

Long live peptides

evolution of peptide half-life extension technologies and emerging hybrid approaches

Among therapeutic modalities, peptides occupy a middle ground between traditional 'small molecule' drugs and the larger injectable biologics, not fitting comfortably into either category.

Peptides, particularly those based on endogenous human hormones, often exhibit unique and powerful pharmacology that has not been possible to effectively mimic with either small molecules or antibodies. However, as therapeutics, they suffer from drawbacks, in particular, the lack of oral bioavailability which necessitates injections. Short half-lives resulting from sensitivity to proteases and rapid renal clearance mean that peptide drugs tend to require frequent doses. Several peptide modification methods have been explored to address the issue of inherently short half-life in an attempt to decrease the frequency of injections. PEGylation was one of the first chemical modification techniques shown to increase peptide half-life by decreasing the rate of renal filtration and has been an important tool for extending half-life and understanding the pharmacology of chronic dosing. Lipidation has also been utilised for the same purpose. An alternative to these chemical modifications has been classical genetic fusions which offer advantages in terms of half-life but also their own severe constraints in terms of drug design. Now, hybrid methods are emerging, which aim to exploit the benefits of the prior approaches while avoiding the major limitations. This article will provide an overview of the various methods currently available in the pharmaceutical industry for peptide half-life extension.

PEGylation

In the 1970s, the first-generation PEGylation processes were pioneered by researchers at

Rutgers University. Basically the attachment of long chains of the hydrophilic polymer polyethylene glycol (Figure 1) to molecules of interest, PEGylation was originally conceived as a modification to prevent the recognition of foreign proteins by the immune system and, thereby, enable their utility as therapeutics. Once formed, antibodies against unmodified drugs can rapidly neutralise and clear protein drugs. Unexpectedly, PEGylation improved the pharmacokinetics of the proteins even in the absence of anti-drug antibodies¹. Simply by making drug molecules larger, PEGylation led to the drug being filtered more slowly by the kidneys. The empirical observation that increasing size or hydrodynamic radius led to reduced renal clearance and increased half-life then became the dominant rationale for the PEGylation of protein and peptide drugs. PEGylation can have a variety of effects on the molecule including making proteins or peptides more water-soluble and protecting them from degradation by proteolytic enzymes. PEGylation can also impact the binding of therapeutic proteins to their cognate cellular receptors, usually reducing the affinity. Changes in the size, structure and attachment mode of PEG polymers can affect the biological activity of the attached drug.

The first-generation PEGylation methods were filled with challenges. However, the chemistry of PEGylation is quite simple. The process involves the covalent attachment of polyethylene glycol chains to reactive side chains of a protein or peptide. For example, PEG is easily attached to the

By Dr Ronald V. Swanson

Figure 1

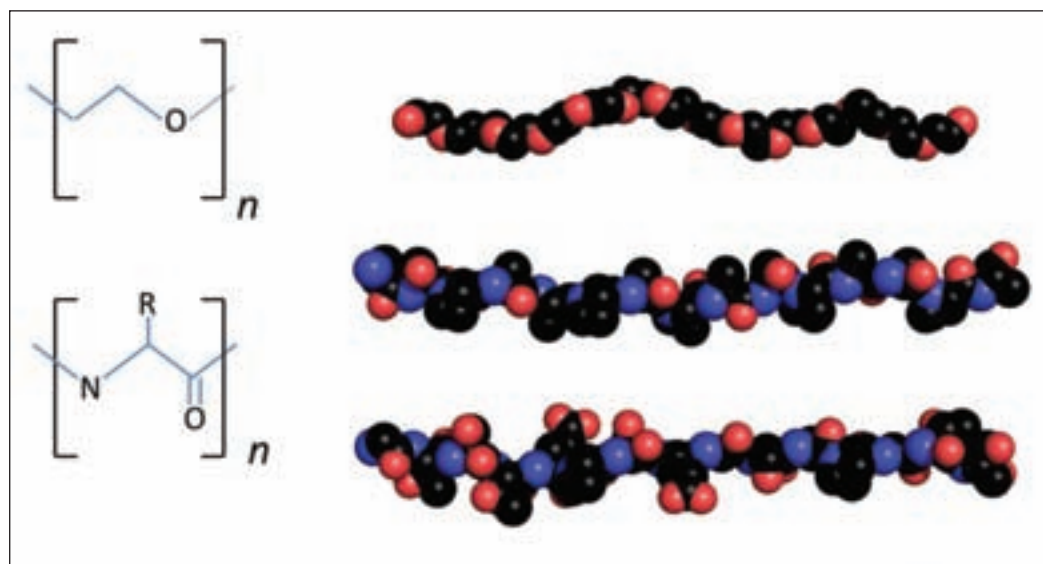
Top left: Chemical structure of PEG. Typical PEGs used in half-life extensions are 20-40 kDa and n is approximately 450-900 although PEGs this large are not discrete and typically contain a mixture of chain lengths.

Top right: Model structure of a short 15 unit fragment of PEG. **Bottom left:**

Polypeptide structure, in XTEN R = Ala, Glu, Gly, Pro, Ser, or Thr; in a typical XTEN sequence $n = 864$; in PAS R = Pro, Ala or Ser and in a typical sequence $n = 600$. Models of short 15 unit representative sequences of PAS **centre**

right and XTEN **bottom right** are shown

Figure courtesy of Mack Flinspach



ϵ -amino groups of lysine on the surface of proteins or peptides². The reaction is pH-dependent. At high pH (8.0 or higher), lysine side chain amino groups are covalently attached to PEG through N-hydroxy succinimides. This method typically results in a family of products containing different numbers of PEG chains attached at different sites on a protein rather than a single discrete product³.

The first approved PEGylated pharmaceuticals were Pegademase bovine (PEGylated bovine adenosine deamidase) as enzyme replacement therapy for severe combined immunodeficiency and Pegasparase (PEGylated asparaginase) for treatment of acute lymphoblastic leukaemia¹. These drugs were complex mixtures of various PEGylated species, but with improved properties for therapy over native enzymes, including increased serum half-life and decreased immunogenicity of the proteins. Due to the inherent polydispersity of the PEG, quality and batch-to-batch reproducibility was difficult. Despite this limitation, two PEGylated interferons, (Peginterferon alfa-2b and Peginterferon alfa-2a) that are heterogeneous populations of numerous mono-PEGylated positional isomers, have been FDA-approved for the treatment of hepatitis C. These drugs were brought to market in 2001 and 2002, respectively.

A variety of enhancements and variations have been made to the fundamental PEGylation technology. Second-generation PEGylation processes introduced the use of branched structures as well as alternative chemistries for PEG attachment. In particular, PEGs with cysteine reactive groups such as maleimide or iodoacetamide allow the targeting of the PEGylation to a single residue within a pep-

ptide or protein reducing the heterogeneity of the final product but not eliminating it due to the polydispersity of the PEG itself.

While the original rationale for PEGylation was to reduce immunogenicity; nevertheless, there have been a few examples of immunogenic PEGylated proteins. One example is PEGylated urate oxidase, an enzyme that lowers the plasma urate level in patients with gout. In clinical trials, a relatively high percentage of patients with gout did not respond to the therapy and developed antibodies that were specific for PEG, but not for the uricase protein². PEGylated liposomes, also generally thought to be non-immunogenic, have been found to be immunogenic in some studies. PEGylated liposomes elicit a strong anti-PEG immunoglobulin M (IgM) response. In addition, multiple injections of PEG-glucuronidase were shown to elicit the generation of specific anti-PEG IgM antibodies, thus accelerating the clearance of PEG-modified proteins from the body.

A major potential drawback of using PEG as a modifier is that it is non-biodegradable. The US Food and Drug Administration (FDA) has approved PEG for use as a vehicle in pharmaceuticals, including injectable, topical, rectal and nasal formulations. PEG shows little toxicity and is eliminated from the body intact by either the kidneys (for PEGs < 30 kDa) or in the feces (for PEGs >20 kDa)¹. Repeated administration of some PEGylated proteins to animals has resulted in observations of renal tubular cellular vacuolation. Recently, vacuolation of choroid plexus epithelial cells has also been seen in toxicity studies with proteins conjugated with large (≥ 40 kDa) PEGs. The

choroid plexus epithelial cells produce cerebrospinal fluid and form the blood CSF barrier. The long-term negative consequences of cellular vacuolation are unclear, but it does represent an undesirable consequence for some potential therapeutics. One possible alternative would be substitution of a biodegradable polymer in place of PEG. Polymers, such as hydroxyethyl starch (HES) are a possible alternative. HES is non-toxic and biodegradable and used as a blood expander. A process of HESylation would function similarly to PEGylation in reducing renal clearance through increasing a peptide's hydrodynamic radius but may confer a lower propensity for accumulation due to biodegradability. However, HES and other proposed biodegradable polymer PEG alternatives are, like PEG, polydisperse making characterisation of the final product and metabolites difficult. One emerging solution which mitigates both concerns is to use defined polypeptides as the polymer component; this approach will be discussed later in the article.

Lipidation

A second major chemical modification method to increase peptide half-life is lipidation which involves the covalent binding of fatty acids to peptide side chains⁴. Originally conceived of and developed as a method for extending the half-life of insulin, lipidation shares the same basic mechanism of half-life extension as PEGylation, namely increasing the hydrodynamic radius to reduce renal filtration. However, the lipid moiety is itself relatively small and the effect is mediated indirectly through the non-covalent binding of the lipid moiety to circulating albumin. A large (67 KDa) and highly abundant protein in human serum (35-50g/L), albumin naturally functions to transport molecules, including lipids, throughout the body. Binding to plasma proteins can also protect the peptide from attacks by peptidases through steric hindrance, again akin to what is seen with PEGylation. One consequence of lipidation is that it reduces the water-solubility of the peptide but engineering of the linker between the peptide and the fatty acid can modulate this, for example by the use of glutamate or mini PEGs within the linker. Linker engineering and variation of the lipid moiety can affect self-aggregation which can contribute to increased half-life by slowing down biodistribution, independent of albumin⁵.

Following the pioneering work with insulin⁶, lipidation of a variety of peptides has been explored, particularly peptides within the diabetes space including human glucagon-like peptide-1 (GLP-1)

analogues, glucose-dependent insulintropic polypeptide and GLP-1R/Glucagon receptor co-agonists among others. Two lipidated peptide drugs are currently FDA-approved for use in humans. These are both long-acting anti-diabetics, the GLP-1 analogue liraglutide and insulin detemir.

A potentially pharmacologically-relevant difference between PEGylation and lipidation is that the therapeutically active peptide is covalently linked to the much larger PEG, whereas the smaller fatty acyl-peptide conjugate is non-covalently associated with the larger albumin, bound and unbound forms existing in equilibrium. This can result in differences in biodistribution that may result in different pharmacology as access to receptors localised in different tissues may elicit differential effects. In some cases, more restricted biodistribution may be desirable, while in others, greater tissue penetration may be important. An interesting variation of the PEG approach which addresses this issue has been developed by Santi et al in which releasable PEG conjugates with predictable cleavage rates are utilised⁷.

PEGylation and lipidation both confer protection against proteases and peptidases by shielding through steric hindrance and extend circulating half-life through increased hydrodynamic radius, directly or indirectly. Both methods utilise chemical conjugation and are flexible in that they are agnostic to the means used to generate the peptide they are modifying, whether biologically or synthetically produced. An advantage of using synthetic peptides is that they can incorporate non-natural amino acids designed to address a number of specific issues including instability due to known proteolytic cleavage liabilities. They can also be more flexible in terms of the choice of attachment site which is critical if activity or potency is highly dependent on the free termini or a modified residue such as a C-terminal amide.

Classical genetic fusions: Fc and HSA

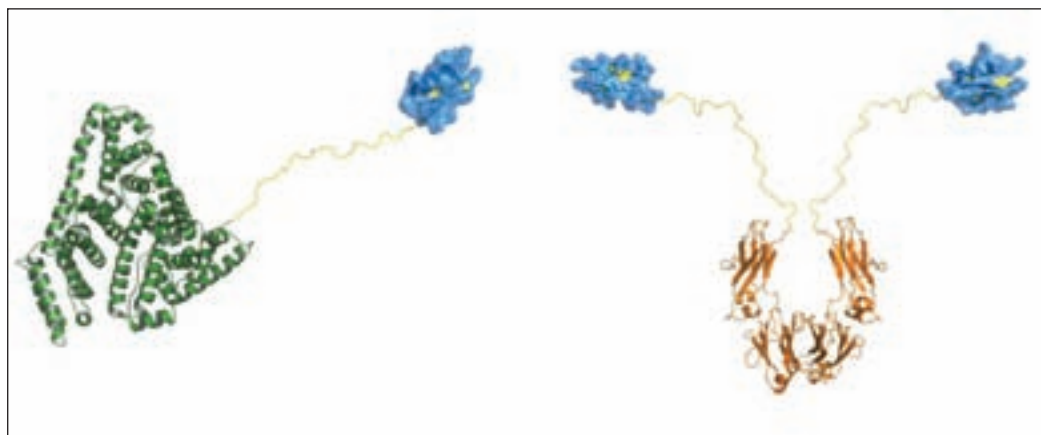
Classical genetic fusions to long-lived serum proteins offer an alternative method of half-life extension distinct from chemical conjugation to PEG or lipids. Two major proteins have traditionally been used as fusion partners: antibody Fc domains and human serum albumin (HAS) (Figure 2). Fc fusions involve the fusion of peptides, proteins or receptor exodomains to the Fc portion of an antibody. Both Fc and albumin fusions achieve extended half-lives not only by increasing the size of the peptide drug, but both also take advantage of the body's natural recycling mechanism: the neonatal Fc receptor,

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Figure 2
Models of classical genetic fusions. Left: Modified scorpion toxin derived Kv1.3 blocking peptide fused at the C-terminus to human serum albumin. Right: Modified scorpion toxin derived Kv1.3 blocking peptides fused at the C-terminus to human antibody Fc
Figure courtesy of Mack Flinspach



FcRn. The pH-dependent binding of these proteins to FcRn prevents degradation of the fusion protein in the endosome. Fusions based on these proteins can have half-lives in the range of 3-16 days, much longer than typical PEGylated or lipidated peptides. Fusion to antibody Fc can improve the solubility and stability of the peptide or protein drug. An example of a peptide Fc fusion is dulaglutide, a GLP-1 receptor agonist currently in late-stage clinical trials. Human serum albumin, the same protein exploited by the fatty acylated peptides is the other popular fusion partner. Albiglutide is a GLP-1 receptor agonist based on this platform. A major difference between Fc and albumin is the dimeric nature of Fc versus the monomeric structure of HAS leading to presentation of a fused peptide as a dimer or a monomer depending on the choice of fusion partner. The dimeric nature of a peptide Fc fusion can produce an avidity effect if the target receptors are spaced closely enough together or are themselves dimers. This may be desirable or not depending on the target.

Designed polypeptide fusions: XTEN and PAS

An intriguing variation of the recombinant fusion concept has been the development of designed low-complexity sequences as fusion partners, basically unstructured, hydrophilic amino acid polymers that are functional analogs of PEG (Figure 1). The inherent biodegradability of the polypeptide platform makes it attractive as a potentially more benign alternative to PEG. Another advantage is the precise molecular structure of the recombinant molecule in contrast to the polydispersity of PEG. Unlike HSA and Fc peptide fusions, in which the three-dimensional folding of the fusion partner needs to be maintained, the recombinant fusions to unstructured partners can, in many cases, be sub-

jected to higher temperatures or harsh conditions such as HPLC purification.

The most advanced of this class of polypeptides is termed XTEN (Amunix) and is 864 amino acids long and comprised of six amino acids (A, E, G, P, S and T) (Figure 1)⁸. Enabled by the biodegradable nature of the polymer, this is much larger than the 40 kDa PEGs typically used and confers a concomitantly greater half-life extension. The fusion of XTEN to peptide drugs results in half-life extension by 60- to 130-fold over native molecules. Two fully recombinantly produced XTENylated products have entered the clinic, namely VRS-859 (Exenatide-XTEN) and VRS-317 (human growth hormone-XTEN). In Phase Ia studies, VRS-859 was found to be well-tolerated and efficacious in patients with Type 2 diabetes. VRS-317 reported superior pharmacokinetic and pharmacodynamic properties compared with previously studied rhGH products and has the potential for once-monthly dosing.

A second polymer based on similar conceptual considerations is PAS (XL-Protein GmbH)⁹. A random coil polymer comprised of an even more restricted set of only three small uncharged amino acids, proline, alanine and serine (Figure 1). Whether differences in the biophysical properties of PAS and the highly negatively charged XTEN may contribute to differences in biodistribution and/or *in vivo* activity is yet unknown but will be revealed as these polypeptides are incorporated into more therapeutics and the behaviour of the fusions characterised.

All the peptide protein fusions, whether the partner is Fc, HSA, XTEN or PAS, are genetically encoded and consequently suffer from similar constraints. One limitation is that only naturally occurring amino acids are incorporated, unlike the methods employing chemical conjugation which

allow the use of synthetic peptides incorporating non-natural amino acids. Although methods to overcome this by expanding the genetic code are being developed by companies such as Ambrx or Sutro, they are not yet in wide use. A second limitation is that either the N- or C-terminus of the peptide needs to be fused to the partner. Oftentimes, the peptide termini are involved in receptor interactions and genetic fusion to one or both termini can greatly impair activity. Since the site of PEG or lipid conjugation can be anywhere on the peptide, it can be optimised to maximise biological activity of the resulting therapeutic.

Hybrid methods merging synthetic peptides with half-life extension proteins

While genetic fusions have historically offered the potential for greater half-life extension, they lack the advantages afforded by the methods utilising chemical conjugation, PEGylation and lipidation, in terms of flexibility of attachment sites and incorporation of unnatural amino acids or modifications to the peptide backbone. One of the first efforts to merge the advantages of the genetic fusions with chemical conjugation for half-life extension was carried out by researchers at the Scripps Research Institute in La Jolla with the technology which later formed the basis for the biotech company CovX^{10,11}. Using a catalytic aldolase antibody, these researchers developed a platform through which the active site lysine of the antibody forms a reversible covalent enamine bond with a beta-diketone incorporated into a peptide or small molecule. The resulting complex is termed a CovX-BodyTM. This approach combines the functional qualities of a peptide drug or small molecule with the long serum half-life of an antibody, not through a genetic fusion but rather through a chemical linkage. Following the initial demonstration of the technology, researchers expanded upon the use of CovX-BodyTM prototype that is based on an integrin targeting peptidomimetic pharmacophore. At least three molecules based on this architecture have entered clinical development: CVX-096, a Glp-1R agonist; CVX-060, an Angiopoietin-2 binding peptide; and CVX-045, a thrombospondin mimetic.

Recently, the XTEN polypeptide has also been used in a chemical conjugation mode¹² making it even more directly analogous to PEG. The first example of an XTENylated peptide that was created using this method is GLP2-2G-XTEN in which the peptide is chemically conjugated to the XTEN protein polymer using maleimide-thiol

chemistry. The chemically conjugated GLP2-2G-XTEN molecules exhibited comparable *in vitro* activity, *in vitro* plasma stability and pharmacokinetics in rats comparable to recombinantly-fused GLP2-2G-XTEN.

The number and spacing of reactive groups such as lysine or cysteine side chains in the completely designed sequences of XTEN or PAS polypeptides can be precisely controlled through site-directed changes due to the restricted amino acid sets from which they are composed. This provides an additional degree of flexibility over methods which might utilise Fc or albumin whose sequences naturally contain many reactive groups and stands in contrast to the CovX technology which relies on a reactive residue in a highly specialised active site. In addition, the lack of tertiary structure of XTEN or PAS should provide more flexibility over the conditions and chemistries used in coupling and in the purification of conjugates.

In summary, hybrid peptide half-life extension methods are emerging that combine the advantages and overcome the individual limitations of chemical conjugation and genetic fusions methods. These methods enable the creation of molecules based on recombinant polypeptide-based partners that impart longer half-life but free the therapeutic peptide moieties from the limitations of being composed solely of natural L-amino acids or configured solely as linear, unidirectional polypeptides fused at either the N- or C-terminus, thus opening the door to a wide range of longer acting peptide based drugs. **DDW**

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As Senior Director of Biologics Research within Janssen R&D, Dr Ronald V. Swanson leads a multi-functional team of more than 40 scientific researchers dedicated to the lead discovery and optimisation of novel antibody and peptide therapeutics within four therapeutic areas: Immunology, Oncology, Cardiovascular/Metabolic and Neuroscience.