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Bioconjugates: The Adaptable Challenge

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Conjugation of a biologic to a carrier molecule can solve problems in solubility and stability, but introduces its own set of challenges.

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With the advent of whole-genome sequence projects, a wealth of information has been attained and continues to be acquired about biomolecules and their biological processes. The information has led to the discovery of numerous new peptide and protein therapeutics based on these entities; however, there a number of challenges facing potential therapeutics that include delivery, compliance, and half-life. The burgeoning field of bioconjugation provides a route to overcoming some of these challenges.

Bioconjugation is the covalent derivatization of biomolecules such as protein, peptides, oligonucleotides, and antibodies. Bioconjugates are gaining popularity because of their versatility in a number of applications. Bioconjugates, such as fluorescent molecules and biotin, can be used as probes and diagnostic aids for imaging. Larger conjugates, such as polyethyleneglycol (PEG), are being used in therapeutic areas to enhance water solubility, reduce immunogenicity, and increase *in vivo* circulation half-life.

Most peptides composed of naturally occurring L-amino acids have short half-lives, often measured in minutes *in vivo*. Considerable effort has been invested in stabilizing peptidic drug substances either by chemical modification or by incorporating the peptide into a matrix that slowly releases the pharmaceutical active into its environment. Chemical modification by introduction of D- and exotic amino acids, as well as *C*- and *N*-terminal capping, are still viable techniques for protecting peptides from enzymatic degradation. The use of conjugation agents such as PEGs, however, has also been used successfully for extending the half-life of proteins and peptides by preventing enzymatic degradation and renal clearance (1, 2). The half-life of bioconjugates is dependent on the *in vivo* rate of degradation of the conjugate (i.e., PEG, HES, XTEN, or HSA), and therefore, for any given dosage, the half-life of the bioconjugate cannot be longer than the half-life of the polymer itself.

PEGYLATED PRODUCTS

ADAGEN (PEG-bovine adenosine deaminase) manufactured by Enzon Pharmaceuticals was the first PEGylated protein approved by FDA in March 1990. It is used to treat X-linked severe combined immunogenicity syndrome, as an alternative to bone marrow transplantation and enzyme replacement by gene therapy. Since the introduction of ADAGEN, a large number of PEGylated protein and peptide pharmaceuticals have followed, and many others are under clinical trial or under development stages (see **Tables I** and **II**). On Mar. 27, 2012, FDA announced the approval of the first PEGylated peptide, Peginesatide, for the treatment of anemia associated with chronic kidney disease.

Although PEG has been successfully used in increasing the half-life of proteins and peptides, there are some challenges and concerns, in particular, risk associated with chronic administration of high dose PEGylated peptides (3). Although, PEGs can be filtered through the kidneys over time, high doses of PEGylated peptides and proteins can cause accumulation of the bioconjugate at the target organ and at other organs in the body. The clearance rate

Table I: Table of PEGylated pharmaceuticals (brand name) currently on the market in reverse chronology by FDA approval year with sponor and indication.

Drug	Sponsor	FDA Approval	Indication
Omontys	Affymax/Takeda	2012	Anaemia associated with chronic kidney disease
Krystexxa	Savient	2010	Treatment of gout
Cimiza	Nektar/UCB Pharma	2008	Crohn's disease and moderate to severe rheumatoid arthritis
Mircera	Roche	2007	Anemia associated with Kidney disease
Macugen	Pfizer	2004	Neovascular age-related macular degeneration
Neulasta	Amgen	2002	Treatment of severe cancer chemotherapy induced neutropenia
Somavert	Pfizer	2002	Treatment of acromegaly
PEGASYS	Roche	2001	Treatment of chronic Hepatitis C and B
Doxil/Caelyx	Ortho Biotech/Schering-Plough	2001	Cancer treatment
Pegintron	Schering-Plough/Enzon	2000	Treatment of chronic Hepatitis C and B
Oncaspar	Enzon	1994	Treatment of acute lymphoblastic leukemia in patients who are hypersensitive to the native unmodified form of L-asparaginase
Adagen	Enzon	1990	Treatment of severe combined immunodeficiency disease (SCID)

of peptides and proteins is lower when they are conjugated with a large steric moiety such as PEG. At high doses of the PEG conjugate, the metabolic system can be overloaded, resulting in poor clearance of the peptide or protein. Ultimately, any observed toxicity due to high doses of the PEG conjugate is dependent on the design of the conjugate (i.e., the location of PEG on the molecule in relation to the active part of the peptide), the target organ, the conjugate's mechanism of action, and the toxicological action of the peptide or protein on nearby organs.

PEGylation of peptides has a number of analytical hurdles that may become more of a concern for regulatory authorities. The economic cost of PEG derivatives and their availability are also of concern. The yield of PEGylation is typically in the range of 45% to 60% and therefore, for every unit of bioconjugate, twice as much PEG would have to be purchased. A contract manufacturer views the purchase of PEG in similar terms to the purchase of other raw materials such as amino acids. Amino acids cost approximately $1 per gram at large scale whereas PEG can cost in the range of $200–$500 per gram. Conversely, the innovator of a PEGylated peptide may view a fair cost comparison to be between the peptide and PEG, in which case the PEG may be lower in cost. As the scale of a peptide project increases from hundreds of grams to several kilograms, the cost of PEG in the manufacturing of PEGylated peptides will probably become the main driving cost of manufacture. Ultimately, the deciding factor on the economies of using a PEG bioconjugate would be dependent on the dosage of the final drug.

Many PEGs are unique and are only available from one PEG vendor. This single sourcing is of concern because as projects mature and commercialize, it is important to have alternative sources of raw materials to mitigate risk. The single source of PEG also raises the question of how generic companies can enter the market with PEGylated biosimilars. From the innovators point of view, the use of a PEG with a proprietary linker may provide exclusive patent protection even though royalties may be required by the PEG raw-material vendor. From a risk-mitigation stand point, a single vendor source would have to provide some sort of contingency plan in transferring their proprietary linker technology to a third party in the event the operations of the single-vendor source shut down for an extended period of time.

ALTERNATIVES TO PEG

Possible alternatives to PEGylation include, for example, HESylation, XTENylation, HSAylation, acylation, PASylation, and glutamylation. The conjugation of peptides to hydroxyethyl starch (HES), XTEN (a polypeptide), human serum albumin (HSA), lipids (acylation), poly-Pro-Ala-

Table II: Peptide Bioconjugates currently marketed or in clinical trials. Data source: reference 4.

Name	Sponsor	Indication	Clinical phase	Coupled moeity
Albiglutide	GSK	Type 2 diabetes	Phase III	Human serum albumin
Albuvirtide	Frontier Biotechnologies	HIV infection	Phase I	Human serum albumin
BRX-0585	Pfizer	Type 1 and 2 diabetes	Phase II	Serum protein transferin
PC-DAC	ConjuChem	Type 2 diabetes	Phase II	Human serum albumin
CBX129801	Cebix	Diabetic nephropathy	Phase II	PEG
CVX-060	Pfizer-CovX	Cancer	Phase I	Antibody
CVX-096	Pfizer-CovX	Type 2 diabetes	Phase I	Antibody
Duglutide	Eli Lilly & Co.	Type 2 diabetes	Phase III	Immunoglobulin
Liraglutide	Novo Nordisk	Type 2 diabetes	Approved	Fatty acid
Peginesatide	Affymax	Treatment of anemia	Approved	PEG
Semaglutide	Novo Nordisk	Type 2 diabetes	Phase III	Fatty acid

Ser (PAS), or polyglutamic acid (glutamylation) avoids the toxic issue of PEG because they all can be biologically degraded and excreted. Like PEGs, most of these reagents can be customized to exhibit different release profiles.

HES has been used as a plasma expander for many years and is considered to have an exceptional safety profile. Acylation usually involves the conjugation of a peptide to a naturally occurring fatty acid (e.g., palmitic acid) and does not seem to present any toxicological issues. Liraglutide (Victoza), a palmitated peptide, was approved in January 2010 for the treatment of type-2 diabetes.

Despite the advantages of some of these other conjugates, they have a number of challenges. The conjugates based on polypeptides (XTEN, HSA, PAS, polyGlu) are potentially immunogenic, but there is substantial evidence that such immugenicity is not realized *in vivo*. A number of the alternative conjugate molecules face similar economic challenges to PEG. Companies involved in the development of these alternative conjugates need to offer them at substantially lower costs than PEG to make them viable alternatives. The availability of identical, activated polymers from multiple sources would be beneficial to mitigate vendor risk and improve economic viability; however, as long as the respective polymers and linkers are patented, most innovators will remain exposed to the well-known risks of single sourcing of raw materials. Contingency plans for secondary supply should not only benefit innovators, but vendors as well by providing a secure supply of activated polymer.

Both HESylation and PEGylation lead to polydisperse bioconjugates that present unique analytical challenges. Polydisperse conjugates have broad peaks and, in the case of PEG and HES, they tend to have low UV adsorption making it difficult to detect peptide impurities generated in the manufacture of the peptide or during conjugation (5). From an analytical stand point, the ability to link a conjugate to a peptide with a reversible linker would be attractive, although a reversible linker may compromise the pharmacokinetics of the bioconjugate.

Currently, PEGylation for peptide and proteins involves two main families: lysine-active PEGs and sulfhydryl-selective PEG reagents. Examples of lysine-active PEGS include NHS esters. The rate of coupling of a lysine-active PEG increases as the pH is raised; however, peptides are not stable at high pH and therefore a balance between peptide stability and rate of coupling has to be met. Coupling involves the formation of a peptide bond between the side chain NH_2 functional group of lysine and the carbonyl portion of the succinimide. All of the lysine-active derivatives, except aldehydes and ketones, can possibly react with other amino acids, such as imidazole groups of histidine and hydroxyl groups of tyrosine, and therefore in the case of site specific PEGylation, a differential protection strategy may be necessary. Aldehyde- and ketone-based lysine-active PEGs are selective for primary amines.

Examples of sulfhydryl-selective PEG reagents involve maleimides, vinyl sulfones, and thioethers. Sulfhydryl-selective PEG reagents attach to the thiol group of a cys-

teine. Because of the lower abundance of cysteine amino acids (i.e., the second least common amino acid) in peptides, more selective PEGylation can be achieved.

Both types of bonds are fairly strong and difficult to reverse. The ultimate reversible linkage would involve conjugation that can be removed *in vitro* but is stable enough *in vivo*. Reversible, disulfide linkages are also selective to thiols; however, they are susceptible to reduction by biological reducing agents such as glutathione. Although disulfide linkages could be reduced chemically to enable analysis of the peptide after conjugation, the possibility of the bioconjugate being reduced *in vivo* presents a major challenge. The use of a conjugate-maleic-anhydride for conjugation to peptide would allow for the later removal of the conjugate by treatment with mild acid at room temperature (6). Bentley et al. and Greenwald et al. have shown that conjugation with PEG NHS esters can be reversed by hydrolysis under mild acid conditions (6). Zalipsky et al. showed the release of the PEG from a PEG bioconjugate using mild reducing conditions (6).

In the case of PEGylated bioconjugates, use of analytical techniques such as enzymatic digestion and Edman degradation may enable selective cleavage of the PEG-peptide bond. This technique may only be applicable for smaller bioconjugates. The PEG conjugate bound to the peptide creates hindrance to the proteolytic enzymes and, thereby, prevents specific cleavage of the PEG-peptide bond. On the contrary, Edman degradation typically results in cleavage of a peptide bond at adjacent amino acids to the PEG, resulting in a missing amino acid. Veronese (2001) has stated these difficulties could be reduced by the use of a PEG conjugate with a methionine in the side arm that is bound to an amine on the peptide. Cyanogen bromide treatment can be used to break the peptide-methionine linkage and allow independent analysis of the peptide (5).

An alternative would be the formation of a bioconjugate linkage that can be enzymatically digested using nonmammalian enzymes. This mechanism would enable the removal of the polymer *in vitro* in order to perform the desired analytical testing on the peptide component. Moreover, the selectivity of degradation of the conjugate-peptide bond by nonmammalian enzymes would not affect the *in vivo* stability of the bioconjugate.

For conjugates that are currently manufactured recombinantly as fusion proteins with XTEN, HES, and other polypeptides, there is the possibility for developing chemical technology that would create a fusion peptide (i.e., linkage through peptide bond) that could potentially be manufactured by both chemical conjugation and by direct recombinant expression. The chemical synthesis of a fusion peptide would involve chemical ligation technologies, which may include click chemistry, native chemical ligation, and Staudinger ligation. This mechanism would create substantial economies in developing and clinically testing pre-proof-of-concept.

THE ADAPTABLE CHALLENGE

Bioconjugates are versatile and can be used for a number of different applications. Most importantly, they are being used for extending the half-life of the peptide. Despite their many uses they face a number of challenges. Depending on the type of conjugate, there may be safety issues with their toxicological profile as is the case with PEGs. In the case of polypeptide-based conjugates, there is concern over immunogenicity. Economic hurdles, although not a major concern at early stages of development, are certainly a concern at larger scale and at later stages of clinical development of the bioconjugates. The ability to purchase these conjugates from several sources is important for risk mitigation. The analytical hurdles encountered with bioconjugates are of paramount concern, especially with regulatory bodies. Advances in linker technology or analytical methods are vital to deal with these analytical challenges. Bioconjugates are the adaptable challenge.

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