

Improving the Pharmacokinetics of Protein and Peptide Drugs: Nature's Way

Gregory Gregoriadis

Lipoxen Technologies Ltd, School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK

Introduction

Peptide and protein drugs, exemplified by insulin, growth hormone and the interferons, have been used therapeutically for some decades but the potentially huge impact of this class of drugs on the treatment of disease has been only recently appreciated, thanks to advances in genomics and proteomics. These have culminated in the discovery of a vast array of new protein and peptide drug candidates, many in clinical trials worldwide, and a significant number already on the market (Walsh, 2003).

However, not all is well with protein and peptide drugs as effective use in the patient is often compromised by limitations that are inherent in their molecular structure. For instance, injected peptides can, depending on size or other characteristics, exhibit rapid rates of clearance from the circulation before therapeutic concentrations in the blood or target tissues are attained. Contributing factors include proteolytic degradation of peptides, premature uptake by the reticuloendothelial system, loss through the kidneys, and complexing to antibodies generated by the peptides on repeated injections (Harris and Chess, 2003).

Early in the development of peptide and protein drugs it was realised that these must be first protected from the vagaries of the biological milieu before they can take their place in the armamentarium of modern therapeutics. Related approaches included changes in the amino acid sequence of the drugs to reduce degradation, fusion with other proteins or glycosylation to improve half-life, encapsulation into microparticles such as liposomes, and conjugation to a variety of polymers (Harris and Chess, 2003). Of the latter, the non-biodegradable polyethylene glycol (PEG) has been the most successful so far. Conjugation of PEG chains to peptides or proteins (PEGylation) offers significant advantages in terms of their circulatory half-lives and pharmacological effect. Several PEGylated drugs, including asparaginase, interferon alpha and tumour necrosis factor are now in clinical use (Mehwar, 2000).

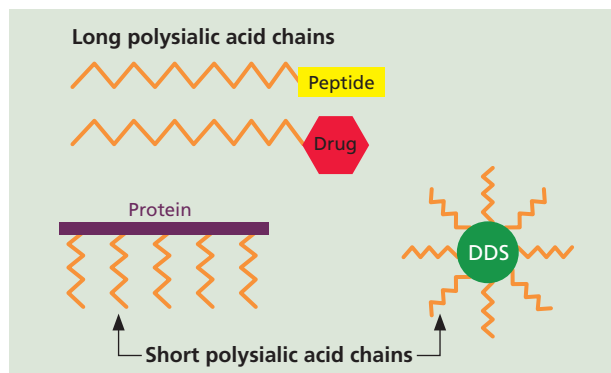
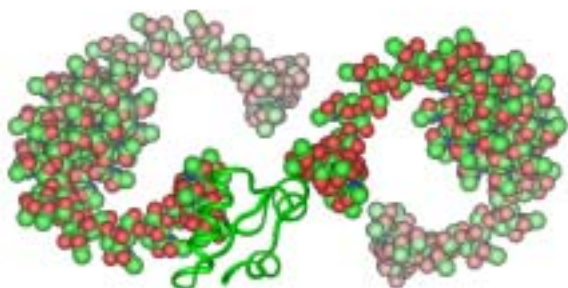


Figure 1 – Schematic representation of polysialylated constructs.

Nature's Way

Molecules or other entities that are “unnoticeable” have the best chance of surviving in the body. For instance, certain bacteria have evolved over millions of years to foil the body's defences by coating their walls with polysialic acid. Nature's ultimate stealth technology, polysialic acids (PSAs), are polymers of sialic acid (a sugar abundantly present on the surface of cells and many proteins) of varying size. Interestingly, their role in protecting invading microbes by interfering with host complement activation and phagocytic activity is extended to such functions as facilitating neural tissue development (by modulating cell-to-cell contact inhibition), and helping cancer cells to metastasise by rendering them less adherent and more prone to migration (Mühlenhoff *et al.*, 1998).

In the early 1990's, it was proposed (Gregoriadis *et al.*, 1993) that the unique ability of polysialic acids to fence off external insults could be used to improve the pharmacokinetics of therapeutic molecules. It was reasoned that, by forming a watery “cloud” around the molecule (PSAs are highly hydrophilic), interaction with other molecules, be it proteolytic enzymes, opsonins, neutralising antibodies or receptors on phagocytic cells, would be prevented allowing the therapeutic to retain its integrity, preserve activity and prolong its presence in the body. For small peptides and drugs, a concomitant increase in size effected by PSA, together with the highly ionic state of the polymer, could also contribute to reduced loss through the kidneys. Figure 1 is a schematic representation of two different types of polysialylated constructs. In the case of peptides and proteins (and, indeed, drug delivery systems such as liposomes), a number of polymer chains of appropriate length, attached randomly or strategically, would ensure protection. With small drug molecules



Polysialylated insulin

including short peptides on the other hand, a long PSA would not only protect, it would also directly determine the circulatory half-life of the molecule: research has shown that the longer the PSA the more it circulates in the blood (up to 40 h half-life in mice) (Gregoriadis *et al.*, 1993). Subsequent work (Fernandes and Gregoriadis, 1996, 1997, 2001; Gregoriadis *et al.*, 2000) with a number of polysialylated therapeutic peptides and proteins and a model small drug molecule confirmed some of the anticipated attributes of PSA.

PolySialic Acids: The Next Generation of Protective Polymers?

Much is known about the structure and function of polysialic acids, of which there are several types (Mühlenhoff *et al.*, 1998). However, in terms of using the polymers for the polysialylation of drugs, the α -(2-8)-linked serogroup B capsular polysaccharide from *Escherichia coli* K1 and its shorter derivatives (also known as colominic acids) are the most suitable. Being chemically and immunologically identical to PSA expressed in the host organism (Mühlenhoff *et al.*, 1998), bacterial PSA is, by virtue of this structural mimicry, completely non-immunogenic, even when coupled to proteins. It is also unable to react with antibodies to the polysialic acid structure, known to exist only at low levels in the blood and to exhibit weak affinity. Moreover, unlike other polymers (e.g. dextran, PEG), polysialic acids are biodegradable and their catabolic products (e.g. sialic acid) are not known to be toxic. This is particularly important where the polymer is used to improve the pharmacological profiles of therapeutics given chronically, especially when relatively large doses of the drug (e.g. insulin, growth hormone) are required. From a practical standpoint, polysialic acids can be easily produced in large quantities from bacterial cultures using, in the case of α -(2-8)-linked PSA, a non-pathogenic, adapted strain of *E. coli* K1. Significantly, cultures could be adjusted to produce PSAs of predetermined average molecular weight, with polydispersities that can be narrowed considerably by appropriate techniques.

PolyXen™ Technology

Lipoxen's PolyXen™ technology, encompassing the technical, theoretical and applied aspects of polysialylation, has been developed mostly by the use of *E. coli*-derived α -(2-8)-linked polysialic acids of two different average molecular weights, namely 22 KDa and 39 KDa, depending on the peptide or protein candidate drug. Conjugation of polysialic acids to drugs is carried out by a variety of straightforward and gentle proprietary techniques that preserve much of the drug's activity. Generally, the techniques involve modification (activation) of either of the terminal units of the polymer to forms that are capable of reacting with pendant groups of the therapeutic, generating constructs with an average of one or more polymer chains per molecule. In practice, it is convenient

to prepare large batches of activated PSA, which can then be freeze-dried and stored (without loss of activity) until required. Polysialylation techniques have been optimised to avoid the generation of unwanted side products and to enable attachment of PSA chains to areas in the molecule away from its active site. Upon their formation, polysialylated constructs can be purified and tested by established procedures.

PolyXen™ Therapeutics: Criteria for Success

PolyXen™ technology has been applied to a wide range of therapeutics to demonstrate that a number of criteria, deemed important for their further development, are satisfied.

Improved Stability

First, it was necessary to show that polysialylation can prevent the loss of activity of a therapeutic in the presence of the biological milieu. This was tested with asparaginase, an enzyme used in the treatment of certain forms of leukaemia. Exposure of asparaginase to blood serum led to rapid loss of its activity which, however, was retained in the polysialylated construct, presumably by PSA-effected restricted access of proteolytic enzymes to vulnerable sites of the protein (see Figure 2).

Preserved Function

Another important consideration was the effect of polysialylation on the functionality of therapeutics. Examples of therapeutics chosen were asparaginase, acting on a low molecular weight substrate (asparagine) that should have little difficulty in reaching the active site of the enzyme through the maze of the polymer chains, and interferon α -2b, the active site of which needs to interact with a large receptor molecule on the surface of cells. In the first case, the enzyme kinetics remained unchanged on polysialylation, confirming unhindered access of the substrate to the active site. It was interesting to note that preservation of function also occurred with interferon, judging from the observation that most (over 80%) of its receptor binding ability was retained when tested with Daudi cells (Figure 3).

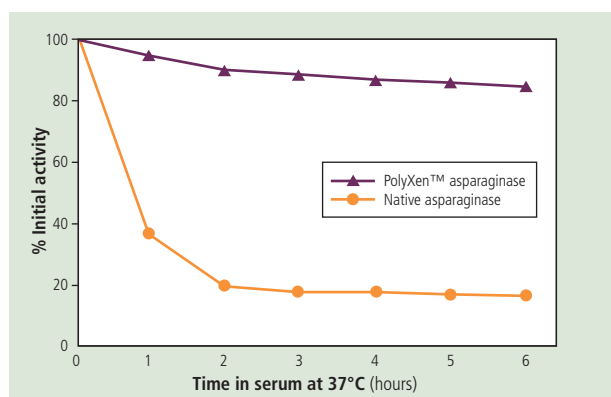


Figure 2 – Stability of intact and polysialylated asparaginase in blood serum (mouse).

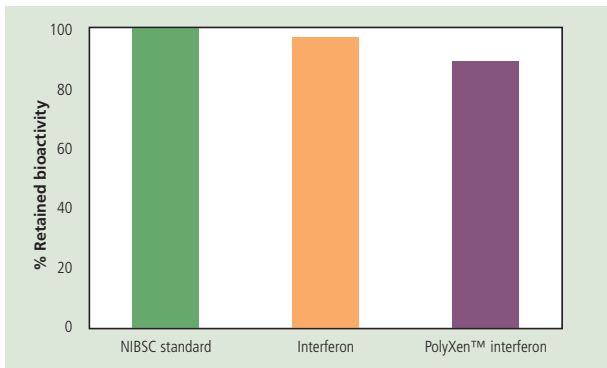


Figure 3 – Receptor (Daudi cells) binding ability of polysialylated interferon α -2b.

Prolonged Presence and Pharmacological Action in Blood Circulation

A crucial feature of polysialylation is that it can extend drug presence in the circulation with concomitant retention of pharmacological activity. This should help to maintain therapeutic concentrations of the drug for longer periods, in turn reducing dosages and frequency of administration. Molecules on which the concept was tested *in vivo* (mice) were a small model drug molecule (fluorescein), a tumour-specific antibody Fab fragment, asparaginase and insulin. As expected, with all drugs where plasma concentrations were measured, circulatory half-lives and areas under the curve increased dramatically when drugs were polysialylated (e.g. an 80-fold increase in the half-life of Fab; rats) (Epenetos *et al.*, 2002). Moreover, in the case of a tumour-specific polysialylated Fab fragment, its prolonged circulation (see Figure 4) was associated with improved localisation in the relevant tumour suggesting that, in spite of PSA chains on the molecule, the antigen-recognising region of the Fab was able to approach and bind to the relevant antigen on the cell surface.

Polymer chain interference with the activity of peptides and proteins causing, for instance, severe loss of activity with PEGylated growth hormone (Clark *et al.*, 1996) and interferon α (Bailon *et al.*, 2001), is a potential drawback that could exclude many important therapeutics from polymer-mediated optimisation. So far, however,

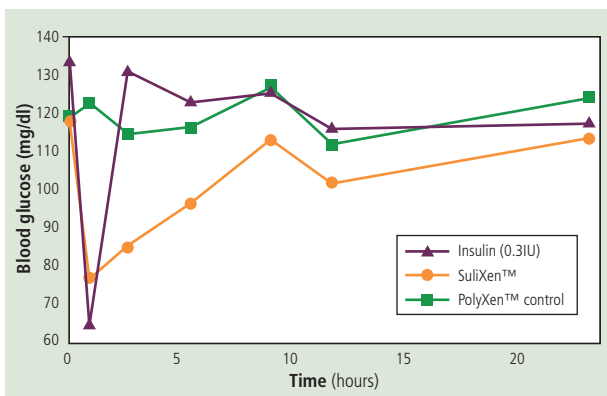


Figure 5 – Hypoglycaemic action of intact and polysialylated insulin (SuliXen™) in subcutaneously injected mice.

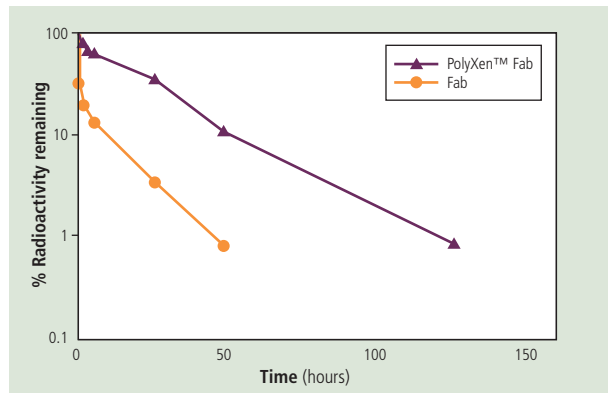


Figure 4 – The effect of polysialylation on the circulatory half-life of an antibody Fab fragment injected intravenously into mice.

results with catalase (Fernandes and Gregoriadis, 1996), asparaginase (Fernandes and Gregoriadis, 1997), interferon α -2b (Hirst *et al.*, 2002), the Fab fragment used here, and several other proteins (confidential information supplied by others) strongly suggest that the presence of PSA chains on the molecules allows retention of much of the activity in polysialylated therapeutics. More importantly, retention of activity also appears to occur *in vivo*. This was clearly demonstrated with polysialylated asparaginase (Fernandes and Gregoriadis, 1997), and with polysialylated insulin (Jain *et al.*, 2003), which not only retained its ability to reduce blood glucose levels in injected animals to levels seen with the intact peptide within the same time period, it also remained active for much longer, presumably as a result of extended presence in the circulation (see Figure 5).

Reduced Immunogenicity and Antigenicity

Effective use of peptide and protein drugs in therapy, especially when serial injections are required, is often marred by the generation of antibodies against the drugs. Such antibodies can neutralise the activity of the therapeutic (thus necessitating higher doses) and also lead to anaphylactic reactions. Antibody-mediated loss of

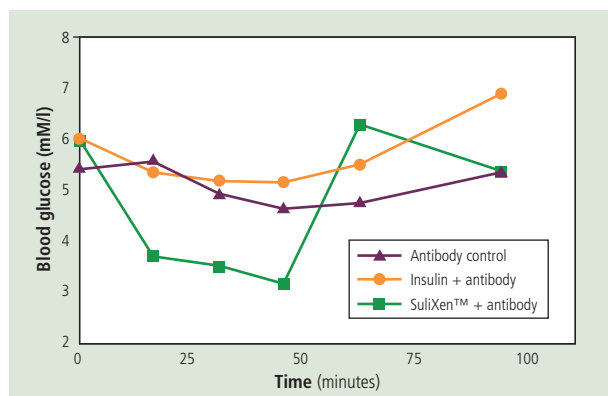


Figure 6 – Hypoglycaemic action of intact and polysialylated insulin (SuliXen™) in subcutaneously injected mice following incubation with anti-insulin antibody. Note retention of activity for the polysialylated hormone.

activity affects several of the currently used peptides and proteins (e.g. insulin, erythropoietin, interleukin-2), even those made recombinantly using the human genes (Caliceti and Veronese, 2003).

It appears that both immunogenicity and antigenicity of peptides and proteins can be abrogated by polysialylation. Thus, whereas one and two subcutaneous injections of insulin were sufficient to generate an immune response in high (C57) and low (Balb/c) responder mice respectively, four injections were necessary for the appearance of a (modest) response to polysialylated insulin (Jain *et al.*, 2003). Moreover, following incubation with an anti-insulin antibody, polysialylated insulin, but not intact insulin, was able to induce hypoglycaemia in mice, presumably because of the antibody's inability to access the relevant antigenic site on the hormone (see Figure 6).

The Future of Polysialylation

Preclinical work has shown that PolyXen™ technology, with its multifaceted proprietary approaches to the synthesis of optimal therapeutic constructs, can circumvent many of the problems encountered in the direct use of peptides and proteins. One challenge for PolyXen™ is to establish its clinical worth in the face of competing technologies. PEGylation, for instance, is widely known with several related products already licenced. On the other hand, PolyXen™ has certain advantages that may prove crucial for its future. First, the technology with all its aspects is protected by an umbrella of granted patents and filed patent applications, owned and controlled by one entity. Secondly, polysialic acid, being a natural material, is completely biodegradable and not expected to be toxic. Moreover, it is totally inert in terms of immunogenicity even when combined with a protein. In contrast, PEG is a non-biodegradable polymer, although there is some evidence of enzyme-mediated low rate oxidation generating aldehydes and ketones in the body (Caliceti and Veronese, 2003). However, this is not a normal detoxification mechanism. When conjugated to a therapeutic protein PEG, regardless of its molecular size, is almost certain to end up in tissues where it will accumulate, especially when given in relatively large amounts (Caliceti and Veronese, 2003) either because of chronic use or because of large drug dosages. Furthermore, PEGylated proteins have been found to generate anti-PEG antibodies that could influence the residence time of the conjugate in the blood circulation. So far, however, no clinical evidence of adverse effects of PEG immunogenicity has been reported, possibly because of the low amounts of injected PEGylated drugs (Caliceti and Veronese, 2003). It may not be so for high doses of peptides and proteins given chronically.

The future of polysialylation as a means to produce new drug entities that will improve the quality of life of patients treated with the wide range of peptide and protein drugs, will depend on whether the technology works at least as well as existing technologies. To that end, evidence amassed so far is promising. There is a huge variety of

therapeutic peptides and proteins either in clinical use or in the preclinical stage, with many of these in need of improvement. It is expected that PolyXen™ will contribute significantly to making drugs perform better.

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Mel Hemamda

Tel: +44 (0) 1865 784 177

Fax: +44 (0) 1865 784 178

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