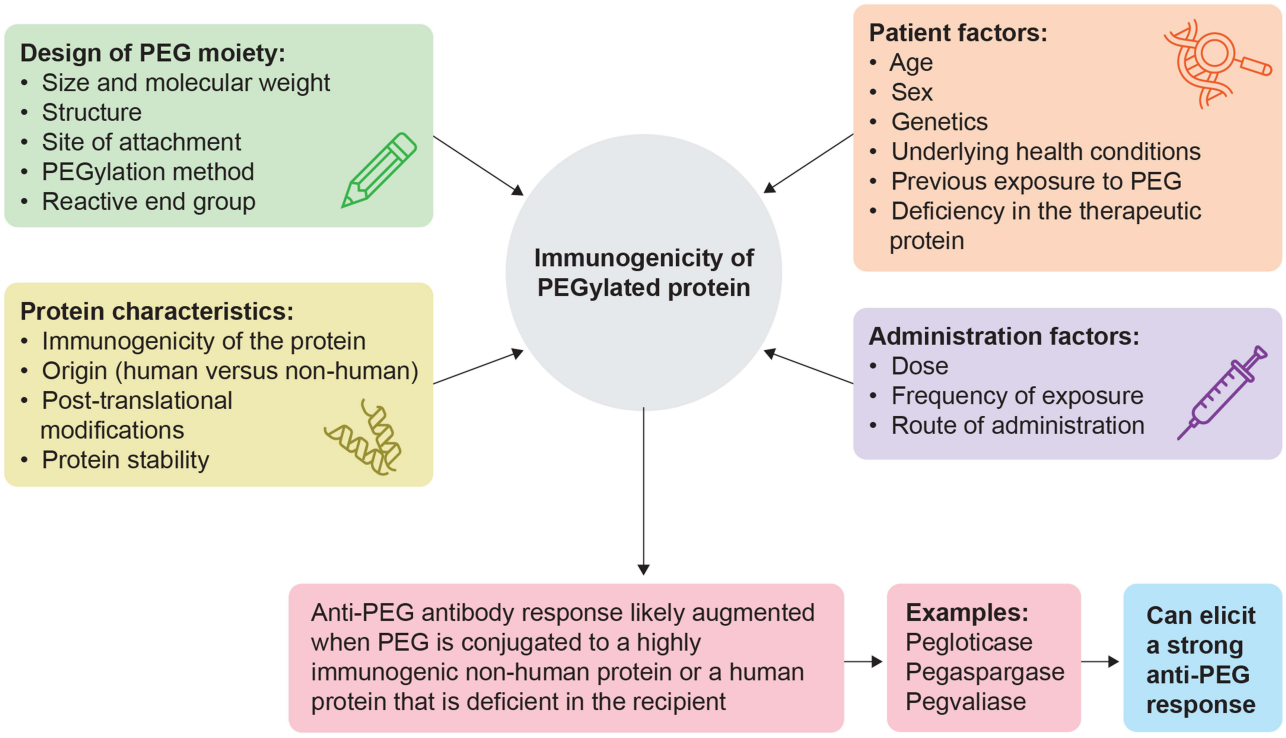


Figure 5 Factors that influence the development of immunogenicity against PEGylated proteins.
Abbreviation: PEG, polyethylene glycol.

The PEG moiety may trigger the development of antibodies via the thymus-dependent pathway when conjugated to a non-human protein/peptide or via the thymus-independent pathway when attached to a non-protein compound or present as a single molecular chain.[19] For PEGylated proteins, the risk of developing anti-PEG antibodies is directly correlated with the immunogenicity of the peptide.[19] In turn, the immunogenicity of the peptide depends on its origin (human or not), post-translational modifications, and the underlying disorder being treated (ie human proteins can be highly immunogenic in patients with a genetic deficiency of these proteins and less immunogenic if the patient has some immune tolerance to residual levels of the endogenous protein).[19] Therefore, higher anti-PEG responses can be expected when using non-human proteins and when the patient is deficient in the therapeutic protein being administered.[19]

Not all individuals who are exposed to PEG will develop antibodies against it, and, at the same time, many healthy individuals may have antibodies against PEG owing to exposure to PEG in cosmetic and over-the-counter products.[93,94] Some factors known to impact the risk of developing an immune response against PEG are described here, but further research is needed to fully understand their interplay and clinical impact.

PEG Size and Molecular Weight

The design of the PEG moiety can influence the degree to which it is recognized as foreign by the immune system. PEGylated structures containing high-molecular-weight PEG have been associated with reactivity to pre-existing antibodies, induction of PEG-specific antibody responses, and hypersensitivity reactions as described with pegloticase for refractory gout (PEG 10 kDa)[10,19,108,109] and pegvaliase (Palynziq) for phenylketonuria (PEG 20 kDa).[19,110] On the other hand, the incidence of anti-PEG antibodies observed in trials of pegunigalsidase alfa (PEG 2 kDa)[24] has been low (5.8–6.7%).[111,112] Thus, PEG size can be a factor in determining immunogenicity; however, the specific threshold or range of PEG sizes implicated in immunogenicity can vary between different therapeutic proteins or peptides and is not universally defined. Interestingly, it has been reported that reactivity in the skin prick test with native PEG decreased over time in patients considered allergic to PEG when lower-molecular-weight PEG was tested, but increased with PEG of 20 kDa or higher. Thus, PEGs with higher molecular weights seem to be more reactive.[113] Nevertheless, PEG antibodies

do not always show a bias for longer PEG molecules,[114] indicating an interplay with additional factors, including PEG attachment site and shape, density, and PEGylation methods.

PEG Structure and Site of Attachment

PEG can exist in different conformations, such as random coils, helices, and linear or branched crystalline forms. The attachment of PEG to a protein can theoretically potentially further impact its structural conformation and expose new epitopes to the immune system. The size and shape of the PEGylated protein can also impact its uptake by antigen-presenting cells and subsequent presentation to the immune system. Understanding the relationship between these factors and the immune response is important for optimizing the efficacy and safety of PEGylated protein therapies. While data on the relative immunogenicity of different PEG structures are still limited, some studies have suggested that certain structural characteristics, including PEG chain length, linker type, and branched versus linear shape, may play a role in the development of antibodies against PEG.[115]

Random PEGylation, which attaches PEG to various sites on the protein, can increase the likelihood of ADA formation owing to the potential for altering the protein's structure[5,36] and exposing new epitopes. In contrast, site-specific PEGylation minimizes these changes,[5] reducing the likelihood of immunogenicity. Site-specific conjugation strategies, such as those targeting Cys residues or terminal amino groups,[5,36] tend to produce more homogeneous and less immunogenic products.

Branched PEGs, which offer higher surface PEG density, have been associated with enhanced immune shielding compared with linear PEGs.[28,63] However, the increased complexity of branched PEGs might also lead to a more pronounced immune response in some cases, depending on how they interact with the immune system.

Higher-molecular-weight PEGs, such as those used in peginterferon alfa-2a (40 kDa branched PEG),[4] generally provide better protection against proteolytic degradation and immune recognition.[70] However, in some cases, large PEG structures might themselves become immunogenic, particularly if they alter the protein's conformation[115] or mask critical epitopes.

PEG molecules typically end with a methoxy group, which is hydrophobic. This hydrophobicity has been associated with increased immunogenicity in some cases,[10] as it may attract immune cells. Newer strategies are exploring alternatives to reduce this effect.

Certolizumab pegol, a TNF-alpha inhibitor, has been PEGylated using a 40 kDa branched PEG[8] to reduce immunogenicity. Despite this, some patients still develop ADAs,[85] which can impact the drug's efficacy. With peginterferon alfa-2a, random PEGylation of interferon alfa-2a with a 40 kDa branched PEG has resulted in the formation of multiple isomers.[70] While effective, the presence of these isomers could contribute to ADA formation in some patients.

Administration

The dose, frequency, and route of administration of PEGylated protein therapy can also affect the immune response. Higher doses or more frequent administration may increase the likelihood of an immune response, although, in some cases, intermittently administered lower doses may be more immunogenic than continuously administered larger doses.[96] In some cases, subcutaneous administration may be more likely to elicit an immune response, theoretically due to the more frequent encounters between the antigen and migratory skin-resident and lymph node-resident dendritic cells compared with the intravenous route. Nevertheless, this is not always the case and is dependent on other co-existent factors, as described in this paper.

Protein Stability

In-vivo protein instability may affect immunogenic potency, as it has the potential to generate neoepitopes. In some cases, PEGylation of the protein can increase its stability, such as PEGylation of human alfa-GAL-A in pegunigalsidase alfa. Alfa-GAL-A is a non-covalently bound homodimeric glycoprotein where dimerization is important for its enzymatic activity and stability.[116,117] Modification with bis-NHS-PEG produces PEGylated covalently linked protein homodimers in the enzyme replacement therapy, pegunigalsidase alfa. This modification improves the stability in vitro under lysosomal and plasma conditions.[51]

Host Factors

Patient age, sex, genetics (especially expression of specific major histocompatibility complex alleles), and underlying health conditions (eg cancer patients receiving chemotherapy) can also affect the immune response.[118,119] Regarding the specific response to the PEG moiety, patients who have previously been exposed to PEG or similar molecules, particularly in the context of vaccine adjuvants, may have an increased likelihood of an immune response.[28,94]

Patients with an atopic constitution, characterized by a heightened immune response to allergens, may be more prone to developing ADAs against PEGylated proteins.[93] This is particularly relevant in individuals with a history of allergies, asthma, or other atopic conditions. These patients may have a more reactive immune system which may recognize PEGylated proteins as foreign, leading to the production of ADAs. For atopic patients, closer monitoring for signs of immunogenicity, such as reduced therapeutic efficacy or allergic reactions, may be warranted. This could include regular assessments of ADA levels or switching to alternative therapies with lower immunogenic risk.

Certain human leukocyte antigen (HLA) types have been associated with an increased risk of immunogenicity to biologic therapies,[120] including PEGylated proteins. Patients with these HLA types might be more susceptible to developing ADAs. Genetic screening could potentially identify patients at higher risk, allowing for more personalized treatment approaches.

Patients who have previously been treated with biologic therapies may have pre-existing antibodies[1] that could cross-react with new PEGylated proteins. This is particularly relevant if the new therapy shares structural similarities with previous treatments. When switching from one biologic to another, particularly within the same class, there is a risk of ADA formation owing to cross-reactivity. Careful consideration of the patient's treatment history and potential for immunogenicity is essential.

Patients with compromised immune systems may be less likely to develop ADAs[120] but, if they do, the consequences could be more severe. Immunosuppressed patients may not mount a typical immune response, leading to atypical presentations of immunogenicity or delayed ADA formation. The aging immune system is typically less responsive,[121] which could reduce the likelihood of ADA development. However, this same reduced responsiveness might also lead to suboptimal therapeutic responses, making the balance between efficacy and immunogenicity critical in elderly populations.

Antigenicity of the PEGylated Protein

The characteristics of the protein to which the PEG moiety is conjugated can impact the development of an anti-PEG response and its mechanism. In healthy individuals, there is immune tolerance toward self-proteins because of an early negative selection process in the thymus; therefore, a thymus-dependent immune response to PEG will only be mounted if PEG is linked to a non-human peptide or protein (eg pegloticase)[19] or a human protein not recognized as self. The latter occurs when a PEGylated protein is administered therapeutically to a patient with a genetic deficiency of the same protein. In this case, the patient's immune system does not recognize it as self, and a thymus-dependent response to any epitope on the conjugated protein can be mounted. On the other hand, heavily PEGylated human proteins can activate B cells via crosslinking of B-cell receptors; however, unless the innate immune response is also activated (eg by aggregates, impurities, or post-translational protein modifications), the anti-PEG antibody response is likely to be weak.[19]

As with thymus-dependent responses, the thymus-independent response to a PEGylated human protein can be stronger if the patient has an underlying genetic deficiency for the protein, resulting in a lack of tolerance. In this case, antibody responses can be generated not just against the therapeutic protein but also against the PEG molecule itself (eg damoctocog alfa pegol [Jivi], a recombinant B-domain-deleted human factor VIII modified with a branched 60-kDa PEG molecule).[122] Overall, it appears that the induction of an anti-PEG antibody response may be augmented when PEG is conjugated to a highly immunogenic non-human protein or a human protein that is deficient in the recipient.[19] Examples include pegloticase, pegaspargase, and pegvaliase, which elicit a strong anti-PEG immunogenic response to conjugated non-human proteins.

Pegloticase, a PEGylated recombinant porcine-like uricase, was approved by the FDA for the treatment of chronic gout, but its use is limited owing to the occurrence of anti-PEG antibodies, leading to increased drug clearance and infusion reactions. Similarly, increased clearance has been observed owing to anti-PEG antibodies in patients receiving

pegaspargase, formulated by PEGylating *E. coli*-asparaginase, for childhood acute lymphoblastic leukemia despite the absence of clinical evidence for allergic reactions.[11] In the case of these PEGylated proteins, the structure and composition of the protein itself likely played a role in the immunogenicity observed, as protein origin, structure, or sequences can trigger immune responses more readily than others when combined with PEGylation, as described in the sections above.

Strategies to Overcome Anti-PEG Immunogenicity

The use of alternative polymers with less or no immunogenicity and antigenicity compared with mPEG is a potential approach. Caliceti et al (2001) studied the immunogenicity and antigenicity of mPEG 5000 g/mol, branched mPEG 10,000 g/mol, polyvinylpyrrolidone 6000 g/mol, and poly(N-acryloylmorpholine) 6000 g/mol conjugated to uricase.[123] They discovered that branched PEG was the least immunogenic and antigenic in this context. Sherman et al (2012) demonstrated that hydroxy-PEG has a lower immunogenicity than mPEG derivatives of porcine uricase and other proteins.[124] Another method to reduce or prevent an anti-PEG response is to administer a PEG-containing compound consisting of a core molecule with short PEG oligomers before administering a PEGylated drug, which may help to avoid the formation of immune complexes.[125]

Pre-Existing Anti-PEG Antibodies in the General Population

Richter and Akerblom (1984) reported a low occurrence of anti-PEG antibodies (0.2%) in the healthy population four decades ago,[126] compared with more recent findings by Yang et al (2016) of a very high occurrence (72%).[94] Yang et al (2016) investigated whether these differences showed a true increase in anti-PEG antibody levels over time by quantifying levels in healthy human serum samples banked from the 1970s, 1980s, and 1990s, finding anti-PEG antibodies in 56% of these historical samples.[94] This suggests that immunological responses to PEG are longstanding.[94] Values reported for PEG antibodies in the literature may differ depending on the assay technique used. Nevertheless, absolute anti-PEG antibody titers remain low in most positive individuals.[19]

Exposure to PEG through various sources, such as medications, cosmetics, and food, can lead to the development of anti-PEG antibodies in some individuals.[94,127] In addition, a study analyzing the entire genome revealed that the occurrence of pre-existing anti-PEG IgM antibodies is linked to a particular variable segment of the immunoglobulin heavy-chain gene, indicating that there may be a genetic factor influencing PEG immunogenicity in some individuals.[97] However, the mere presence of these antibodies does not necessarily imply a clinical sensitivity or adverse reaction to PEGylated drugs.[127]

Despite the reported high prevalence of anti-PEG antibodies in the general population,[94] PEGylated COVID-19 vaccines have been safely administered to most patients.[128] For example, a post-hoc analysis of clinical trial data found that the administration of a COVID-19 mRNA vaccine did not increase the risk of infusion-related reactions or ADAs in patients treated with pegunigalsidase alfa.[129] With dose adaptation and monitoring, a PEGylated COVID-19 mRNA vaccine was administered to 12 children with anti-PEG antibodies and a known allergy to pegaspargase without any post-vaccination allergic reaction.[130] Furthermore, differential induction of anti-PEG antibodies by the mRNA-1273 (Moderna) and BNT162b (Pfizer-BioNTech) COVID-19 vaccines has been observed, and no obvious association between PEG antibodies and adverse reactions to these vaccines has been reported.[127] This scenario parallels the situation where individuals may test positive for antibodies to certain food compounds but do not exhibit clinical sensitivity or adverse reactions upon consuming these foods.

As more research is conducted and data are gathered, a better understanding of the clinical significance of pre-existing anti-PEG antibodies and their impact on PEGylated drug therapy will be gained. Regulatory agencies, such as the FDA, actively monitor and assess the potential risks associated with pre-existing anti-PEG antibodies. This includes the ongoing evaluation of adverse events and the development of guidelines and recommendations for the use of PEGylated drugs in individuals with pre-existing anti-PEG antibodies.

PEGylated Liposomes and Nanoparticles

In addition to protein conjugation, PEG can be incorporated into liposomes. One example is PEGylated liposomal doxorubicin, a chemotherapy drug that uses PEGylated liposomes to enhance drug delivery to the target site and reduce side effects.[131–133] Several mRNA-based COVID-19 vaccines, such as Pfizer-BioNTech and Moderna vaccines, utilize PEGylated lipids in their lipid nanoparticle formulations.[134,135] These PEGylated lipids help to protect mRNA and improve its delivery into cells for efficient translation of the viral spike protein. Repeated administration of PEGylated liposomes may trigger an immune response, leading to accelerated blood clearance and an increase in hepatic and splenic accumulation of the second dose of PEGylated liposomes following the first injection.[133,136,137] The characterization of PEGylated liposomes is beyond the scope of this review.

Conclusions

In conclusion, PEGylation is an established technology for translation of pharmacologically active proteins into effective therapeutics by enhancing their pharmacokinetic, immunological, and biophysical behavior; however, it can also have certain drawbacks that limit its application. Immune response toward PEG in some individuals or triggering of hypersensitivity reactions, in particular, represents one of the main limits to its application. However, it is important to note that immunogenicity and potential adverse events associated with PEGylated proteins can vary widely based on the specific characteristics of the PEG moiety, immunogenicity of the native protein, dose and route of administration, and host factors. The design and optimization of PEGylation strategies that consider parameters such as polymer size and shape, density, and conjugation sites are paramount to minimize the generation of immune responses. Nevertheless, according to the dependence of the immune system on genetic and/or individual aspects, additional uncontrolled and unknown factors can affect the immunogenic profile of PEGylated proteins. Thorough preclinical and clinical assessments and post-marketing surveillance are necessary to identify and understand the immunogenic potential of PEGylated proteins. There have been reports of a high prevalence of anti-PEG antibodies in the general population, likely owing to exposure to PEG from different consumer and medicinal products. While these antibodies tend to occur at low titers and their clinical significance is not well understood, it is noteworthy that PEGylated COVID-19 mRNA vaccines, for example, appear to be safe based on findings from ongoing surveillance.[128]

The clinical implications of PEGylation are substantial, particularly in improving the therapeutic efficacy and safety profiles of protein-based drugs. PEGylation has enabled the development of drugs with prolonged half-lives, reduced dosing frequency, and improved patient compliance. However, the potential for immunogenic responses necessitates careful consideration in the clinical use of PEGylated therapeutics. Clinicians must be aware of the potential for hypersensitivity reactions and monitor patients accordingly, especially those with a known history of allergies or repeated exposure to PEG-containing products. Equally, clinicians should be aware that the properties of PEGylated proteins, including immunogenicity, depend on several different factors as described in this review, and clinicians should not extrapolate attributes from one PEGylated molecule to another.

Further research is essential to address the remaining challenges and enhance the therapeutic potential of PEGylated proteins. Key areas for future investigation include:

1. Understanding immunogenicity: Elucidating the factors that impact the risk of PEG-induced immunogenicity and identifying strategies to mitigate these responses.
2. Optimizing PEGylation: Developing novel PEGylation techniques that maximize therapeutic efficacy while minimizing adverse immune reactions.
3. Factors influencing long-term safety: Conducting long-term studies to assess the safety and efficacy of PEGylated therapeutics, particularly in populations with high anti-PEG antibody prevalence. Assessing the impact of pre-existing ADAs on PEGylated proteins and how the impact is influenced by host- and drug-related factors.
4. Personalized medicine: Exploring the potential of personalized PEGylation strategies tailored to individual patient profiles to optimize therapeutic outcomes and minimize adverse effects.

With this review we have aimed to demonstrate the range of different effects of PEGylation and the need to further investigate what drives these differences. Overall, the PEGylation of therapeutic proteins represents a significant biotechnological advancement, and methods continue to be optimized. A comprehensive understanding of the factors impacting immunogenic responses to PEGylated proteins is crucial for deriving the greatest therapeutic benefit.

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Author Contributions

Both authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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