Structural Modification of Proteins and Peptides

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ABSTRACT

Proteins and peptides are ubiquitous in every cell and thus are vital for various biological functions. In recent years, there is an extensive growth in the development of various biologicals and large molecules like proteins and peptides. Now, they are replacing the market of existing organic based pharmaceuticals. This review article intends to summarize various structural modifications that are carried out in order to alter basic properties of peptides and proteins so that their solubility, absorption, permeability and various other problems associated with their delivery can be resolved. Thus they can offer significant therapeutic potential.

Keywords: Proteins, peptides, Prodrugs, PEGylation, Polysialation, Lipidization, Hydrophobic ion pairing, Cylcodextrins, Nobex technology, Emisphere Technology

INTRODUCTION

The progress in peptides and proteins have given a boost to drug innovation and discovery, and challenge the skill of pharmaceutical developers to develop novel techniques and delivery methods for present and future therapies. Thus, the potential of proteins and peptides should not be surprising. The range of chemical diversity offered by this industry is not being possessed by any other class of biological molecules. They are like nature's tool box. The more we can use such natural proteins and peptides or their closely associated analogs, the higher will be its specificity and safety. The benefit of such structurally developed proteins and peptides derived from their physiologically active parent molecules over other molecules is that they substantially reduce the risk of unsought side effects.

Market Value

Owing to complexity and instability associated with protein and peptide drugs, they are mostly delivered by parenteral route. But with progressive development in industry, scientist have been able to develop other routes of delivery such as transdermal, inhalational, intranasal and oral but the preferred one is the oral route. The emergence of more advanced technologies will help in achieving the desired outcomes. The global market for protein drugs increased from $86.8 billion in 2007 to $95.2 billion in 2008 and was estimated to be $160.1 billion in 2013, a compound annual growth rate (CAGR) of 10.9%.[1] However, BCC Research projects the market to grow to nearly $179.1 billion by 2018 with a five-year CAGR of 5.6% from 2013 to 2018.[2] Several emerging proteins and peptides are in preclinical stage. In addition, there are fewer molecules which are already in final stage of clinical development and would be available commercially within the next few years. While a large number of peptides are undergoing clinical trials. Peptides can be used as the active ingredients of new drugs as well as "add-ones" to other pharmaceutical agents at their target sites to modify their biological action.

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Drawbacks associated with proteins and peptides

As such, proteins and peptides are chemically and enzymatically instable. Enzymatic degradation affects both the fraction absorbed and also the half lives of peptides in the body.[7] The cleavage of peptide bond occurs either at the amino or carboxyl terminus of the chain by exopeptidases (i.e. aminopeptidase and carboxypeptidase). Proteins tend to aggregate themselves and interact with various components in biological environment which affect their biological activity, absorption and biodistribution. They are highly soluble with log $P < 0$ and unstable in acid environment of gastrointestinal tract.[6] They have poor absorption through biological membranes. Proteins and peptides are charged molecules that cannot penetrate through the lipophilic plasma membrane by transcellular transport. Also, they have a molecular size >500 Da which restricts its paracellular transport through the tight junctions.[7] Hence, their delivery through oral route becomes difficult. The contact time with the absorbing surface for the delivery system of proteins and peptides are too short to allow therapeutic level to be maintained for a long period of time. This results in incomplete absorption and failure to maintain sustained action with rapid plasma clearance and peculiar dose response curves.[5] They are prematurely uptake by tissues like reticuloendothelial system. Due to their unique physical and chemical properties, they pose both physical and chemical issues and thus their isolation, purification, formulation, and delivery of proteins represent significant challenges to pharmaceutical scientists. Thus, modifying such proteins and peptides not only solves various problems associated with them but surely increases therapies and cures for various diseases and ailments.

COMMERCIALLY AVAILABLE PROTEINS AND PEPTIDES

Commercially marketed proteins and peptides and their mode of action are summarized in Table 1.

Table 1: Commercially Marketed Proteins and Peptides[8]

TRADE NAME	MODE OF ACTION
Angiotensin II	Vasoconstriction, 8 peptide involved in blood Pressure Regulation
Atrial natriuretic peptide	vasodilator, 28 peptide increases glomerular filtration and diuresis
Bradykinin	9-peptide which are produced during tissue damage, inflammation and viral infections
Denileukindiftitox (Ontak)	Used to treatcutaneous T-cell lymphoma.
Deslorelin (Somagard)	GnRH super agonist used to treat central precocious puberty.
Interferon alfa- 2 a (Referon A)	Used for a number ofmalignancies: leukemia, Renal- cell carcinoma, AIDS related Kaposi's sarcoma
Interleukin- 2 (Tecaleukin)	Used for renal- cell carcinoma
Pentagastrin (Peptavion)	Used to diagnose gastric secretory problems
Secretin (Secretin Ferring)	27- peptide in powder form, used to diagnoseZollinger- Ellison syndrome
ThymopentinTimunox (Timunox)	5-peptide used as immunomodulating investigation drug in HIV positive patients

STRUCTURAL MODIFICATION

For viable delivery of proteins and peptides via oral route, the physicochemical properties like (molecular weight, pH stability, hydrophobicity, molecular size, and ionization constant) and biological barriers (proteolysis in stomach, variable pH, poor permeation and membrane efflux) must be considered. This can be done by: (i) Modification of the physicochemical property like lipophilicity and enzyme susceptibility of the macromolecules; (ii) the addition of novel functionalism (e.g. receptor recognition or cell permeability) to macromolecules; or (iii) the use of a delivery carrier system.[6] The combination of these approaches might lead to a successful result.

The modification of the primary structure of a peptide leads to improvement on enzymatic stability and mucosal penetration. The bonding involved in such modifications can be covalent and non-covalent. Cova-

lent modifications involve the chemical derivatization of the available functional groups of amino acids, peptides and proteins. Non-covalent modifications, on the other hand, involve the process by which the polymer chain acquires its proper three-dimensional structure. To modify proteins and peptides structurally not only improves delivery of such drugs but it would also increase patient compliance. Also the structural changes of such drugs are economically viable as compared to developing newer carrier systems. The different ways of structural modification of proteins and peptides are shown are shown in Figure 1.

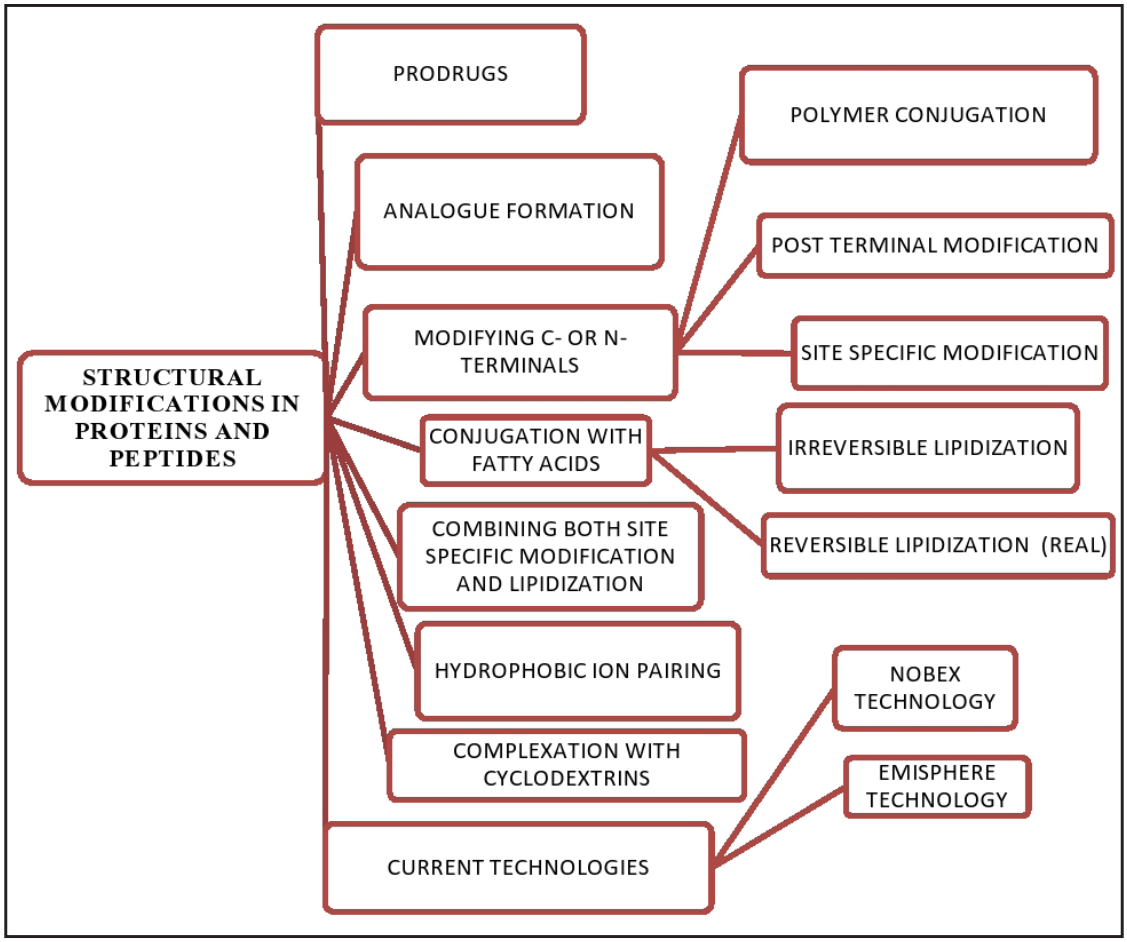

Figure 1: Structural Modification of Proteins and Peptides

The importance of such structural modification are:

solubility, stability, crystallinity, targetability and taste of drug are altered.

- The hydrolytic and enzymatic stability is enhanced.
- The permeability across intestinal epithelia will improve.
- Plasma half life of protein will increase.
- Residence time in the body is prolonged.
- Protein immunogenicity is reduced or eliminated.
- Adverse reactions are few.
- Shelf life is prolonged with increase in systemic circulation and systemic exposure of drug.
- Renal clearance is lowered.
- Patient-compliance is improved.

TECHNIQUES FOR STRUCTURAL MODIFICATION PRODRUG CONCEPT

Prodrugs are pharmacologically inactive entities which are made active by structural chemical modifications. Prodrug strategies consist of a transient or reversible modification of the physicochemical properties of a given compound through chemical derivatization.[9] Solubility, stability, crystallinity, targetability and taste

are some of the parameters which can be altered by prodrug. Thus, a prodrug increases chemical stability, alters aqueous solubility, improves bioavailability and provides hydrolytic stability to the drug. Prodrug maintains intrinsic pharmacological properties of the parent drug. Once the biological barrier is crossed, enzymatic or non-enzymatic reactions convert it into the parent drug (Figure 2).

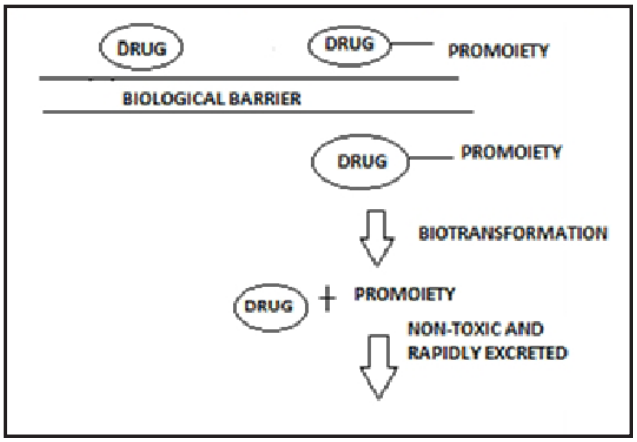

Figure 2: Schematic representation of prodrug formation

Cyclization

"Chemical linkers" are used for cyclization. They stabilize oligopeptides and improve their lipophilicity by reducing their charges and hydrogen bonding potential. The linkers were designed to be susceptible to esterase metabolism leading to release of the peptides. The cyclic prodrug was predicted to be possible for peptides with up to 8 to 9 amino acids. Many cyclic peptides and some of the newer experimental HIV protease and renin inhibitors have high inherent lipophilicity and thus poor aqueous solubility at physiological pH. The formulation of these compounds as aqueous parenteral dosage forms is difficult due to erratic oral bioavailability in animal models. Balkoves *et al.* introduced an ionisable moiety by synthesizing a water soluble phosphate monoester derivative of a lipopeptide by phosphorylation of the phenolic hydroxyl group of a homotyrosine residue.[10] The prodrug exhibited excellent hydrolytic stability and had comparable in vivo activity compared to the parent cyclic peptide, indicating that the prodrug undergoes enzymatic hydrolysis to generate the parent drug. The prodrug now possesses improved aqueous solubility with more sustained concentration in plasma. Borchard t*et al.* prepared acyloxyalkoxy-, phenyl propionic acid- and coumarinic acid-based cyclic prodrugs of [Leu]-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) and its metabolically stable analog DADLE (H-Tyr-D-Ala-Gly-Phe-D-Leu-OH) and determined their biopharmaceutical and metabolic properties.[11] The cyclic prodrugs of these opioid peptides were shown to have:

(i) Unique solution structures that reduce their hydrogen bonding potential;

(ii) Favourable physicochemical properties (e.g., increased lipophilicity) for membrane permeation; (iii) Metabolic stability to exo- and endopeptidase.[11]

Phosphorylation

Chong *et al.* prepared the phosphate monoester prodrugs of a series of a threonine and serine containing peptidomimetic HIV protease inhibitors. The introduction of an ionizable phosphate moiety significantly improved the aqueous solubility of these peptidomimetics.[12] William *et al.* and Kearney *et al.* found that these prodrugs showed the potent antiviral activity in a cell culture assay similar to corresponding parent molecules. Both serine and threonine linked phosphate prodrugs after intravenous administration in rats, showed slow rates of metabolic dephosphorylation.[13,14] This can be due to the steric environment surrounding the phosphate group.

N, O-acyl migration reaction

Oliyai *et al.* and Bundgaard *et al.* used N,O-acyl migration reaction to enhance the aqueous solubility of polypeptides. O-peptide prodrug is formed when β-hydroxyl containing peptides can undergo acid-catalyzed N,O- acyl transfer. A protonated amine group is formed when rearrangement to the O-peptide takes place which impart more desirable solubility properties to the molecule.[15–18]

N-alkylation

An alternative approach to increase the aqueous solubility of a polypeptide is N-alkylation of the peptide bond. Proline replaces alanine or leucine residue which has been shown to substantially improve the water solubility of a cyclic pentapeptide. The solubility enhancement has been attributed to the diminished structural regularity and lowering the intramolecular hydrogen bonding capability of the polypeptide.[19–21]

Thiolation

A feasible site for prodrug modification is the thiol function of a polypeptide. Thioesterification, phosphorylation or disulfide formation are used for formation of thiol prodrugs of conventional xenobiotics and peptide mimetic compounds. Fournie *et al.* showed that the pharmacological effect of the drug entity is restored once the prodrugs are biologically converted to parent thiol.[22–24]

ANALOGUE FORMATION

It involves substitution of existing amino acids with new amino acids. Here, modifications are carried out by interchanging of amino acids preferably by substitution of L- amino acid with D- amino acids or by replacement with different amino acids[25] eg. (i) A vasopressin analogue-Desmopressin (1-desamino-8-D-argininevasopressin), in this, synthesis is done by deamination of N-terminal amino acid and replacement of the C-terminal L649 arginine by D-arginine in vasopressin. This enhances the permeability across intestinal epithelia and improves enzymatic stability of vasopressin. Vasopressin, a nonapeptide, is orally active at large doses but its analogue, desmopressin have twice activity as that of vasopressin at 1/75 fraction of dose. (ii) A thyrotropin releasing hormone, Thytropar (tripeptideGlu-His-Pro) analogue- MK-771: It is synthesized by substituting nitrogen atom with sulphur in pyrrolidine ring 2 of the proline residue of THR . It has two hundred times more CNS activity and is equipotent of THR in causing release of TSH. The oral bioavailability of thytropar is poor and has high metabolic clearance. Though the oral bioavailability of its analogue was only 2% due to inefficient membrane transport, its metabolic clearance is low. (iii) Oxytocin (cyclic nonapeptide) analogue–Carbetocin: 1-Butanoic acid-2-(O-methyl-L-tyrosine)-1-carbaoxytocin) and I-deamino-2-tyrosine(o-ethyl)-oxytocin: Oxytocin, as such, have shorter duration of action and half life of 3-5min. Its derivative has longer half life of 40 min and longer duration of action.

MODIFICATION IN N- OR C-TERMINUS OF THE PEPTIDE CHAINS

By conjugation with polymers *PEGylation*

PEGylation is considered as a gold standard for chemical modifications of protein drugs. It involves one or more polyethylene glycol (PEG) chains linked to a protein, peptide or non-peptide molecule (Fig. 3). PEGylation is the term given to conjugation with monomethoxy poly (ethyleneglycol) or mPEG. PEG polymer, a polyether compound, or a polymer of ethylene oxide is highly soluble in water and is FDA approved.

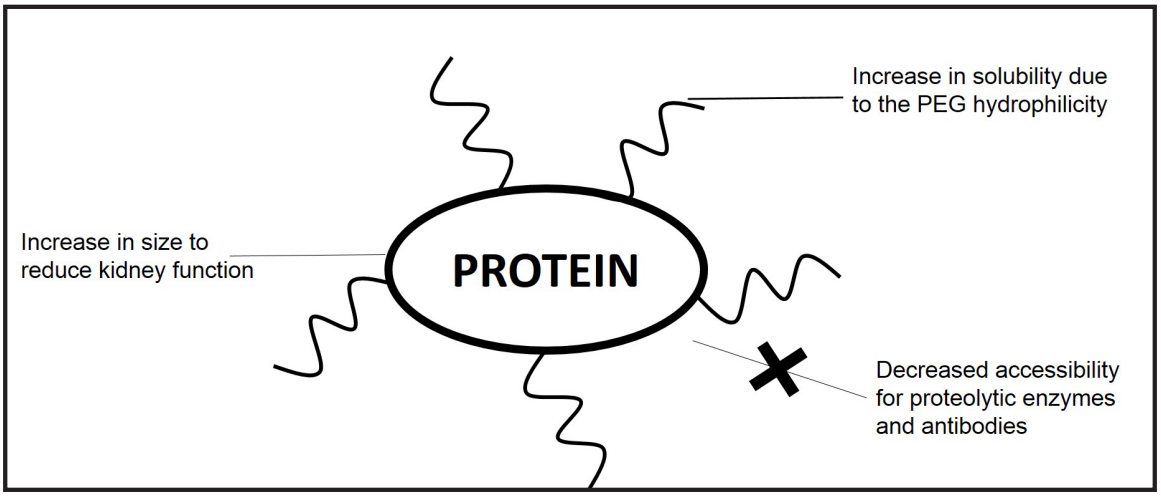

Figure 3: PEGylated protein

Advancements by Pegylation (Table 2)

It improves the pharmacokinetics of drugs and increases the plasma half life of protein. This may be due to PEGylated protein is too large for glomerular filtration. It thus, sterically hinders the protein's interaction with cellular receptors required for metabolism and elimination. eg. PEGylated adenosine deaminase (PEG-ADA) which is the first PEGylated approved product and used for ADA-deficiency. It is non-antigenic, non-immunogenic and non-toxic. The PEG-drug conjugates have prolonged residence time, decreased degradation by metabolic enzymes, reduction or elimination of protein immunogenicity and increased protein stability. It also decreases protein aggregation, few adverse reactions, long shelf life. Thus increasing the systemic circulation and systemic exposure. It also improves patient-compliance.[27,28]

PEGylation enhances the potential of peptides and proteins as therapeutic agents and plays an important role in drug delivery. For maximum pharmacological benefits, a stable bond is formed between PEG and polypeptide.[28] To prepare an active PEG derivative with a functional group—such as active ester, active carbonate, tresylate or aldehyde suitable for coupling to a given target

Table 2: Advancements by Pegylation

PROPERTIES	EFFECT
PHYSICAL	• Hydrodynamic size increases • Molecular weight increases
PHYSICOCHEMICAL	• The protein conformation changes • Loss in binding affinity due to steric hindrance with the drug-target binding interaction • The physiological distribution behaviour altered due to increased hydrophilicity • Change in electrostatic binding dueto an altered charge distribution.
PHARMACOKINETICS	• Increase in elimination half life • Reduced renal clearance

molecule a variety of chemical modifications are used. The polypeptide drug is then covalently linked by PEG derivative. Preferred positions for PEGylation are the N-terminal amino group of the polypeptide backbone and the ε-amino group in the side chain of the amino acid residue lysine. Being highly labile, mild chemical reaction conditions are used by proteins and peptides for the conjugation at the hydrophobic sites.[27,28]

Cazalis*et al.* prepared a strategy for site-specific PEGylation of a thrombomodulin (TM) derivative at the C- terminus. A shortened TM mutant which consists of epidermal growth factor (EGF)-like domains 4-6 was expressed in Escherichia coli with a C-terminal azido-methionine. The TM mutant was site-specifically conjugated to a methyl-PEG-triarylphosphine compound via the Staudinger reaction. Enzymatic activity of the TM so formed before and after PEGylation remain unchanged, confirming the efficacy of this site-specific PEGylation scheme.[29]

PEGylation improves the properties of several classes of protein drugs, such as enzymes, cytokines and antibodies. In general, the improvements are reduction of immunogenicity, increased retention time in the body and increased stability towards metabolic enzymes. The drawback associated with the PEGylation of proteins involves loss of biological activity and especially with PEGylation at N-terminus. But this is compensated by the increased stability, higher hydrodynamic volume and the prolonged body-residence time. An exemplary example is the PEGylated α-interferon Pegasys®, in which though only 7% of the antiviral activity of the native protein is retained, but because of the improved pharmacokinetics it shows a greatly improved performance *in vivo* compared with the unmodified enzyme. PEGylation, thus, helps in paracellular transport of proteins and peptides.[36]

Drawbacks of PEGylation

Some drawbacks associated with PEGylation are: Loss of pharmacological activity and loss of binding affinity with the drug target. PEG is non- biodegradable and thus, there is some evidence of enzyme driven low rate oxidation generating aldehydes and ketones. But it is not a normal detoxification mechanism. PEG end up in the tissues participating in the uptake of the PEGylated constructs where it will accumulate intralysomally when it is conjugated to therapeutic proteins which are large enough to escape kidney clearance. Additionally, PEGylated proteins generate anti-PEG antibodies that could influence the residence time of the conjugate in the circulating blood. Though, no adverse effects of PEG immunogenicity have been observed, possibly because of the very small amounts of injected PEGylated drugs currently in use.[34] Some pegylated drugs are mentioned in table 3.

Poly(styrene-co-maleic acid anhydride) or SMA: It is a synthetic polymer, made up of styrene and maleic anhydride polymer, with molecular weight of 1.5 kDa, which on conjugation with proteins and peptide increases systemic circulation time and elimination half life of the conjugated drug to natural long-circulating blood plasma components such as serum albumin or lipoproteins.[27]

Polysialation: It involves the conjugation of peptides and proteins to the naturally occurring, biodegradable α-(2→8) linked polysialic acid[3]. Polysialation is illustrated in Figure 4.

Polysialic acid is highly hydrophilic in nature which increases the systemic circulation. It has the advantage of being biodegradable and its catabolic products are not toxic (e.g. NeuNAc). It markedly reduces proteolysis, prolongs the half life in systemic circulation (up to 40 h) and retains the activity of protein and peptide *in-vivo*.It also reduces immunogenicity and antigenicity. Polysialic acids are linear polymers of *N*-acetylneuraminic acid (sialic acid) abundantly present on the surface of cells and many proteins.[3] Polysialylation has been tested and are used in therapeutics for the treatment of various diseases. It includes preservation of stability and function, optimal pharmacokinetics and pharmacodynamics, and

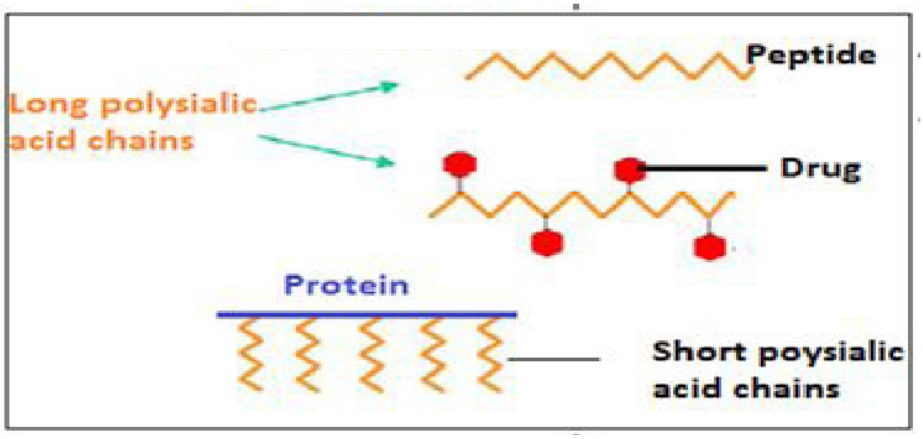

Figure 4: Polysialation

reduced immunogenicity or antigenicity.[3] Gregoriadis *et al.* coupled covalently a low molecular weight polysialic acid, colominic acid with Erwinia carotovora L- asparginase (enzyme for treating leukemia) thus prolonging the circulation of the protein. The activity of the enzyme is retained and it is protected from inactivation in the presence of blood plasma with increase residence time in the circulation.[34] Jain *et al.* reacted the amino group of recombinant human insulin with the oxidized form of polysialic acids (colominic acid, 22 and 39 kDa) forming an aldehydic group**.** Polysialylationthus enhanced the therapeutic value of insulin with improved pharmacological properties with long term control of blood glucose level.[35]

Post Translational Modification

It is a step of protein synthesis which involves covalent addition of functional groups or polypeptide sequences to increase the functional diversity of the proteome.[37] The post translational modifications in proteins are of great importance for protein folding and function, especially in higher organisms. Besides shortening of the nascent polypeptide chain at the N- or C-terminus, phosphorylation and glycosylation are by far the most common types of PTM found in proteins. The main post-translational modifications associated with proteins are: Acetylation, acylation, ADP-ribosylation, Amidation, γ-Carboxylation and β-hydroxylation,

Table 3: PEGylated drugs

BRAND NAME	DRUG NAME (PEGylated)	PARENT DRUG	INDICATION	HALF LIFE		PEG MOL WT.	YEAR OF FDA APPROVAL
				PEGylated drug	Drug		
Adagen	Pegadmase	Adenosine deaminase	Severe combined immunodeficiency disease	3 to > 6 days	80 h[31]	5000	1990
Oncaspar	Pegaspargase	Asparaginase	Leukemia	5.8 days	14–22h	5000	1994
PEG-intron	Peginterferon-α2b	IFN- α2B	Hepatitis C	13 h	0.98 h	12000	2000
Pegasys	Peginterferon-	IFN- α2A	Hepatitis C	51 h	0.7h	40000	2001
Neulasta	Pegfilgrastim	Granulocyte-colony stimulating factor (GCSF)	Neutropenia	7 h	1.8h	20000	2002
Somavert	Pegvisomant	Growth Hormone antagonist	Acromegaly	10 h	0.34h	4–5* 5000	2003
Macugen	Pegaptanib	Anti-VEGFcaptamer	Age-related macular degeneration	10 days	2h[30]	40000	2004
Mircera	Epoetin beta-methoxy polyethylene glycol	Erythropoietin (EPO)	Anemia associate with Kidney disease	140h	4–12h	40000[32]	2007
Cimzia	PEG-Certolizumabpegol	Anti-TNF Fab	Rheumatoid arthritis and Crohn's disease	70h	4–6h	40000[33]	2008

Disulfide bond formation, Glycosylation, Phosphorylation, Proteolytic processing, Sulfation. The different types of post translational modification are shown in Figure 5. It increases the biological half-life (or elimination half-life), reduces immunogenicity and improves the solubility. The advantage over PEGylation is the use of endogenous substances that are natural and biodegradable .[26]

Site Specification Modification

Some polypeptides have low aqueous solubility and high tendency to aggregate. Biomolecules which have high therapeutic potential are deserted due to these properties. Various strategies which can be approached are: (i) Fusion of a target polypeptides to a solubilising protein fusion partner such a maltose-binding protein (MBP), glutathione S-transferase (GST), green fluorescent protein (GFP) (ii) Glycosylation with hydrophilic carbohydrates, (iii) PEGylation with water soluble poly(ethylene glycol) (PEG) chains.

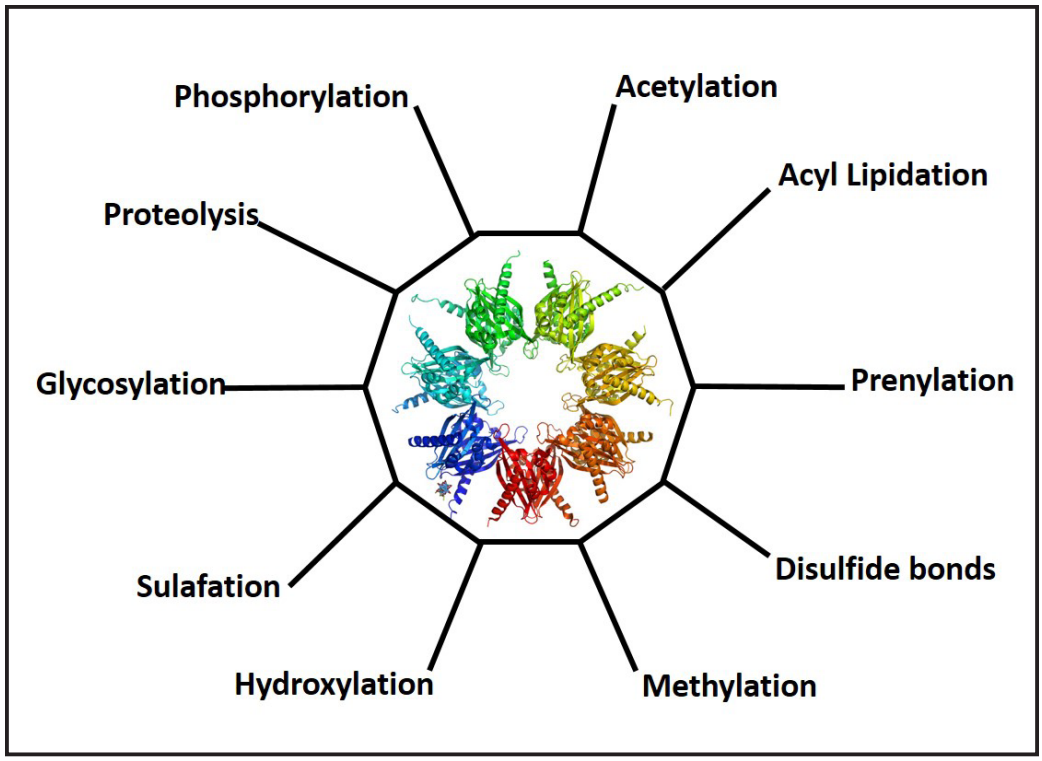

Figure 5: Post translational modification

But the drawback of these strategies is that they require introduction of large molecules onto proteins and peptides which can perturb their structure and function. This can be overcome by addition of short solubility enhancement peptide tags containing 5–10 positively charged amino acids to N- or C-terminus of proteins and peptides. Such peptide tags are small relative to large proteins but are in significant fraction for smaller proteins. Tolbert *et al* used a betaine moiety, a small molecule, containing a positively charged quaternary ammonium group (MW-100) is introduced as a small solubility enhancement tag onto the N-terminus of polypeptides by chemical ligation or expressed protein ligation. The modified forms of polypeptides are more water soluble eg. Modification of 153-residue enzyme xanthine-guanine phosphoribosyltransferase (GPRT) and 38-residue CG-T20 with the betaine moiety. The strategy which is involved is first time generating an N-terminal cysteine by tobacco etch virus NIa protease (TEV protease) and then ligating a betainethioester onto the N-terminal cysteine by native chemical ligation/expressed protein ligation to form a native peptide bond. Note that the cleavage by TEV protease is not so easy due to hydrophobic nature of the peptides. Thus fusion with small ubiquitin related modifier (SUMO) to N-terminal of CG-T-20 with TEV protease cleavable linker between SUMO and CG-T-20 as SUMO has the ability to enhance protein expression and solubility.[38] GPRT is a hydrophobic protein which aggregates and precipates in acidic pH below 6 and is stable at neutral pH whereas CG-T 20 is a hydrophobic peptide (a modified version of Enfuviritide) which aggregates at both neutral and acidic pH. Once the site specific modification is done, aggregation is reduced and solubility

is increased at acidic pH with no effect on structure and function. GPRT solubility increases at about 4.8 times the original protein at pH 5 and CG-T 20 peptide solubility increased to 6.2 times at pH 6.

CONJUGATION WITH FATTY ACIDS (LIPIDIZATION)

Irreversible Lipidization Technique - Conventional Lipidization Approach

It improves the lipophilicity of proteins and peptides. Lipidization is a post transcriptional modification of proteins which improves membrane permeability, metabolic stability, bioavailability and changes pharmacokinetic and pharmacodynamic properties of several peptide therapeutics.[2,39] It involves acylation of the ε-amino group of lysine residues or acylation of N - terminal α - amino groups.[40] The presence of a lipid group in peptides changes their secondary structures, hydrophobicity and self-assembling propensities but retains its ability to bind to target receptors. Cheng *et al.* produced the analog, Mal-sCT water-soluble (-maleimido lysine derivative of palmitic acid) of salmon calcitonin, a 32-amino acid peptide drug by lipid modification.[41] It was found to have greater stability in-vivo with no compromise in the activity of the peptide. Disadvantages of this technique are that a lipid and a polypeptide are usually incompatible in reaction media, a directly linked lipid–polypeptide conjugate is very likely insoluble in water and lipidized polypeptides in general are of low biological activity.[42,43]

Reversible Aqueous Lipidization Technology (REAL):

This is a new method for developing fatty acids-polypeptide conjugates which is carried out in aqueous solution and can regenerate the original active polypeptides in tissues or the blood and thus called REAL Technology. Lipidization has been carried out at cysteine residues following cleavage of intra -disulfide bonds with a reducing agent. This type of lipidization is typically reversible.[42]

REAL modified peptides provides increase in git stability, plasma half life and plasma absorption. Reversible lipidization is a simple and effective approach for improving the efficacy of peptide drugs[44]. Shen *et al.* carried out reversible lipidization of salmon calcitonin, a 32-amino acids polypeptide drug. sCT itself is susceptible towards enzymatic degradation with plasma concentration of 4.6 ± 2.8pg/0.2 mL plasma after 12 hours of oral administration. sCT was reversibly lipidized using a lipidizing agent, N-palmitoylcysteinyl 2-pyridyl disulfide. REAL-sCT formed has modified properties like enhanced oral bioavailability, improved pharmacokinetic and pharmacodynamic behaviour and prolonged release. It has plasma concentration of 7pg/mL plasma after 12 hours of oral administration. REAL-sCT also showed anti-bone resorption activity in ovariectomized (OVX) rats.[42]

Wang *et al.* carried out reversible lipidization of opioid peptide leu-enkephalin (ENK). It has poor oral absorption and is susceptible to enzymatic hydrolysis in small intestine mucosal homogenate (half life-12 min) and liver homogenate (half life- 80min).They are devoid of antinociceptive effect when injected intravenously unless suprapharmacological doses (170–320 mg/kg) are used. Lipidizing agent used here was 3,4-bis(decylthiomethyl)-2,5- Furandione (a novel amine-reacting lipophilic dimethylmaleic anhydride analog. REAL product has enhanced stability towards enzymes with 12 fold increment in half life (93 hours).It also has higher an sustained plasma peptide levels upto 24 hours in mice (4.4 times higher) and AUC 21 times higher. It showed a significant and sustained anti-nociception action in rodent inflammatory model.[43] Shen *et al.* prepared and characterized the reversible lipidized-dipalmitoyldesmopressin and compared its anti-diuretic efficacy and biodistribution with unmodified desmopressin (DDAVP). The intramolecular disulfide bond in DDAVP was reduced using Dithiothreitol (DTT), and the reduced DDAVP was then treated with a lipidization reagent, thiopyridine-containing disulfide, Pal-CPD. Acid precipitation and then size –exclusion was used to purify the product, DPP. The potency of the anti-diuretic activity of DDAVP was enhanced to more than 250-fold when DPP when administered to Brattleboro rats subcutaneously. When DDAVP is administered intravenously, the plasma concentration decreased rapidly during the first 20 min followed by slow elimination rate. But in case of DPP administration, the plasma concentration increased in first 20 min followed by slow elimination rate similar to DDAVP injected mice. DDAVP was regenerated and was detected in the plasma of mice treated with DPP. It was found that there was a 250-fold increase in anti-diuretic activity of DDAVP once modified.[42]

Combination of site specific modification and lipidization technique

Chang *et al.* used the potential of combining site-specific amino acid substitution and lipid modification to increase the circulating half-lives of ACTH. Cys was used to substitute Phe39 of ACTH which has a free sulfhydryl group. This sulfhydryl group react specifically with iodoacetamide derivatives of lipophilic groups. The biological activities of lipophilized ACTH were higher

than native ACTH as it bound more tightly to human serum albumin and cell membranes in vitro and had longer serum half-lives in vivo than native ACTH. These results indicate that the pharmacokinetic properties of peptides can be improved by site-specific substitution with cysteine residues and subsequent conjugation with lipophilic moieties.[45]

HYDROPHOBIC ION PAIRING

Modification of protein by adding an opposite charged surfactant that binds to protein to obtain neutral hydrophobicity entity. Solubility of ionic compounds is high due to ease of solvation of the counter ions with ionic detergents. Positively charged proteins and negatively charged surfactants should be employed, since cationic surfactants might have toxic side effects.[46] Replacement of counter ions decreases the aqueous solubility and increases the lipophilicity. Thus, it changes the partition coefficient by orders of magnitude. HIP method is inexpensive and reversible. The process involves replacement of counter ions (e.g. chloride, acetate, nitrate) with an ionic detergent of similar charge. For many proteins, dissolution in organic solvents occurs with retention of native-like structure and maintenance of enzymatic activity without any chemical modification.[47] Thus, ion-pairing is a useful method for increasing the bioavailability of drugs and enhancing permeation of certain drugs.[48] Figure 6 shows the general scheme of hydrophobic ion formation.

HIP complex display increases ability to cross biological membrane. Dai *et al*., Koul *et al*., Sun *et al*. prepared insulin hydrophobic ion paired complex using different anionic surfactants like sodium lauryl sulphate, surfoplex, sodium deoxycholate and sodium oleate respectively in 1: 6 ratio (drug : surfactant).[49–52] The hydrophobic ion paired complex so formed has new properties like increase solubility and partitioning in 1-octanol, retained complex tertiary and secondary structures of insulin. In-vivo activity of insulin is similar to free insulin. Nanoparticles formed using HIP has high entrapment efficiency and higher drug loading as shown by Sun *et al*

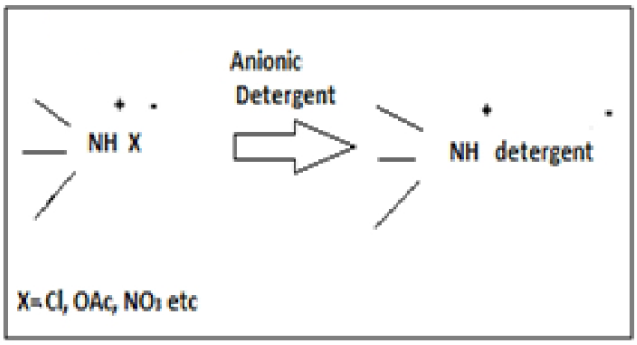

Figure 6: Hydrophobic ion formation

and Kendrick *et al* prepared HIP complex of subtilisin BPN' protein using sodium dodecyl sulphate in 1: 50 ratio (Drug : Surfactant).[51] The new HIP complex of drug so formed maintains native structure of protein, it prevents intermolecular β sheet formation, increased activity as a suspension in iso-octane.[53] Yang *et al*. prepared HIP complex of water soluble peptide melittin using sodium dodecyl sulphate in 1:6 ratio. The solubility of drug after complexation increases in organic solvent significantly. Its bioactivity is similar to free melittin and entrapment efficiency of drug in PLGA nanoparticles become high. (about 90%).[54] Park *et al*. prepared HIP complex of leuprolide acetate (an analogue of luteinizing hormone-releasing hormone (LH-RH)) with sodium oleate in 1:1 ratio. Newer features of the complex thus formed is that it becomes more lipophilic. The complex suppresses the immediate burst release of leuprolide from the microspheres in contrast to that prepared from free leuprolide. Both the formulations of microspheres showed constant release profile.[55]

Park *et al*. prepared HIP complex of Salmon calcitonin using sodium oleate as complexing agent. The complexes when incorporated into nanoparticles are readily taken up by Caco2 cells. In-vivo experiments showed that they are readily absorbed orally.[56]

Park *et al*. formed the HIP complex of hen egg white lysozyme which is small protein composed of 129 amino acids residues in 1:6 ratio with SDS or sodium oleate at pH 10.[57]

COMPLEXATION WITH CYCLODEXTRINS

Proteins and peptides are hindered by unfavourable solubility, aggregation irreversibly or instability. Cyclodextrins are circular oligosaccharides composed of α- (1,4)-linked α-D-glucosyl units. The exterior of cycolodextrin is hydrophilic and its cavity is hydrophobic. This cavity gives it the ability to accommodate hydrophobic molecules/moieties in the cavity. Such an arrangement of molecules provides multiple favorable effects cyclodextrins have on proteins, which is commonly used in pharmacological applications. The most common and industrially utilized cyclodextrins are α-, β- and γ-cyclodextrin, which consist of six, seven and eight glucosyl units. Cyclodextrins are hollow, toroidal, shortened cones. Cones have hydrophilic rims crowned by hydroxyl primary group at 6^{th} position on the narrow rim and secondary hydroxyl groups on 2^{nd} and 3^{rd} position on the wide rim. In contrast, the internal cavity is lined with ether oxygen at 4^{th} position and hydrogens at 3^{rd}, 5^{th} and 6^{th} position. Various effects of cyclodextrins on different proteins

are: protection against degradation (if point of attack of a protease is sterically `masked' by cyclodextrin), aggregation suppression (if residues responsible for aggregation are highly solvent accessible), alteration of function (if residues involved in function are `masked' by cyclodextrin)[7].

Cyclodextrins' have the ability to use the hydrophobic part present on protein surfaces. This makes them valuable in two areas. Firstly, it helps in the delivery of proteins and small molecules to target site or systemic circulation. For example, hydroxypropyl-β-cyclodextrin enhances the affinity and antagonist potency of a neuromedin B receptor antagonist, the peptide PH 168368, by increasing its solubility while dimethyl- β-cyclodextrin increases the nasal absorption of leucine enkephalin. Secondly, it helps in the stabilization of proteins against aggregation, thermal denaturation and degradation. Thus, cyclodextrins increase the shelf-life of therapeutic proteins, such as growth hormones and insulin.[58]

Cyclodextrins have also been suggested to act as `chaperone mimics' by enhancing protein refolding from denatured or even aggregated states. Busrelin acetate, a synthetic nonapeptide, is pyro Glu-His-Trp-SerTyr-D-Ser(tert-butyl)-Leu-Arg-PrO-ethylamide, is a highly potent agonist of lueitnizing hormone-releasing hormone. Drug release from a vehicle is influenced by various factors: Drug-vehicle interactions, solubility, partition coefficient and the particle size of drug in vehicle. Busrelin acetate is practically insoluble in peanut oil and solubility increased slightly on complexation with DE-β- CD ((2,6-di-O-ethyl)-beta-cyclodextrin) or TA-CD's (triacetyl CD). In contrast, the solubility of the CDs in the vehicle increased isin the order of TA-β-CD<TA-γ-CD<DE-β-CD corresponding with the retardation order of buserelin release. Within the oily matrix drug might be dispersed through a weak interaction between CDs and drug. Cyclodextrins play an important role in delivery of oligonucleotides by improving their resistance to nucelases and cellular uptake and by reducing their immunostimulatory properties.[59] The structure of β- CD and formation of inclusion complex is shown in Figure 7.

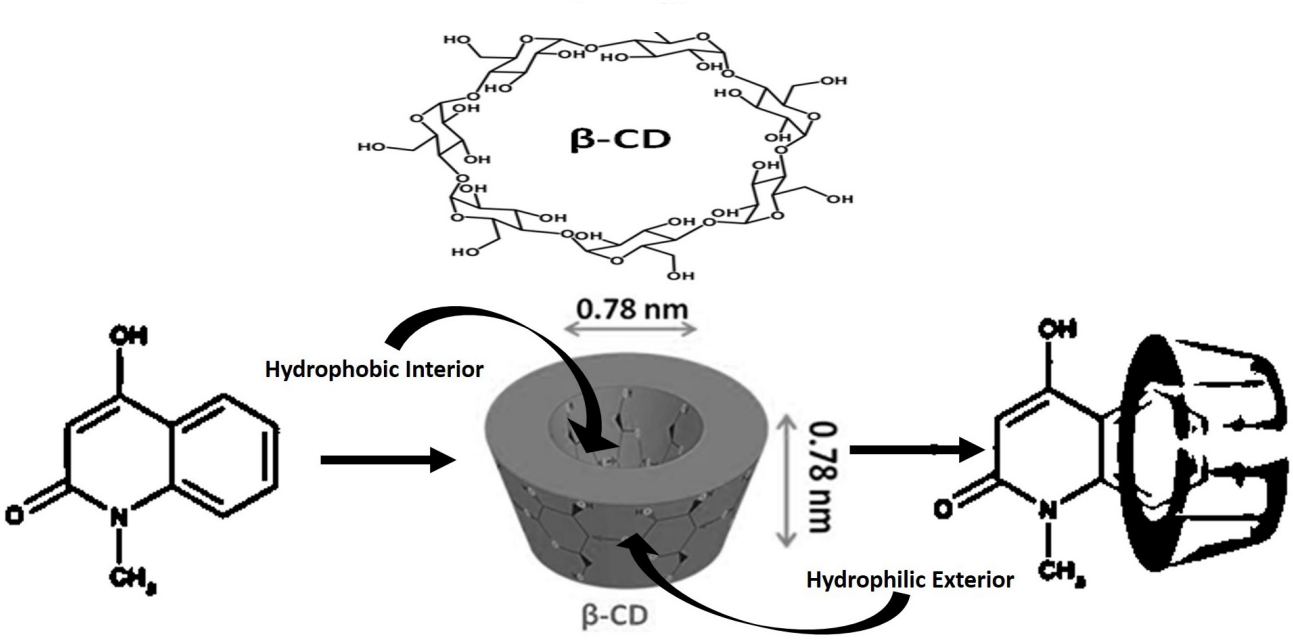

Figure 7: Structure of Cyclodextrin and inclusion complex

CURRENT TECHNOLOGIES

Nobex Technology:

NOBEX oral drug delivery technology focuses on modification of proteins, peptides and small organic molecules by attaching one or more amphiphilic oligomers. Once these oligomers (water and fat-soluble) are attached, it results in improved solubility to allow optimized formulation, modification of pharmacology to prolong circulating half-life and activity and stability to enzymatic degradation. (Figure 8).

Creation of amphiphilic oligomers is accomplished by binding a lipophilic alkyl unit to a hydrophilic polyethylene glycol (PEG) unit. The resulting oligomer is then covalently bound to a specific site or sites on the target molecule to facilitate the desired changes in properties

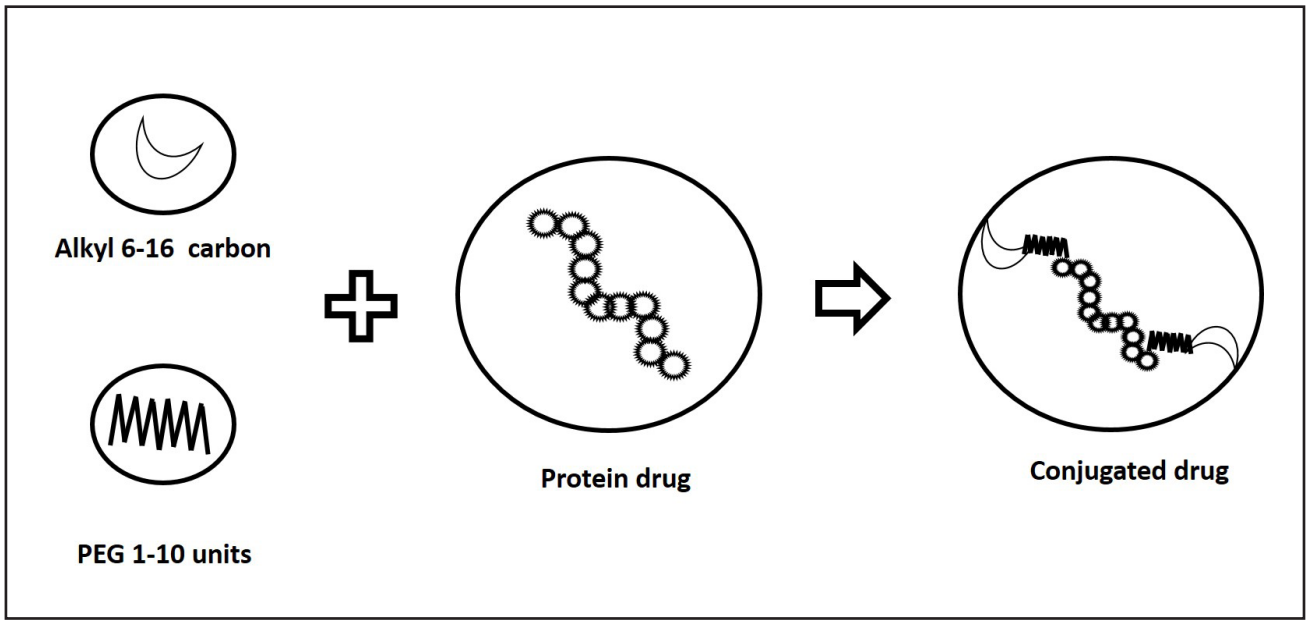

Figure 8: Nobex Technology

of the native drug.The two types of bonds are involved in this technology are hydrolyzable bond at the site of attachment, thus resulting in a pro-drug and non-hydrolyzable bond to create a 'micro-pegylated' drug resulting in inhibition of metabolism of the drug or changes in tissue penetration characteristics, such as facilitation of passage across the blood–brain barriereg. using NOBEX technology, hexyl-insulin-monoconjugate-2 (HIM2) has been developed by covalent attachment of PEG-amphilic oligomer to free amino group on the lysine residue at 20th position in the β-chain of recombinant human insulin. This modification has enhanced the stability of insulin in presence of gastrointestinal enzymes and facilitate greater absorption.[60]

Emisphere Technology

In Emisphere'seligen™ technology, macromolecules like proteins and peptides are made to interact with small hydrophobic organic compounds non-covalently. This increases their lipophilicity and enhances their absorption.[6] The technology uses of proprietary delivery agents, known as EMISPHERE® delivery agents or carriers with a molecular weight of 250 to 350, and almost all of them are amido acids. The non-covalent interaction transiently alters the physicochemical properties of the macromolecules (e.g., hydrophobicity, conformation, stability, etc.).[61] Several macromolecules have been delivered in humans orally, safely and effectively using this technology .[6]The unique potential of emisphere technology has been tried with macromolecules heparin, insulin, recombinant human growth hormone, salmon calcitonin, and parathyroid hormone leads to significantly higher absorption rates through transcellular route compared to drug alone. They process of carrier-mediated absorption is reversible and thereby ensures that the macromolecule regains its native state and maintains its original therapeutic activity following absorption.[61]

CONCLUSION

The peptide and protein based pharmaceuticals thus are rapidly becoming very important class of therapeutic agents. The peptides and proteins based drugs effectively treats various diseases and life-threatening conditions. The improvement in therapies and cure is definite by increasing the alteration in the properties of peptides and proteins. For this various strategies can be used one of which can be structural modification. Modification in the structural moieties of these drugs alters solubility, lipophilicity, stability, crystallinity, targetability, taste and enzymatic susceptibility. For this reason, pharmaceutical and biotechnological companies are doingdifferent researches and testing new techniques for the modifications. By applying these techniques the delivery of such drugs may be possible, as they provide high specificity, bioreactivity, safety, and overall higher success rate.

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CONFLICT OF INTEREST

None declared

ABBREVIATIONS

Abbreviation	Full form
CAGR	Compound annual growth rate
Log P	Logarithm value of partition coefficient
GnrH	Gonadotropin releasing hormone
AIDS	Aquired Immunodeficiency syndrome
HIV	Human Immunodeficiency virus
PEG	Polyethylene glycol
ADA	Adenosine deaminase
PED-ADA	Pegylated adenosine deaminase
TM	Thrombomodulin
EGF	Epidermal growth factor
SMA	Poly(styrene-co-maleic acid anhydride)
NeuNAc	N-acetylneuraminic acid
MW	Molecular weight
GPRT	Guanine phosphoribosyltransferase
TEV	Tobacco etch virus
SUMO	Small ubiquitin related modifier
sCT	Salmon calcitonin
Mal	Maleimido
REAL	Reversible aqueous lipidizable technology
OVX	Ovariectomized
ENK	Enkephalin
AUC	Area under curve
DDAVP	Unmodified desmopressin
DTT	Dithiothreitol
PAL-CPD	Thiopyridine-containing disulfide
ACTH	Adrenocorticotropic hormone
HIP	Hydrophobic ion pairing
LH-RH	Luteinizing hormone-releasing hormone
PLGA	Poly(lactic-co-glycolic acid)
SDS	Sodium dodecyl sulphate
CD	Cyclodextrin
HIM2	Hexyl-insulin-monoconjugate-2

Abbreviation	Unit
Da	Dalton
$	Dollar
h	hours
min	minutes

REFERENCES

1. Hu G. Understanding the fundamentals of peptides and proteins. BioProcessing Journal. 2011; 10: 12–4.
2. Renukuntla J, Vadlapudi AD, Patel A, Boddu SHS, Mitra AK. Approaches for enhancing oral bioavailability of peptides and proteins. Int. J. Pharm. 2013; 447: 75–93.
3. Gregoriadis G, Jain S, Papaioannou I, Laing P. Improving the therapeutic efficacy of peptides and proteins: A role for polysialic acids. Int. J. Pharm. 2005; 300(1): 125–30.
4. Degim IT, Celebi N. Controlled Delivery of Peptides and Proteins. Curr. Pharm. Des. 1998; 13: 99–117.
5. Marshall SA, Lazar GA, Chirino AJ, Desjarlais JR. Rational design and engineering of therapeutic proteins. Drug Discov. Today. 2003; 8: 212–21.
6. Morishita M, Peppas NA. Is the oral route possible for protein and peptide delivery. Drug Discov.Today. 2006; 11: 905–10.
7. Irie T, Uekama K. Cyclodextrins in peptide and protein delivery. Adv. Drug Delivery Rev. 1999; 36: 101–23.
8. Rishabh P, Singh AV, Awanish P, Poonam T, Majumdar SK, Nath LK. Protein and Peptide Drugs: A Brief Review. Research J. Pharm. and Tech. 2009; 2: 228–33.
9. Oliyai R, Stella VJ. Prodrugs of peptides and Proteins for improved Formulation and Delivery. Annu. Rev. Pharmacol. Toxicol. 1993; 32: 521–44.
10. Balkovec JM, Black RM, Hammond ML, Heck JV, Zambias RA. Synthesis. stability and biological evaluation of water-soluble prodrugs of a new echinocandin lipopeptide. Discovery of a potential clinical agent for the treatment of systemic candidiasis and *fneumocystis carinii* pneumonia (PCP). J. Med. Chem. 1992; 35: 194–8.
11. Borchardt RT. Optimizing oral absorption of peptides using prodrug strategies. J. Controlled Release. 1999; 62: 231–8.
12. Chong KT, Ruwart M, Hinshaw RR, Wilkinson KF, Rush DB, Yancey MF. Strohbach JW, Thaisrivongs S. Peptidomimetics HIV protease inhibitors: Phosphate prodrugs with improved biological activities. J. Med. Chem. 1993; 36: 2575–7.
13. Williams A, Naylor RA. Evidence for SN2(P) mechanism in the phosphoryiation of alkaline phosphatase by substrate. J. Chem. Soc. B. 1971; 10: 1973–9.
14. Kearney AS, Stella VJ. The in vitro enzymatic Iabilities of chemically distinct phosphomonoester prodrugs. Pharm. Res. 1992; 9: 497–503.
15. Oliyai R, StellaVJ. Kinetics and mechanism of isomerization of cyclosporin A. Pharm. Res. 1992; 9: 617–22.
16. Oliyai R, Safadi M, Meier PG, Hu MK, Rich DH, Stella, VJ. Kinetics of acid catalyzed degradation of cyclosporin A and its analogs in aqueous solution. Int. J. Peptide Protein Res. 1994; 43: 239–47.
17. Oliyai R, Siahaan T, Stella VJ. The importance of structural factors on the rate and the extent of N,O-acyl migration in cyclic and linear peptides. Pharm. Res. 1994; 12: I–7.
18. Bundgaard H, Friis GJ. Prodrugs of peptides. Isocyclosporin A as a potential prodrug of cyclosporin A. Int. J. Pharm. 1992; 82: 85–90.
19. Ishikawa K, Fukami T, Nagase T, Fujita K, Hayama T. Cyclic pentapeptide endota helio antagonists with high ETA selectivity. Potency-and solubility-enhancing modifications. J. Med. Chem. 1992; 35: 2139–42.
20. Gordee RS, Zeckner DJ, Howard LC, Alborn WE, Debono M. Anti-candida activity and toxicology of LY121019. A novel semisynthetic polypeptide antifungal antibiotic. Ann. N.Y. Acad. Sci. 1988; 544: 294–301.
21. Blaakmeer J, Tijsse-Klasen T, Tesser GI. Enhancement of solubility by temporary dimethoxybenzyl- substitution of peptide bonds. Int. J. Peptide Protein Res. 1991; 37(6): 556–64.
22. Fournie-Zaluski MC, Coric P, Turcaud S, Lucas I, Nobel, F. 'Mixed-inhibitor-prodrug' as a new approach toward systemically active inhibitors of enkephalin-degrading enzymes. J. Med. Chem. 1992; 35(13): 2473–81.
23. Fournie-Zaluski MC, Coric P, Turcaud S, Bruetschy L. Lucas E. Potent and systemically active aminopeptidase N inhibitors designed from active site investigation. J. Med. Chem. 1992; 35: 1259–66.
24. Swynnerton NF. McGovern EP, Mangold DJ, Nino JA, Gouse EM, Fleckenstein L. HPLC assay for S-2-(3-aminopropylamino) ethylphophorothioate (WR-2721) in plasma. J. Liquid Chromatogr. 1983; 6:1523–34.
25. Vilhardt H, Lundinx S. In vitro intestinal transport of vasopressin and its analogues. Acta physiol. Scand. 1986; 126(4): 601–7.
26. Wold F. In vivo chemical Modification of proteins (Post-Translational Modification). Ann. Rev. Biochem. 1981; 50(1): 783–814.
27. Schiffter HA. The Delivery of Drugs – Peptides and Proteins. 1st(Ed), UK, Oxford: Elsevier; 2011. pp.587–604.

28. Harris JM, Chess RB. Effect of pegylation on Pharmaceuticals. Nature Reviews: Drug Discovery. 2003; 2: 214–21.
29. Cazalis CS, Haller CA, Sease-Cargo L, Chaikof EL. C-terminal site-specific PEGylation of a truncated thrombomodulin mutant with retention of full bioactivity. Bioconjug. Chem. 2004; 15: 1005–9.
30. Esposito CL, Catuogno S, Franscisis VD, Cerchia L. Discovery medicine. 2013, Nov 14.
Available from: URL: http://www.discoverymedicine.com/Carla-Lucia-Esposito/2011/06/13/new-insight-into-clinical-development-of-nucleic-acid-aptamers/.
31. Davis S, Abuchowski A, Park Y.K, Davist FF. Alteration of the circulating life and antigenic properties of bovine adenosine deaminase in mice by attachment of polyethylene glycol. Clin.exp. Immunol. 1981; 46: 649–52.
32. Greindl A, Kessler C, Breuer B, Haberl U, Rybka A, Emgenbroich M, Pötgens AJG, Frank HG. AGEM400 (HES), a Novel Erythropoietin Mimetic Peptide Conjugated to Hydroxyethyl Starch with Excellent *In Vitro* Efficacy. The Open Hematol. Journal.2010; 4: 1–14.
33. Jevševar S, Kunstelj M, Porekar VG. PEGylation of therapeutic proteins. Biotechnology Journal. 2010; 5: 1–50.
34. Fernandes AI, Gregoriadis G. Polysialylated asparaginase: preparation, activity and pharmacokinetics. Biochimica et Biophysica Acta. 1997; 1341(1): 26–34.
35. Jain S, Hreczuk-Hirst DH, McCormack B, Mital M, Epenetos A, Laing P, Gregoriadis G. Polysialylated insulin: synthesis, characterization and biological activity in vivo. Biochimica et Biophysica Acta. 2003; 1622: 42–9.
36. Mahato RI, Narang AS, Thoma L, Miller DD. Emerging Trends in Oral Delivery of Peptide and Protein Drugs. Crit. Rev. Ther. Drug Carrier Syst. 2003; 20(2,3): 153–214.
37. Foder E. Molecular Virology group. 2013, Nov 14.
Available from: URL: http://fodor.path.ox.ac.uk/research/post-translational-modifications-of-influenza-virus-proteins/
38. Xiao J, Burn A, Tolbert TJ. Increasing Solubility of Proteins and Peptides by Site-Specific Modification with Betaine. Bioconjugate Chem. 2008; 19: 1113–21.
39. Zhang L, Bulaj G. Converting peptides into drug leads by lapidation. Curr. Med. Chem. 2012; 11: 1602–18.
40. Jorgensen L, Nielson HM. Delivery Technologies for Biopharmaceuticals: Peptides, Proteins, Nucleic Acids and Vaccines. 1st(Ed), UK,Wiley John & Sons Inc.; 2009. pp.171–91.
41. Cheng W, Satyanarayanajois S, Lim LY. Aqueous-Soluble, Non-Reversible Lipid Conjugate of Salmon Calcitonin: Synthesis, Characterization and In Vivo Activity. Pharm Res. 2006; 24(1): 99–110.
42. Wang J, Chow D, Heiati H, Shen WC. Reversible lipidization for the oral delivery of salmon calcitonin. J. Control Release. 2003; 88(3): 369–80.
43. Wang J, Hogenkamp DJ, Tran M, Li WY, Yoshimura RF , Johnstone TBC, Shen WC, Kelvin WG. Reversible lipidization for the oral delivery of leu-enkephalin. J. of Drug Target. 2006; 14: 127–36.
44. Wang J, Shen D, Shen WC. Preparation, purification and characterization of a reversibly lipidized desmopressin with. potentiated anti-diuretic activity. Pharm. Res. 1999; 16(11): 1674–79.
45. Wan L, Chen YH, Chang TS. Improving Pharmacokinetic Properties of Adrenocorticotropin by Site-Specific Lipid Modification. L Pharm Sci. 2002; 92(9): 1882–92.
46. Bilati U, Mann EA, Doelker E. Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. Eur. J. Pharm. Biopharm. 2005; 59(3): 375–88.
47. Manning MC, Meyer JD. Hydrophobic Ion Pairing: Altering the solubility of Biomolecules. Pharm Res. 1998; 15(2): 188–93.
48. Guerrero DQ, Allemann E, Fessi H, Doelker E. Application of the Ion-pair Concept to Hydrophilic Substances wit h Special Emphasis on Peptides. 1996; 14(2): 119–27.
49. Dai WG, Dong LC. Characterization of physiochemical and biological properties of an insulin/laurylsulfate complex formed by hydrophobic ion pairing. Int. J. Pharm. 2007; 336: 58–66.
50. Rastogi R, Anand S, Koul V. Evaluation of pharmacological efficacy of 'insulin–surfoplex' encapsulatedpolymer vesicles. Int. J. Pharm. 2009; 373: 107–15.
51. Sun S, Liang N, Kawashima Y, Xia D, Cui F. Hydrophobic ion pairing of an insulin-sodium deoxycholate complex for oral delivery of insulin. Int. J. Nanomed.2011; 6: 3049–56.
52. Sun S, Liang N, Piao H, Yamamoto H, Kawashima Y, Cui F. Insulin-S.O (sodium oleate) complex-loaded PLGA nanoparticles: Formulation, characterization and in vivo evaluation. J Microencapsul. 2010; 27: 471–8.
53. Kendrick BS, Meyer JD, Matsuura JE, Carpenter JF, Manning MC. Hydrophobic Ion Pairing as a Method for Enhancing Structure and Activity of Lyophilized Subtilisin BPN' Suspended in Isooctane. Arch. Biochem. Biophys.1997; 347: 113–8.
54. Yang L, Cui F, Shi K, Cun D, Wang R. Design of high payload PLGA nanoparticles containing melittin/sodium dodecyl sulfate complex by the hydrophobic ion-pairing technique PLGA nanoparticles containing melittin/SDS complex. Drug Dev. Ind. Pharm. 2009; 35(8): 959–68.
55. Choi SH, Park TG. Hydrophobic ion pair formation between leuprolide andsodium oleate for sustained release from biodegradable polymeric microspheres. Int. J. Pharm. 2000; 203: 193–202.
56. Yoo HS, Park TG. Biodegradable Nanoparticles Containing Protein-Fatty Acid Complexes for Oral Delivery of Salmon Calcitonin. J. Pharm. Sci. 2003; 93: 488–95.
57. Yoo HY , Choi HK, Park TG. Protein±Fatty Acid Complex for Enhanced Loading and Stability within Biodegradable Nanoparticles. J Pharm Sci. 2001; 90: 194–201.
58. Aachmann FL, Otzen DE, .Larsen KL, Wimmer R. Structural background of cyclodextrin - protein interactions. Protein Engineering. 2003; 16: 905–12.
59. Redentia E, Pietra C, Gerloczy A , Szente L. Cyclodextrins in oligonucleotide delivery. Adv. Drug Del. Rev. 2001; 53: 235–44.
60. Still JG. Development of oral insulin: progress and current status. Diabetes Metab. Res. Rev. 2002; 18 (Suppl 1): S29–S37.
61. Singh B, Majuru S. Oral Delivery of Therapeutic Macromolecules: A Perspective Using the eligen™ Technology; 2014, Feb 2,
Available from: URL:http://www.paigekeller.com/ME2/dirmod.asp?sid=HYPERLINK "http://www.paigekeller.com/ME2/dirmod.