

**Summaries Veronese (ed.):
PEGylated Protein Drugs: Basic Science and Clinical Applications**

Ruth Duncan and Francesco M. Veronese
Preface: PEGylated protein conjugates: A new class of therapeutics for the 21st century

Summary
Francesco M. Veronese, Anna Mero and Gianfranco Pasut
Protein PEGylation, basic science and biological applications

A historical overview of protein-polymer conjugation is reported here, demonstrating the superiority of poly(ethylene glycol) (PEG) among other synthetic or natural polymers, thanks to its unique properties like the absence of toxicity and immunogenicity, and a high solubility in water and in organic solvents. Furthermore, PEG is approved by the FDA for human use. Relevant physicochemical and biological properties of PEG and PEG-conjugates, as the basis of the pharmacokinetic and pharmacodynamic improvements, are reported here and discussed in view of successful therapeutic applications. The chapter also highlights that, although PEGylation is well studied and exploited by many researchers from both academia and industry, it remains difficult to forecast its effects on a predetermined bioactive molecule. The use of PEG-enzymes in bioconversion, which is of interest in drug discovery and production, is also briefly described.

Keywords: PEG, protein PEGylation, polymer therapeutics, protein immunogenicity, protein pharmacokinetics, biocatalysis

Summary
Gian Maria Bonora and Sara Drioli
Reactive PEGs for protein conjugation

Poly(ethylene glycol) (PEG) derivatives are the first choice of the water soluble, biocompatible polymers on hand for conjugation to proteins and polypeptides. This chapter deals with the PEG reagents that are available for the preparation of bioconjugates. The opportunities of different reactive groups on PEG are described and their different activities against the functional moieties of the amino acids are illustrated. Some attention is also given to the modification of the PEG backbone to increase its loading capacity and to eventually modify the stability of the conjugating bonds.

Keywords: PEG, protein PEGylation, reactive PEG, functional groups, PEG modifications

Summary

Ji-Won Choi, Antony Godwin, Siby Balan, Penny Bryant, Yuehua Cong, Estera Pawlisz, Manuchehr Porssa, Norbert Rumpf, Ruchi Singh, Keith Powell and Steve Brocchini
Rebridging disulphides: site-specific PEGylation on natural amino acids by sequential bis-alkylation

Site-specific PEGylation reagents have been developed that undergo thiol-specific bis-alkylation with the two cysteine sulphur atoms from a native accessible disulphide in proteins. The process for this approach of site-specific PEGylation involves two steps: (1) disulphide reduction to release the two thiols and (2) bis-alkylation of the PEG reagent to the two sulphur atoms to give a three-carbon bridge to which PEG is covalently attached. Mechanistically, the conjugation is thought to occur by a sequential, interactive bis-alkylation that requires functionalised PEG reagents that have a α, β -unsaturated α -mono-sulphone moiety. Competitive reactions can be effectively suppressed to achieve high yield PEGylation with a stoichiometric equivalence of the reagent. The reagents are easily prepared and precursor forms of our PEG reagents can be used to control the rate to form the reactive PEG mono-sulphone in situ that undergoes conjugation with the protein. Purification is often a simple process where unPEGylated protein can be easily recycled to further increase yields. Many classes of therapeutically relevant proteins possess accessible native disulphide. Our studies have shown that peptides, proteins, enzymes and antibody fragments can be site-specifically PEGylated by bis-alkylation using a native, accessible disulphide.

Summary

Mauro Sergi, Francesca Caboi, Carlo Maullu, Gaetano Orsini and Giancarlo Tonon
Enzymatic techniques for PEGylation of biopharmaceuticals

Modification of therapeutic proteins and peptides by polyethylene glycol conjugation is a well known method to improve the pharmacological properties of such drugs. Here we describe an alternative way of PEGylation from classic chemical methods, taking advantage of enzymes able to specifically modify some amino acid side chains, in particular glycosyltransferases and transglutaminases. A few examples are here described, in particular granulocyte-colony stimulating factor, which has been successfully PEGylated by enzymatic methods leading to a new long-lasting compound presently under evaluation in clinical studies.

Keywords: PEGylation, transglutaminase, glycol-PEGylation, glycosyltransferase, G-CSF

Summary

Angelo Fontana, Barbara Spolaore, Anna Mero and Francesco M. Veronese
The site-specific TGase-mediated PEGylation of proteins occurs at flexible sites

Transglutaminase (TGase) is able to catalyse the acyl transfer reaction between the γ -carboxamide group of a protein-bound glutamine (Gln) residue and an amino-derivative of poly(ethylene glycol) (PEG-NH₂), thus leading to a PEGylated protein. Several proteins of therapeutic interest have been PEGylated by means of TGase, among them interleukin-2, granulocyte colony-stimulating factor, human growth hormone and erythropoietin. Surprisingly, PEGylation occurred at specific Gln residue(s), despite the fact that these proteins contained several Gln residues. An analysis of the TGase-mediated reactions in terms of structure and dynamics of protein substrates revealed a correlation between sites of TGase attack and chain regions of enhanced backbone flexibility, as detected by the crystallographic profile of the *B*-factor along the protein polypeptide chain. Moreover, the TGase-mediated reactions often occurred at chain regions characterized by missing electron density, indicating that these regions are disordered. In particular, it was noted that in a number of cases the sites of TGase attack occurred at the same chain regions prone to limited proteolysis phenomena. Since chain flexibility or local unfolding was earlier found to dictate the sites of limited proteolysis of proteins, it is concluded that both TGase and a protease require an unfolded polypeptide substrate in an extended conformation for the site-specific enzymatic attack.

Keywords: PEG, PEGylation, protein drugs, drug delivery, human growth hormone, interleukin-2, granulocyte colony-stimulating factor, erythropoietin, apomyoglobin, transglutaminase

Summary

Conan J. Fee
Protein conjugates purification and characterization

Methods for separation and characterization of PEGylated proteins are reviewed in this chapter. It is explained that these methods are challenging because PEG itself is a relatively inert, neutral, hydrophilic polymer and the starting point for PEGylation is a pure protein. Other than changes to molecular weight and size, differences between the properties of the PEGylated forms of a pure protein are relatively small, since they arise only from the addition to the protein of relatively inert, neutral polymer chains, which tend to shield interactions.

Physicochemical properties that are routinely used to characterize and purify proteins are discussed with regard to their applications for PEGylated proteins, including molecular mass, size and shape (mass spectrometry, size exclusion chromatography, membranes, capillary electrophoresis, gel electrophoresis), electrostatic charge (cation and anion exchange chromatography, isoelectric point gel electrophoresis, capillary electrophoresis) and relative hydrophobicity (hydrophobic interaction, reversed phase).

Keywords: separation, purification, characterization, analysis, PEG, PEGylated proteins, ion exchange, size exclusion, membranes, electrophoresis, hydrophobicity

Summary

Rob Webster, Victoria Elliott, B. Kevin Park, Donald Walker, Mark Hankin and Philip Taupin

PEG and PEG conjugates toxicity: towards an understanding of the toxicity of PEG and its relevance to PEGylated biologicals

PEG is used to improve pharmacokinetic properties of biologicals. Concern has been expressed about the toxicological effect and/or fate of the PEG. This paper reviews the available toxicity, metabolism and clearance data of PEG and PEGylated products in order to place such concerns in to appropriate context. The available data demonstrates that PEG itself only shows toxicity at high, parenteral doses and the usual target organ is the kidney as this is the route of excretion for unchanged PEG. A large therapeutic window (approximately 600-fold) exists between the maximum PEG burden from a current biological agent and the doses of PEG associated with human toxicity. Pathological changes which results in no functional deficit, PEG containing vacuoles in cells, have been observed with PEGylated biologicals. There is evidence that these PEG vesicle can resolve with time. In conclusion the doses used clinically for current and many future PEGylated biologicals are low and will result in exposures to PEG significantly lower than that required to elicit PEG toxicity. In all cases the routine regulatory toxicology studies would identify relevant pathology should it occur.

Keywords: PEGylation, PEG, toxicity, clearance, metabolism, parenteral administration, biological burden, therapeutic index, vesicles

Summary

Jonathan K. Armstrong

The occurrence, induction, specificity and potential effect of antibodies against poly(ethylene glycol)

Specific antibodies against poly(ethylene glycol) (anti-PEG) were induced in animals following exposure to PEG-conjugated proteins and particles, resulting in rapid clearance of PEG-conjugated agents. In humans, induction of anti-PEG was observed following exposure to a PEG-conjugated drug, and pre-existing anti-PEG was identified in over 25% the healthy population. In clinical studies, the presence of anti-PEG was strongly associated with rapid clearance of PEG-asparaginase and PEG-uricase. PEGylation of therapeutic agents will continue to be of significant value in medicine to reduce immunogenicity, antigenicity and toxicity as well as markedly reducing renal clearance, while maintaining drug efficacy. It is important to recognize that PEG itself may possess

antigenic and immunogenic properties. Further comprehensive studies are warranted to fully elucidate the effect of anti-PEG on PEG-conjugated agents and if confirmed in a prospective trial, patients should be screened and monitored for anti-PEG, and strategies developed to overcome the potential negative effect of anti-PEG on drug clearance to improve the effectiveness of therapy.

Keywords: antibody, anti-PEG, IgG, IgM, PEGylation, PEG-protein, liposome, red blood cell, immunogenicity, antigenicity, oxyethylene, polyoxyethylene, poly(ethylene glycol), poly(ethylene oxide), epitope

Summary

Graham Molineux

Pegfilgrastim - designing an improved form of rmetHuG-CSF

rmethuG-CSF is the recombinant version of natural granulocyte colony-stimulating factor, the dominant stimulator in the production of neutrophilic leukocytes (neutrophils). Neutrophils represent the first line of defense against invading pathogens and when neutrophil numbers are suppressed by cancer chemotherapy, patients become liable to life-threatening infections.

The clearance of rmetHuG-CSF is effected by a combination of neutrophil mediated degradation and renal filtration. Site-directed addition of a single, linear PEG molecule yielded a form of G-CSF (pegfilgrastim) that was shown to be resistant to renal elimination yet remained sensitive to neutrophil-mediated destruction. This semi-synthetic cytokine drug can persist in the plasma for extended periods in neutropenic conditions, yet is cleared rapidly when neutrophils recover. This lends a degree of automation to the therapeutic control of neutrophil numbers which has been exploited in clinical practice since its approval for human use in 2002.

Keywords: G-CSF, granulocyte colony-stimulating factor, pegfilgrastim, Neulasta®, neutropenia

Summary

Rory F. Finn

Pegylation of human growth hormone: strategies and properties

Recombinant human growth hormone (hGH) is a well characterized molecule with broad acceptance as a treatment for growth hormone deficiencies (GHD). However, treatment with hGH requires daily injections due to the drug's short duration of action. Many groups have focused on PEGylation of hGH as a means to extend its half-life and generate less frequent dosage forms. This chapter provides a review of the preclinical and clinical results obtained from the many approaches directed towards modification of hGH

with PEG. The chapter will describe a historical progression of PEGylation strategies and results. The first half of the chapter will discuss initial studies that utilized multiple 5 kDa PEG attachments for extension of hGH half-life and the subsequent development of a PEGylated hGH receptor antagonist, pegvisomant, a successful therapy for acromegaly. The latter half of the chapter will summarize more recent and current work focusing on site selective mono-PEGylation of hGH.

Keywords: human growth hormone, somatotropin, growth hormone receptor antagonist (GHRA) pegvisomant, Somavert®, acromegaly, growth hormone deficiency (GHD), B2036, Insulin-like growth factor-1 (IGF-1), multi-PEGylation, Mono-PEGylation

Summary

Gianfranco Pasut

PEGylated α interferons: two different strategies to achieve increased efficacy

Conjugation of poly(ethylene glycol) to proteins is a well known technique used to prolong half-life and reduce immunogenicity. In the case of interferon α , improving pharmacokinetics to reduce dosing frequency was the driving force in the development of two long-acting derivatives: PEG-Intron® by Schering-Plough and Pegasys® by Roche Pharmaceuticals. These conjugates, even though developed with a similar approach and for the same clinical use, present several differences that offer the possibility for an interesting and unique comparison. The basic PEGylation chemistry and characterization of the conjugates will be described together with an analysis of the pharmacokinetic/pharmacodynamic behaviors.

Summary

Michael S. Hershfield, John S. Sundry, Nancy J. Ganson and Susan J. Kelly

Development of PEGylated mammalian urate oxidase as a therapy for patients with refractory gout

Gout is a form of arthritis caused by inflammatory crystals of monosodium urate, which deposit in joints when the plasma concentration of uric acid chronically exceeds the limit of solubility, ~7 mg/dL (0.42 mM). The human species is predisposed to hyperuricemia and gout by mutation of the urate oxidase gene during evolution. Urate oxidases from various sources have been used as a model to investigate the effects of PEGylation in animals. More than 15 years ago we initiated a project to develop a PEGylated recombinant mammalian urate oxidase as an Orphan Drug for treating patients with refractory gout. Clinical testing of this PEG-uricase, now called pegloticase, began in 2001. Pegloticase was found to have a half-life in plasma of about two weeks, and when infused at 2–4 week intervals to rapidly correct hyperuricemia. PEGylation was effective in limiting immune recognition of the recombinant uricase protein, but antibodies to PEG

develop in some patients, resulting in the rapid clearance of pegloticase and loss of efficacy. However, in many patients with refractory gout, treatment with pegloticase maintains plasma urate at well below saturating concentrations, leading to elimination of tissue urate deposits and control of disease.

Summary

Andrew M. Nesbitt, Sue Stephens and Elliot K. Chartash

Certolizumab pegol: a PEGylated anti-tumour necrosis factor alpha biological agent

Tumour necrosis factor (TNF) α is a proinflammatory cytokine involved in systemic inflammation that mediates chronic inflammatory diseases such as rheumatoid arthritis (RA), Crohn's disease (CD) and psoriasis. Recognition of TNF α as a primary mediator of inflammatory disease has driven the development of monoclonal antibodies (mAbs) against TNF α as potential novel therapies for these disorders. Certolizumab pegol is a novel, polyethylene glycol (PEG)-conjugated, humanised, antigen-binding fragment (Fab') of an anti-TNF α mAb that does not mediate apoptosis or neutrophil degranulation. Preclinical studies have shown excellent bioavailability, with preferential distribution and retention in inflamed tissue, which could be due to the low diffusion rate of PEGylated molecules and/or the lack of an Fc, which prevents FcRn-mediated transport. Pharmacokinetics are linear and predictable. Certolizumab pegol is a potentially valuable new treatment option for several inflammatory diseases. It has shown promising efficacy and tolerability results in Phase II and III trials for RA, CD and psoriasis.

Keywords: anti-TNF α monoclonal antibodies, Fab' fragment, certolizumab pegol, infliximab, adalimumab, Crohn's disease, rheumatoid arthritis, psoriasis, pharmacodynamics, pharmacokinetics, clinical efficacy

Summary

Anna Mero, Gianfranco Pasut and Francesco M. Veronese

PEG: an useful technology in anticancer therapy

Cancer chemotherapy dates back to the 1940s with the first use of nitrogen mustards and antifolate drugs. The use of small molecule and biopharmaceutical drugs is today the acceptable approach to cancer treatment in both ambulatory and in patient care. Drug delivery of these drugs has given rise to safer and more efficacious options. Today, the use of polymers for sustained and targeted delivery has allowed oncologists to deal with the earlier limitations of chemotherapy. In this chapter the focus is on polymer conjugation of anticancer drugs, such as high molecular weight proteins and low molecular weight compounds. Examples will be presented to demonstrate an increase in the pharmacological therapeutic index by targeting the drug molecules to the diseased sites with corresponding reduction in drug related side effects. The focus will be on the attachment of polyethylene glycol (PEG) to oncolytic drugs, a process sometimes

referred to as PEGylation. This technology has been completely validated in the area of protein modification, but is very much in its infancy in the modification of small molecular weight drugs. However, increasing and encouraging efforts have recently been made and will be presented. This chapter will also discuss recent achievements in PEGylation processes with a particular emphasis on the application of PEG to non-conventional therapies such as oxidation therapy, photodynamic therapy and radiopharmaceutical therapy.

Keywords: PEG conjugation, antitumour drugs, enzyme modification

PEGylated Protein Drugs: Basic Science and Clinical
Applications

Veronese, F.M. (Ed.)

2009, IX, 290 p., Hardcover

ISBN: 978-3-7643-8678-8

A product of Birkhäuser Basel