Review

Discussion about Several Potential Drawbacks of PEGylated Therapeutic Proteins

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PEGylation changes the physical and chemical properties of the biomedical molecule, such as its conformation, electrostatic binding, and hydrophobicity, and results in an improvement in the pharmacokinetic behavior of the drug, while it also causes some disadvantages of which cannot be neglected. The available data manifests that polyethylene glycol (PEG) itself shows potential risk, such as immunogenicity of the PEG and PEG-containing vacuoles in cells observed with PEGylated biologicals. Decreased activity and heterogeneity are also the negative aspects of PEGylation. The unfavorable impacts which are brought by the PEGylation are described here with examples of modified therapeutic proteins on the market and used in the clinical trials.

Key words PEGylation; therapeutic protein; potential risk

1. INTRODUCTION

Polyethylene glycol (PEG) has been used as a biocompatible modifier for a variety of enzymes and proteins to obtain an increased *in vivo* retention time, reduction in toxicity, antigenicity and immunogenicity.[1)] PEGylation, the addition of PEG molecules to a protein, which is a triumphant strategy for changing the physical and chemical properties of the biomedical molecule through the covalent attachment of PEG chains becomes widely used across biological industry. The unremitting interest in PEGylation is well documented by a number of papers and patents that have appeared since the discovery of this methodology by Davis and Abuchowski in the late 1970s.[2)] PEGylation has delivered sustained success of many therapeutic proteins, see Table 1, together with the number of protein conjugates entering clinical trials.

Although PEG conjugation to protein has been used as a method for extending the circulating half-life of many therapeutic proteins, PEG itself does carry some potential safety risks, such as the antibody formation against PEG (anti-PEG), hypersensitivity to PEG and vacuolation, which restrict more extensive use of the PEGylation.

2. THE ANTI-PEG AND HYPERSENSITIVITY TO PEG

2.1. The Increasing Occurrence of Anti-PEG In contrast to the accepted general assumption that PEG has long been claimed to be non-antigenic and weakly immunogenic,[14–18)] animal studies clearly indicated that PEGylated agents can elicit anti-PEG.[19–24)] And a great number of publications have claimed that the PEG moiety of these drugs in itself may be immunogenic and can induce anti-PEG antibodies. Richter and Akerblom[19)] determined that the occurrence of anti-PEG in the healthy population was 0.2% two decades earlier, compared with a very high 22–25% occurrence in the recent finding.[25–27)] Of course, because of the lack of reference sera and a lack of data on the validation and specifications of the assay, it is impossible to draw the conclusion of an increasing incidence of anti-PEG antibodies in the healthy donor population.[28)] The different results may be due to an improvement of the limit of detection of antibodies during the years or to greater extensive exposure to PEG in everyday products, industrial applications, as well as pharmaceutical industry as surfactants, dispersing agents or medications.[29)] In summary, it is significant to recognize that PEG is both immunogenic

Table 1. Some Marketed PEGylated Biopharmaceuticals

Brand name	PEG conjugates	Indication	Approved year	Ref.
Adagen	Pegademase bovine	Severe combined immunodeficiency disease (SCID)	1990 (U.S.A.)	3
Oncaspar	Pegaspargase	Leukemia	1994 (U.S.A.)	4
PegIntron	Peginterferon alfa-2b	Hepatitis C	2000 (EU) 2001 (U.S.A.)	5
Pegasys	Peginterferon alfa-2a	Hepatitis C	2002 (U.S.A., EU)	6
Neulasta	Pegflgrastim	Neutropenia	2002 (U.S.A.) 2003 (EU)	7
Somavert	Pegvisomant	Acromegaly	2002 (U.S.A.) 2003 (EU)	8
Macugen	Pegaptanib sodium	Neovascular (wet) age-related macular degeneration	2004 (U.S.A.) 2006 (EU)	9
Mircera	mPEG-epoetin beta	Anemia associated with chronic kidney disease	2007 (U.S.A., EU)	10
Cimzia	Certolizumab pegol	Rheumatoid arthritis and Crohn's disease	2008 (U.S.A.)	11
Krystexxa	Pegloticase	Chronic gout	2010 (U.S.A.)	12
Omontys	PEGinesatide	Anemia associated with chronic kidney disease	2012 (U.S.A.)	13

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and antigenic.[30,31] Further comprehensive researches are warranted to fully evaluate the impact of anti-PEG antibodies on PEG conjugates. The increasing use of PEG and PEGylated therapeutic proteins does really increase the likelihood of encountering potential side reactions. Firstly, polymer itself can lead to hypersensitivity indirectly by side products formed during synthesis. Secondly, some adverse changes in the pharmacokinetic behavior appear with the PEGylated drugs. What's more, due to the non-biodegradability of PEG, there are some unforeseen effects of the remains. All these potential drawbacks will be discussed in the following.

2.2. The Likely Impact of Immunological Response There are emerging reports of delayed and immediate hypersensitivity reactions to PEG-containing substances and immune reactions to PEG are becoming more evident: De Groot *et al.* reported three cases of anaphylactic shock as a reaction to SonoVue (commercial contrast agent containing PEG).[32] Similarly, there are 7 cases of anaphylaxis to the PEG-containing sulphur hexafluoride that have been described in Europe, while none of these reactions have been reported in similar non-PEG-containing agents.[33] Later, Pérez-Pérez *et al.* published a paper described a patient that developed biologic-induced urticaria due to polysorbate 80 (a PEG-containing polymer).[34] These cases highlight the consideration of hypersensitivity to PEG. PEGs are multivalent and potentially large enough to elicit an immune response without haptenation. The mechanism by which development of PEG allergy occurs is not entirely clear, so further comprehensive studies are required to fully elucidate the essence of the phenomenon.

The accelerated blood clearance (ABC) phenomenon results from the potential immune reaction to the presence of PEG. It is generally believed that liposomes modified with PEG have no or lower immunogenicity. However, based on many recent literatures, when the PEGylated liposomes were repeatedly applied to the same animal, the immune responses occurred. The first injection of PEGylated liposomes resulted in a reduction in the circulation time and an increase in hepatic and splenic accumulation of the second dose of PEGylated liposomes in a time-interval, which was called "ABC" phenomenon.[35–37] It has been shown that the effect of prolonged circulation of intravenously injected PEGylated liposome disappears, if the second dose is injected in a few days later, due to the production of anti-PEG immunoglobulin M (IgM).[38] The mechanism of ABC is still not fully understood, but based on the available reports, the ABC phenomenon can be explained: the formation of anti-PEG IgM in the spleen occurs upon the first injection; then the IgM binds to the second dose and subsequently activates the complement system, thereby resulting in opsonization of PEG with C3 fragments and an enhanced uptake by Kupffer cells.[39,40] This phenomenon has been induced by pre-treatment with not only PEGylated liposomes but also PEGylated proteins.[41] Such immunogenicity of PEG presents a barrier in the research of their use in the clinics because it affects the bioavailability of the drug, decreases the therapeutic efficacy of encapsulated drugs and may cause adverse effects due to the altered biodistribution of the drug.

Uricase (EC 1.7.3.3, UC) is an liver enzyme that metabolizes uric acid into allantoin, acting in the purine degradation pathway.[42,43] All mammals produce uricase, except humans and certain primates.[44,45] Administration of uricase has proved to be a good alternative to treat gout, however, since humans do not express uricase, the enzyme would be expected to be by the immune system as a foreign protein. So PEGylation is a good choice, which is used to widen the therapeutic and biotechnological uses of proteins and to enable a more convenient dosing regimen because of its high solubility, low toxicity, and low immunogenicity.[46–50] In the initial phase 1 trial, PEG-UOX was administered by subcutaneous injection to human subjects with refractory gout, however, in several subjects the circulating life and efficacy of PEG-UOX was foreshortened by the induction of anti-PEG,[51] which was specific against the PEG residue rather than against the uricase itself.[52] In 2010, the U.S. Food and Drug Administration (FDA) approved pegloticase (trade name Krystexxa), a PEGylated recombinant porcine-like uricase, for the treatment of chronic gout in adult patients refractory to conventional therapy. In the current situation, pegloticase is probably the most powerful drug to treat the disease, but its use is hampered by occurrence of anti-PEG, leading to increased drug clearance and may bring about risk of subsequent infusion reaction.[53] PEG-asparaginase (PEG-ASNase) is an active agent in the treatment of childhood acute lymphoblastic leukemia (ALL). But the phenomenon of increased drug clearance has been observed in a subgroup of pediatric patients treated for ALL without any clinical evidence of an allergic reaction.[54,55] Armstrong *et al.*[56] showed that the presence of anti-PEG was very closely associated with rapid clearance of PEG-ASNase.

2.3. The Strategies to Overcome Anti-PEG a. Choosing appropriate polymers with less or no immunogenicity and antigenicity as candidates for drug conjugation compared to methoxy-PEG (mPEG) is an alternative approach: Caliceti *et al.*[57] investigated the immunogenicity and antigenicity of mPEG 5000 g/mol, branched m-PEG 10000 g/mol, polyvinylpyrrolidone 6000 g/mol and poly(*N*-acyloylmorpholine) 6000 g/mol conjugated to uricase. They found the branched PEG was the least immunogenic and antigenic.[57] Sherman *et al.*[58] showed that hydroxy-PEG has lower immunogenicity compared with mPEG derivatives of porcine uricase and other proteins.

b. There is another way to decrease or suppress anti-PEG by infusing a PEG-containing compound which is comprised of a core molecule with short PEG oligomers, prior to administration of a PEGylated drug. It can avoid forming immune complex.[59]

3. THE PEG-ASSOCIATED CYTOPLASMIC VACUOLATION

There are reports of increased vacuolation in tissues from animals administered PEGylated proteins. In 1998, using Sprague-Dawley rats, Bendele *et al.* showed that PEG-tumor necrosis factor (TNF)-binding protein (bp) is potential to form vacuoles in the renal tubular epithelium.[60] They found that TNF-bp conjugated to a larger PEG (PEG_{50K}) produced less severe renal tubular vacuolation than TNF-bp conjugated to PEG_{20K}.[60] Not come singly but in pairs. Recently, Rudmann *et al.*[61] reported that the vacuolation observed with unconjugated high molecular weight (HMW) PEGs are markedly influenced by the molecular weight of the PEG. The molecular weight of the PEG can impact the tissue distribution.[61,62] Yamaoka *et al.*[62] drew important inclusions: Firstly, clearance of PEG decreases markedly as molecular weight increases. Urinary clear-

ance is the major excretion pathway for PEGs, with markedly reduced urinary clearance observed as the molecular weight exceeds 20 kD. Secondly, Biliary excretion is also molecular weight-dependent. Hepatic clearance reaches a minimum at about 50 kD molecular mass and lower or higher molecular weight PEGs show increased hepatobiliary clearance. But there is a different mechanism between them. The increasing number of Kupffer cells absorb PEGs when PEG molecular weight increases over 50 kD. Thirdly, although both the liver and kidney play a significant role in the excretion of PEG, urinary excretion of PEG is still the major route of elimination when the molecular weights up to and including 190 kD, compared with hepatobiliary clearance representing a minor pathway. Maybe the conclusions can explain the phenomenon in Alison Bendele's experiment, which is TNF-bp conjugated to a larger PEG (PEG_{50K}) produced less severe renal tubular vacuolation than TNF-bp conjugated to PEG_{20K}. There are some marketed PEGylated drugs which could cause cytoplasmic vacuoles in cells. For example, Cimzia that is approved by the FDA in 2008 for chronic administration to control Crohn's disease can give rise to vacuoles in macrophages and other multiple organs in monkeys and rats; Somavert is an another drug which can cause vacuoles in lymph nodes and spleens and similarly the development of vacuoles were observed in adrenal gland and aortic endothelia in dogs treated with pegloticase.[63–65] Rudmann *et al.*[61] indicated that PEG immunoreactivity was demonstrated in the cytoplasm of vacuolated cells by developing an Immunological Histological Chemistry (IHC) procedure. The vacuoles might include PEGs or the accumulation of PEGs results in the vacuolation. Although severe side effects of the accumulation have not been reported and PEG-associated vacuolization *in vitro* cells is without apparent toxicologic significance, consequences of life-long therapies with high dosages of PEG–protein conjugates containing PEG conjugates of high molecular weight are hardly predictable. Occasional warnings that significant PEG–protein accumulation in the liver may increase the risk of toxicity have appeared.[31,66,67] For the drug pegloticase, due to the high prevalence of cardiovascular diseases in the intended patient population, the potential accumulation of the drug in vacuoles in the endothelial cells may increase the incidence of the patient developing a cardiovascular event, such as atherosclerosis.[64] And accumulation of vacuoles in the proximal tubules of the renal epithelium is of particular concern in patient populations that have a high incidence of renal failure, such as diabetics.

On the one hand, although the recently researches show that vacuolization don't produce apparent effect on cell function or viability, the absence of pathology associated with PEG-related vacuolization in animals and extensive clinical use over many patient years provides evidence of safety to support the use of PEG for PEGylated therapeutic proteins. We should perform systematic long-term studies on the influence of vacuoles at the sites of accumulation. The genesis of vacuolization and the potential impact of slow or no reversibility requires further understanding; On the another hand, some more secure PEGylated techniques, for example, which cannot lead to vacuolation, are necessary. Polytherics Ltd. designed a sort of new PEGs named PolyPEGs[68] which differ from linear or branched PEGs in that they comprise a poly(methacrylate) backbone with short pendent PEG teeth attached, stretching out in parallel like teeth along a comb. The researchers said that PolyPEG does not accumulate in vacuoles in the liver and kidney when administered repeatedly.[68]

4. HETEROGENEITY OF PEGYLATED DRUGS

PEGylation is not a simple process: if you want to ensure the benign effect of it on the pharmacokinetic properties and biological activity of modified compounds, some significant parameters must be considered, which include the molecular weight and architecture of the PEG, the number of attached PEGs (monoPEGylated, diPEGylated, *etc.*), and the possible location of PEG attachment sites. A number of techniques can be used to address these problems, such as targeting specific amino acids by advances in the area of PEG-coupling chemistries. However, the importance of nonspecific coupling chemistries in existing commercial processes and technologies renders the analysis of heterogeneity in products resulting from this type of coupling reactions an important challenge.[69] And it is difficult to separate different monoPEGylated isomers from each other and some isomers may vary in biologic activity so it is a tough work to control or predict the properties of the mixture.

5. OTHER DISADVANTAGES OF PEGYLATION

The influences of PEGylation on the biological activity and bioavailability of the PEGylated product have been reviewed according to the references. PEG chains can surround the protein aiming at shielding and protecting it from the environment, but they also modify the interaction capabilities of the protein that answer for its biological function. So it can reduce the binding affinity and biologic activity of the molecule. Depending on the method of PEGylation and the weight of bound PEG, activity retained in the PEGylated product may very widely, from 7% to 98%.[16] Another unexpected behavior was observed for PEGylation: addition of PEG yielded stronger viscosity. Kerwin *et al.*[70] showed that a mixture including PEG and TNF receptor1 had a five times higher viscosity than that of PEG alone or TNF receptor1 alone. PEG can be single-chained or branched. The latter increases protein solubility, decreases viscosity, and could further increase protein half-life, but it is more expensive. We can choice a suitable type in line with our purposes.

6. CONCLUSION

Promising applications of the PEGylated proteins and peptides have been found in biotechnological and biomedical fields due to the improved solubility, thermal and mechanical stability, reduced antigenicity and immunogenicity, enhanced circulating half-lives, optimized pharmacokinetic and pharmacodynamic properties. However, because of the existence of anti-PEG, vacuoles related to PEGs and other disadvantages, the extensive use of the PEGylation is limited in some extent. Certainly, the Review does not wish to create the impression that PEGylation should be avoided. On the contrary, this method should be used more appropriately and widely. To solve these problems, on the one hand, we should research the different modified reaction conditions for various PEGs, which aimed at confirming the optimal modification degree and the molecular weight of product. By doing so, we can minimize

the toxicity and immunogenicity, and could get the right balance between extending the time of blood clearance and keeping a suitable diffusion speed for conjugates. On the other hand, designing to synthesize new PEG reagents, which could achieve the site-directed PEGylation modification, is very essential. And it will also eliminate or reduce the unfavorable influence on the biological activity. Of course, to say the least, PEGylation is not the only approach for protein in conjugation and new techniques are now emerging as alternatives using polymers like PASylation,[71] XTEN sequences[72] and polysialic acid (PolyXen[67]).

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