



## Conjugative Post-Translational Modifications for Pharmacological Improvement of Therapeutic Proteins

Diego de Carvalho Carneiro<sup>1\*</sup> and Suzana Telles da Cunha Lima<sup>2</sup>

<sup>1</sup>Health Sciences Institute, Federal University of Bahia, Salvador, Bahia, Brazil

<sup>2</sup>Institute of Biology, Federal University of Bahia, Salvador, Bahia, Brazil

### ABSTRACT

Therapeutic proteins can be pharmacologically improved by conjugative post-translational modifications (PTMs) through the rational design of their structure and production process. N- and O- linked glycosylation can confer advantages to proteins and can be controlled by the process conditions, producing cell line and enzymatic expression or activity. The resulting glycosidic profile influences their pharmacological features such as in velaglucerase alfa. Conjugation of polyethylene glycol (PEG) onto therapeutic proteins has been used to overcome pharmacological limitations. They can be site-specifically or randomly linked through the reaction between a terminal group of PEG and an amino acid residue group of the protein. Both protein and PEG components participate in pharmacological mechanisms, enabling a longer half-life for peginterferon beta-1a, for example. Fatty acylation of proteins occurs in cells via some known reactions that may involve different linkages, fatty acids, modified residues, and enzymes. Insulin degludec is a fatty acylated biopharmaceutical formulated to solve the problem of variability in insulin exposure associated with other products. Other potential uses of conjugative PTMs for pharmacological improvement of therapeutic proteins are proposed in this paper.

**Keywords:** PTMs; Biopharmaceuticals; Glycosylation; PEGylation; Fatty acylation

### INTRODUCTION

Therapeutic proteins regularly undergo post-translational modifications (PTMs) in order to consistently form their functional structure, especially when produced by eukaryotic cell lines. Some PTMs are processed by the cellular metabolism during industrial production of protein biopharmaceuticals while others are artificially engineered into the protein structure to improve their pharmacological features [1]. PTMs are additional chemical modifications taken after mRNA templates are readout by ribosomes. They are usually executed by enzymes through peptide bond cleavages or modification of amino acid residues. The functionality of many proteins requires the conjugation of molecules (e.g. glycosidic PTMs) that take part in the protein's chemical activity, intracellular localization, or tertiary structure [2].

Tandem mass spectrometry (MS/MS) has been used to delve the composition and structure of proteins. In this method, a previous mass spectrometry (MS) analysis of the precursor ions is performed before MS analysis of the dissociated ions produced by gas-phase activation. Since the amide bonds in proteins require collision energies higher than e.g. glycosidic or phosphate bonds present in some PTMs, ion activation by electron transfer dissociation (ETD) or electron capture dissociation (ECD) can be used to prevent the loss of PTMs during MS/MS analysis, giving means to elucidate their composition and structure [3]. PTMs are able to change the chemical and physical properties of proteins such as shape, charge, molecular weight, and interactions with other molecules, enabling the exploitation of those changes through some techniques to separate, identify, and characterize proteins and their PTMs (e.g. two-dimensional electrophoresis, liquid chromatography, western blotting, immunoaffinity-based approaches, etc.) [4]. Mammalian cell lines are preferentially selected in processes involving biopharmaceutical manufacturing since they are the best expression systems for production of proteins with proper post-translational modifications. Chinese hamster ovary (CHO) cells are the most

regularly utilized mammalian cells for industrial manufacturing of therapeutic proteins. CHO cells are more advantageous than other cell lines because they can vigorously grow in suspension culture with chemically-defined or serum-free media, they are safe for pathogenic viruses, and they are able to express proteins with PTMs similar to those found in humans. Moreover, generating engineered mammalian cells expressing a specific gene for production of proteins with quality and high-yield is easily achieved by CHO cell cultures [5]. Modification of therapeutic proteins by PTMs can have an impact on the protein's yield, stability, pharmacokinetics, pharmacodynamics, and immunogenicity. Understanding the association between structural modification of proteins and their function can give means to enhance pharmacological features and processing controls to secure product quality through the rational design of therapeutic proteins. Preselected modifications and improved properties may result in the development of new generations of biopharmaceuticals [6]. This article intends to review the conjugative post-translational modifications used by pharmaceutical industries to improve the pharmacological features of therapeutic proteins for humans. Table 1 summarizes the therapeutic proteins for treatment in humans approved by the FDA since 2002 that were pharmacologically improved by conjugative PTMs [7,8].

**Table 1: Therapeutic proteins pharmacologically improved by PTM conjugates and approved by the FDA for human treatment since 2002**

Product	Company	Therapeutic Indication	PTM
TRESIBA	Novo Nordisk	Diabetes type 1 and 2	Fatty Acylation
PLEGRIDY	Biogen	Relapsing forms of multiple sclerosis	PEGylation
KRYSTEXXA	Savient Pharmaceuticals	Chronic refractory gout	PEGylation
VPRIV	Shire Pharmaceutical	Gaucher Disease	Glycosylation
PEGINTRON	Schering-Plough	Chronic hepatitis C	PEGylation
CIMZIA	UCB	Crohn's disease	PEGylation
MIRCERA	Hoffmann-La Roche	Anemia associated with chronic renal failure	PEGylation
LEVEMIR	Novo Nordisk	Diabetes type 1 and 2	Fatty Acylation
SOMAVERT	Pharmacia Corp.	Acromegaly	PEGylation
PEGASYS	Hoffmann-La Roche	Chronic hepatitis C	PEGylation
NEULASTA	Amgen	Febrile neutropenia	PEGylation

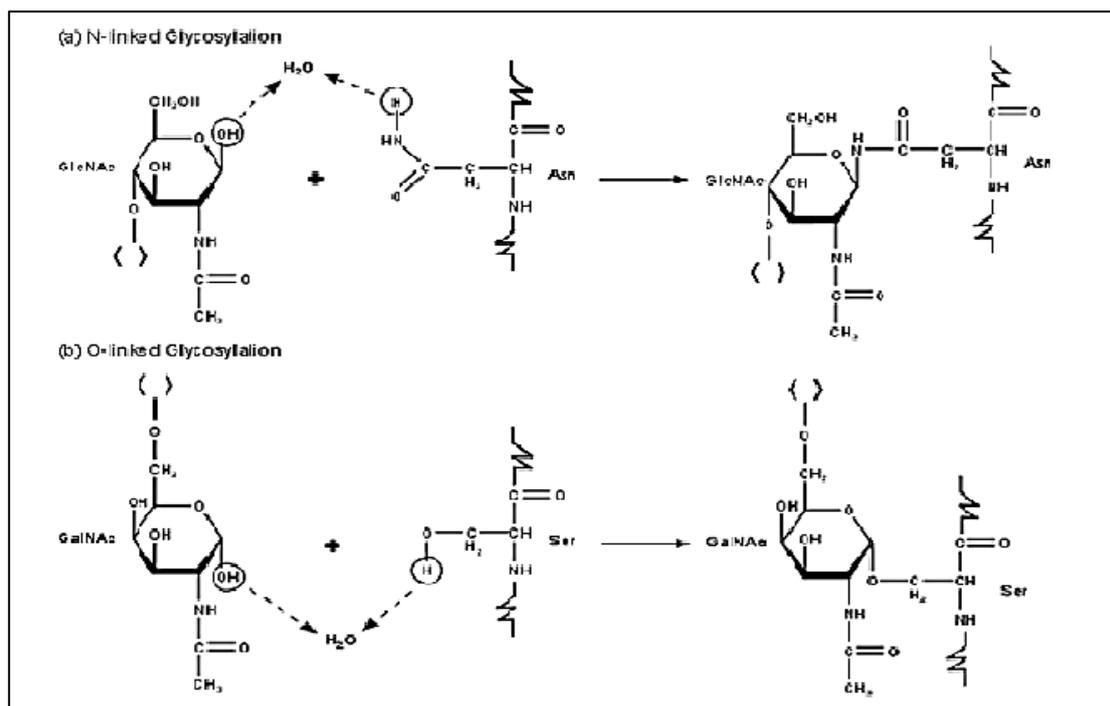
## METHODOLOGY

This review used 2 books and 41 articles searched via the PubMed engine using the following builder: post-translational modification OR PTM AND therapeutic protein OR biopharmaceutical OR pharmacology AND glycosylation OR PEGylation OR fatty acylation, among others as needed. The articles were filtered through the PubMed engine without time limit although the most recent, available and relevant for this review were preferentially chosen. The database *BIOPHARMA*<sup>®</sup>, which is specialized in biopharmaceutical products, was used as the source for FDA approved therapeutic proteins, while their therapeutic indications and presence of conjugative PTMs were verified using the products' labels retrieved from Drugs@FDA, the database for approved drug products from the U.S. Food and Drug Administration. All the chemical drawings were performed using the desktop application MarvinSketch<sup>®</sup> version 16.8.8 developed by ChemAxon Ltd. Copyright<sup>®</sup>.

## RESULTS AND DISCUSSION

### Glycosylation

Glycosylation is the PTM most commonly found in proteins, and above 60% of therapeutic proteins are glycosylated. This PTM consists on the attachment of glycans to amino acid residues and takes place via complex pathways in the endoplasmic reticulum and Golgi apparatus, generally by a N-linked glycosylation or an O-linked glycosylation. N-linked glycosylation occurs through the nitrogen atom of asparagine side chain, and O-linked glycosylation occurs through the oxygen atom of either serine or threonine side chain [9]. Figure 1 shows a simplified chemical reaction representation of O-linked and N-linked glycoprotein formation. The biological advantages of attaching glycans to proteins have been uncovered. For instance, hydrophilic clusters of oligosaccharides are able to alter the protein's polarity and solubility, and oligosaccharides added after protein synthesis work as markers for its destination in the cell (along the secretory pathway, for example) and for the degradation of misfolded proteins. Glycans can also contribute to tertiary structure formation of proteins, confer protection against proteolytic enzymes, and provide specific biologic activity glycoproteins [10].



**Figure 1:** N- and O-linked glycosylation of proteins. GlcNAc and GalNAc are the monosaccharides at the reducing end of the glycans. The chevrons indicate the sites where the oligosaccharide chains continue. Asparagine (Asn) and Serine (Ser) are amino acid residues from the primary structure of a protein. Ser has been used to illustrate the O-linked glycosylation reaction although Thr can also be involved. H<sub>2</sub>O is formed during the N- and O-linked glycosylation reactions as shown

Nutrient constitution, pH, temperature, oxygenation, and ammonia content are some of the culture conditions that may contribute to the distribution of carbohydrate moieties of therapeutic proteins. For instance, specific glutamine and glucose levels in the culture medium may produce less sialylated or more mannosylated glycans. The presence of ammonia can decrease the formation of terminal sialic acid content in O-linked glycans. The medium's pH can interfere in the pH of the Golgi apparatus, causing reduced activity of enzymes participating in glycosylation, and a glycoform profile change may occur at high dissolved oxygen levels [11]. Glycosylation profile and level can also vary with the producing cell line and the culture production mode (e.g. CHO cell's increased sialylated glycoforms in perfusion mode rather than fed-batch mode). Overexpressing enzymes such as  $\beta(1,4)$  GalT and  $\alpha(2,3)$  SiaT can render higher terminal sialylation of glycans [12]. In addition, mannosidase cleavage of O-linked glycans with mannose as reducing end (Man-O-Ser/Thr) may be used to manufacture more mammalian homologous counterparts of therapeutic proteins using *P. pastoris* [13]. The pharmacology of therapeutic glycoproteins relies on their oligosaccharide constituents as determinants for their pharmacological properties. Glycosylation is involved in modulation of interactions between proteins such as the affinity of erythropoietin (EPO) to its receptor. Glycosylated EPOs have lower association rate constants, which decreases as the amount of sialic acid in their glycans increase. As another example, B-cell activating factor receptors with a Fc region have decreased clearance rate as its content of sialic acid increases. The amount of sialylated glycans can also induce production of neutralizing antibodies by T- and B- cells against therapeutic proteins. Therapeutic Fc antibodies constituted of glycans with low fucose content are able to enhance cytotoxicity mediated by mononuclear antibody-dependent cells while recruitment of polymorphonuclear cells is more efficient with high fucosylated glycans. Also, therapeutic protein's immunogenicity may be related to a glycosylation pattern different from that generated in the human body or the lack of a specific glycosylation pattern [14]. In this section, a discussion about an example of therapeutic protein and its pharmacological improvements using a glycosylation modification is presented below.

Gaucher Disease (GD) is caused by a hereditary deficiency of  $\beta$ -glucocerebrosidase, which is responsible for cleaving the glucose and ceramide molecules that form glucocerebroside, resulting in its accumulation in the lysosomes of macrophages. VPRIV® or velaglucerase alfa has the normal human sequence of  $\beta$ -glucocerebrosidase and provides a more advantageous enzyme replacement therapy [15]. A long-term study of phase III clinical trial data has shown that VPRIV® has satisfactory clinical response and good safety and tolerability standards [16]. A complete characterization has demonstrated that velaglucerase alfa has high-mannosylated glycans whose exposed mannose residues increase its cellular uptake by macrophages while targeting their endocytotic system mediated by mannose receptors. This glycoprofile is achieved by adding a mannosidase I inhibitor called kifunensine in the culture medium during the production of velaglucerase alfa while imiglucerase, which is another biopharmaceutical used to treat GD, has its mannose residues exposed by

using exoglycosidases. However, it is contentious if differences in the glycosylation of those therapeutic proteins in fact impact on their uptake into macrophages [17,18].

### PEGylation

PEGylation is a PTM strategy that has been developed to overcome pharmacokinetic (PK) and pharmacodynamic (PD) limitations of therapeutic proteins. It consists of the attachment of polyethylene glycol (PEG) chains to the protein's structure. PEG is a polymer of ethylene oxide monomers that has been considered safe for pharmacological use in humans. Besides proteins, PEG can also be used for pharmacological improvement of low molecular weight drugs and drug delivery systems such as hydrogels, liposomes, microparticles, and nanocarriers [19]. Even though this method has become the most widely used to improve the pharmacological features of therapeutic proteins, it has presented concerns like signs of vacuolation caused by PEGylated proteins in animal models, lack of degradation in systemic circulation for conjugated PEGs with molecular weight higher than renal clearance, and detection of anti-PEG antibodies in animal models and patients. For this reason, an effort to search for PEG alternatives that solve those limitations or bring additional benefits has been growing. Non-degradable and degradable alternative polymers have been investigated such as poly(N-vinylpyrrolidone) (PVP), polyglycerol (PG), poly[oligo(ethylene glycol) methyl methacrylate] (POEGMA), poly(zwitterions), poly(amino acid)-based hybrid materials, and others [20]. The first generation of PEGylated therapeutic proteins is characterized by linear PEGs with low molecular weight. To conjugate PEG to a protein, it is necessary the activation of the PEG through a functional group from one of its terminal sides that is suitable for reaction with an available reactive group on the protein (usually the amino groups on the side chain of lysine and N-terminus). Other end groups can also be part of the PEG's structure when it is prepared with a suitable initiator or termination reagent. Monomethoxy PEG (mPEG) is more suitable to conjugate proteins since it can produce reactive PEGs that prevent formation of crosslinked polypeptides. Figure 2 lists the scope of first generation PEG derivatives of PEGylated proteins with a PEG and a protein's amino acid residue linked through either an alpha or epsilon amino group. It is suggested that the PEG's ability to precipitate proteins, minimize immunogenicity and antigenicity, keep away proteins and cells from surfaces, and avoid degradation by enzymes or mammalian cells is related to its amphiphilic property, capacity to bind to water molecules, and backbone chain's flexibility [21].

The second generation has been developed to solve the problems of the first generation such as impurities, limitation to low molecular weight, weak linkages, side reactions, and deficiency of site-specificity. Site-specific PEGylation can form a homogeneous product with PEG chains attached to specific amino acid residues that do not impact on the biological activity of the therapeutic protein while maintaining the PEG's pharmacological benefits. Encoding single amino acid residues or sequences is a method used for site-specific modification available to create specific PEG conjugates. For example, a free cysteine can be encoded into a protein so that a terminal maleimide group attached to PEG can react with the cysteine forming a covalent bond (Figure 3a). In the same fashion, when a polyhistidine tag (His-tag) is encoded into a protein, it can be PEGylated using the reagent Ni-nitrilotriacetic acid (Ni-NTA) covalently bound to a PEG, which forms a complex between its nickel ion and two of the histidine residues (Figure 3b).

Furthermore, incorporating a functionality that does not exist naturally in the cell (i.e. bio-orthogonal group) can be used to form a covalent bond with a complementary functional group at the terminal side of PEG. An enzyme that recognizes a specific amino acid sequence, amino acid residue, glycan's terminal monosaccharide, or bio-orthogonal group can be used to catalyze the linkage of PEG chains. For instance, transglutaminase (TGase) can catalyze the link between a PEG molecule with a terminal primary amine and the carboxamide group of a glutamine that must be positioned in a flexible loop in order for the acyl transfer reaction occurs (Figure 3c) [22]. Ultimately, noncovalent PEGylation is a prospective approach to overcome the affinity reduction of PEGylated therapeutic proteins. While linking PEG in a nonpermanent way, the PEG chains can be released from the protein at a particular moment in order to allow the protein to perform its pharmacological activity without steric hindrance or folding alteration [23].

The oral absorption rate of polyethylene glycol decreases as its molecular weight (MW) increases until 3350 kDa (when absorption becomes almost absent) while, after parenteral administration, the metabolic clearance decreases and the elimination in urine increases with higher MW. Also, comparing to non-PEGylated proteins, the PEGylated ones usually have slower absorption rate after parenteral administration and a range of 1 to 3 days of time to maximum concentration ( $T_{max}$ ). Initially, the PK of PEGylated proteins is governed by both the protein and PEG components. Then, their PK and biodistribution are driven by mechanisms associated to the PEG only. Cellular mechanisms of denaturation and proteolysis are involved in removing the protein part from circulation whereas target-mediated uptake and pinocytosis participate in PEG-related processing mechanisms of clearance.

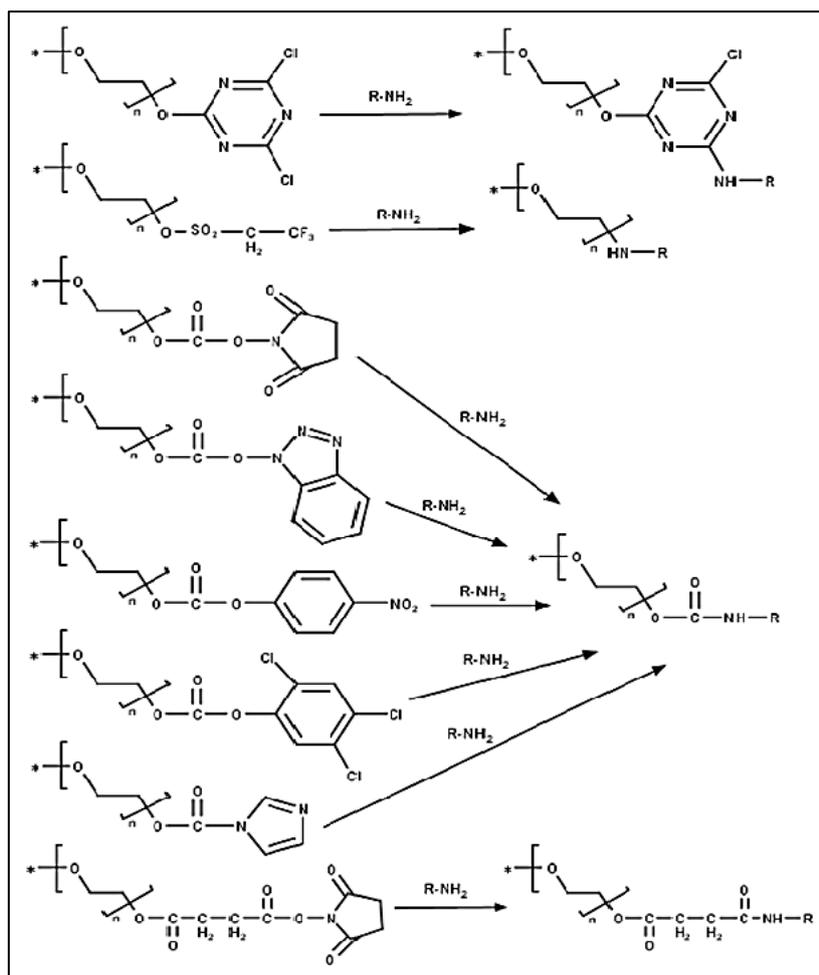


Figure 2: First generation PEG derivatives of PEGylated proteins. R-NH<sub>2</sub> represents an amino group from either the side chain or the N-terminus of a therapeutic protein's amino acid residue.

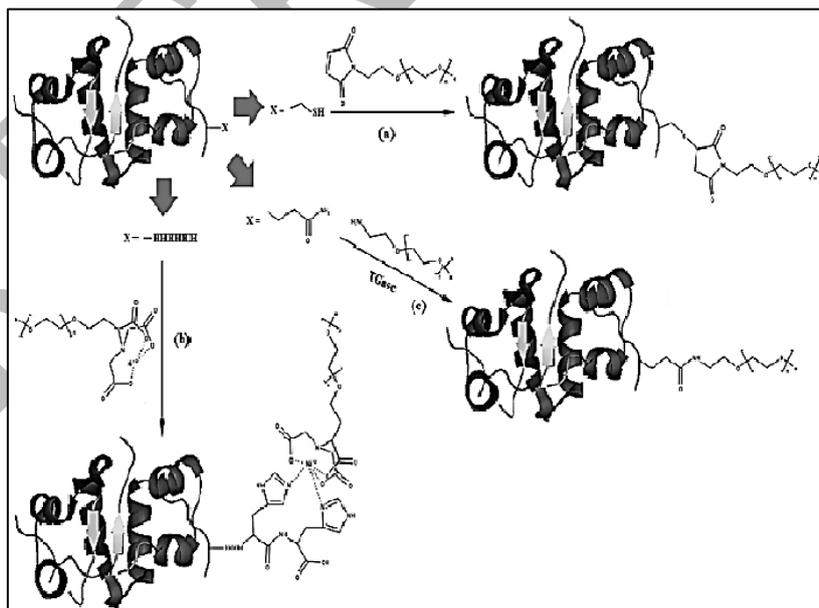


Figure 3: Site-specific PEGylation of therapeutic proteins. X generically represents a specific site of a therapeutic protein used for PEGylation. (a) Linkage of PEG through the reaction of the maleimide group on the terminal side of PEG and the sulfide group of a free cysteine previously encoded into the protein. (b) PEGylation of a therapeutic protein using the reagent Ni-NTA-PEG, which is able to form a complex between two histidine residues of a His-tag and the nickel ion. (c) Enzymatic linkage of PEG via TGase catalysis of a primary amine on the terminal side of PEG and the carboxamide group of a glutamine.

Since renal filtration of PEG from PEGylated proteins is the last elimination pathway of high MW PEGs ( $\geq 30$  kDa), it is proposed that rod-shaped and rigid molecules are able to pass through renal glomeruli, rendering their observed clearance. PEG and/or PEGylated proteins may be cleared directly by the kidney or through a fluid-phase pinocytosis, so the tissues and cells where the PEG components of the proteins are not processed by lysosomal enzymes may form lysosomal distension and vacuolation [24]. Some biopharmaceuticals have been associated with anti-PEG antibodies and PEG-induced complement activation although many therapeutic proteins have been considered safe for a long time. A response against their protein component is usually explained as the reason for the immunogenic reactions observed, and a validated standard anti-PEG assay needs to be developed to screen for anti-PEG antibodies [25]. A discussion about an example of therapeutic protein and its pharmacological improvements using PEGylation is described below.

Interferons type 1 (mainly IFN- $\alpha$  and IFN- $\beta$ ) belong to a family of related proteins that play a variety of biological functions such as antiretroviral, antiproliferative, and immunomodulatory activities, which provide the basics for the pharmacological benefits of IFN therapies. As many therapeutic proteins with low molecular weight, INFs have short serum half-life, so PEGylation has been used as a strategy to improve its PK and PD properties. Peginterferon beta-1a is a recombinant IFN- $\beta$ -1a therapeutic protein with a N-terminus PEGylation, which is specific to a site that does not participate in receptor binding, used for treatment of relapsing-remitting multiple sclerosis (RRMS). RRMS is a complex pathogenesis that encompasses inflammation, demyelination, and axonal damage. Conjugating PEG to IFN- $\beta$ -1a resulted in growth of systemic exposure during different routes of administration. Comparing to the non-PEGylated form, PEG-IFN- $\beta$ -1a has longer terminal half-life and elevated PD markers for receptor activation by IFNs type 1. Subcutaneous administration of 125 $\mu$ g of PEG-IFN- $\beta$ -1a every two weeks during 48 weeks can significantly reduce relapses and annualized relapse rate, risk of disability progression, T2 lesions counts, and tertiary magnetic resonance imaging measures [26,27]. Dosing every 2 weeks was demonstrated to be more efficient than every 4 weeks. Maximum serum concentration was observed after 1-1.5 days of administration, and mono-phasic decrease and median half-life were found between 2 and 3 days. The sustained neopterin growth during 10-14 days indicated cumulative duration of peginterferon beta-1a's activity. The increase of induced neopterin and reduction of reversible lymphocyte count are consistent with the pharmacology of the IFN type 1 family [28,29].

### Fatty acylation

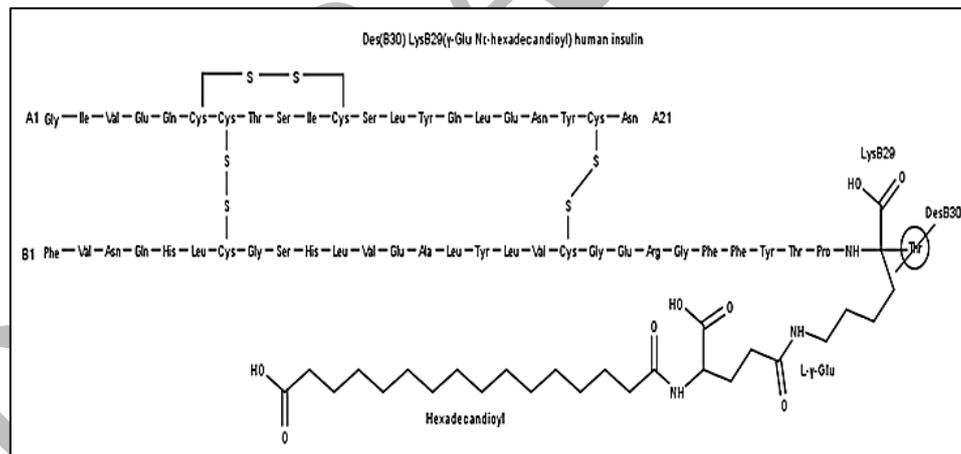
The major cellular fatty acylation reactions of proteins are N-myristoylation, S-palmitoylation, and membrane-bound O-acyl transferase (MBOAT) fatty acylation of secreted proteins. Fatty acylation can improve membrane binding and form interactions between proteins through a hydrophobic pocket insertion. Table 2 summarizes the major cellular fatty acylation reactions of proteins currently known [30]. N-myristoylated proteins generally have the sequence Met-Gly-X-X-X-Ser/Thr on N-terminus. Once, Gly is exposed as the N-terminus via the removal of Met by methionine aminopeptidases, N-myristoyl transferase (NMT) catalyzes the transfer of a myristate molecule (through myristoyl-CoA) to the N-terminal Gly, forming an amide covalent bond. NMT has a hydrophobic binding pocket that accommodates 14 carbons, which confers specificity for myristate [31]. S-palmitoylation is catalyzed by palmitoyl acyltransferases, which are members of the DHHC protein family (named after its conserved sequence motif Asp-His-His-Cys). In *S. Cerevisiae*, it initiates with the reaction between palmitoyl-CoA and the cysteine of the DHHC sequence, forming an acyl-DHHC intermediate. Then, this intermediate transfers the palmitate molecule to a cysteine positioned near the N- or C- terminus or within the acceptor protein substrate [32]. MBOAT enzymes such as Hedgehog acyltransferase (Hhat), Porcn (Porcn), and ghrelin O-acyl transferase (GOAT) catalyzes fatty acylation of secreted proteins, also by intermediation of CoA. Hhat couples a palmitate molecule to a N-terminal Cys of hedgehog family proteins via amide linkage (N-palmitoylation) [33]. Porcn catalyzes an oxyester linkage between *cis*- $\Delta^9$ -palmitoleate (palmitoleic) and a conserved serine residue (Ser209) in Wnt proteins (O-palmitoleoylation) [34]. GOAT links an octanoate molecule to the serine on the third position of the N-terminal sequence GSSFL of the protein ghrelin (O-octanooylation) [35].

Table 2: Major cellular fatty acylation reactions of proteins

Reaction	Molecule	Linkage	Modified Residue	Enzyme
N-myristoylation	Myristate (14:0)	Amide	Gly	NMT
S-palmitoylation	Palmitate (16:0)	Thioester	Cys	DHHC
N-palmitoylation	Palmitate (16:0)	Amide	Cys	Hhat
O-octanooylation	Octanoate (8:0)	Oxyester	Ser	GOAT
O-palmitoleoylation	Palmitoleic (16:1 $\Delta^9$ )	Oxyester	Ser	Porcn

Chemical reporters have been developed to assay the biological functions of protein fatty acylation using bio-orthogonal linkage approaches. The reporter alk-12 can label N-myristoylated proteins and other types of fatty acylated proteins such as S-palmitoylated ones. Hence, hydroxylamine is used to hydrolyse the ester and thioester linkages found in the other forms of fatty acylation, selecting the N-myristoylated proteins for

detection and profiling. In addition, a combination of alk-12 and in-gel fluorescence has been developed to study N-myristoylation in cancer cells and NMT as an antimalarial drug target. N-palmitoylation has been shown to have a significant role in Sonic Hedgehog signaling in human carcinoma cells using the alk-15 reporter. S-palmitoylation can be detected and profiled using the alk-16 reporter in several applications such as revealing S-palmitoylated proteins and their roles. Alk-16 can also label the O-palmitoleoylation of Ser209 by porcupine in Wnt proteins [36]. Since S-acylation is a reversible protein modification (i.e. carried out by acyltransferases and reversed by acylthioesterases), it is used as a dynamic control of many ligand- and voltage-gated ion channels. It may regulate channel trafficking and control the kinetics and modulation of ion channels by other PTMs. S-acylation may modulate ion channel function through the modification of pre-forming and regulatory subunits and control of signaling, scaffolding, and adapter proteins [37]. Reversible lysine fatty acylation (e.g. Lys-myristoylation) has been reported as a regulator of sTNF- $\alpha$  secretion by helping to target TNF- $\alpha$  for degradation in lysosomes and reducing its placement in recycling endosomes [38]. Numerous proteins have been identified to be palmitoylated such as TLRs 2, 5, and 10 (toll-like receptors), which may represent a new mechanism for modulatory regulation of inflammatory activity [39]. The fatty acylation of insulin discussed below is an example of the potential for pharmacological improvement of therapeutic proteins by fatty acid conjugation. Insulin degludec (IDeg) or Tresiba® is a long-acting human insulin analog for basal glycemia control of diabetes type 1 and 2 produced through recombinant DNA technology for subcutaneous injection and engineered to solve the problem of variable insulin exposure associated with other basal insulins. The name “degludec” addresses three of its features: absence of Thr<sup>B30</sup> (“de”), addition of a glutamic acid on the side chain of Lys<sup>B29</sup> through a non-standard peptide bond (“glu”), and attachment of a dicarboxylic acid (thapsic or hexadecanedioic acid) to the  $\alpha$ -amino group of the added Glu (“dec”). Figure 4 illustrates the structure of insulin degludec. Since IDeg loses the positive charge of Lys<sup>B29</sup> and gains one negative charge from the hexadecanedioic acid, it can be formulated into a solution with phenol at pH 7.4, which is more suitable for the subcutaneous neutral environment, forming stable dihexamers. After subcutaneous injection, these dihexamers cluster into long multihexamers that are absorbed slowly because of their large molecular weight, creating a hypodermic depot. The insulin degludec’s monomers gradually dissociate from the multihexamers into the circulatory system, where the conjugated fatty acyl group also mediates binding to serum albumin, resulting in a slow and even delivery with consistent day-to-day exposure and no peaks. IDeg has a half-life of 25.4 hours and can be found in the bloodstream for more than 120 hours. Its exposure reaches a stable and steady state after 2 or 3 doses, and the dose does not need to be adjusted every day [40-42].



**Figure 4: Structural composition of insulin degludec. A1 and A21 indicate the 1st and 21st amino acids of insulin chain A. B1 and B29 (Lys) indicate the 1st and 29th amino acid of insulin chain B. ThrB30 (30th amino acid of chain B) has been omitted in insulin degludec. L- $\gamma$ -Glu indicates the location of the Glu residue linked to the  $\epsilon$ -amino group of LysB29. Hexadecandioyl is the fatty acid side chain attached to the  $\alpha$ -amino group of Glu.**

Insulin degludec has been confirmed to be safe and effective in patients with diabetes mellitus type 1 and 2. It is able to significantly reduce insulin dose and frequency of hypoglycemia, but not in diabetes type 2 although, comparing with other long-acting insulin analogs, IDeg presents better glycemic control. Also, IDeg can minimize hyperglycemic peaks along the day, including dawn and dusk, which lowers the risk of nocturnal episodes of hypoglycemia [43,44]. The combination of insulin degludec and insulin aspart (IAsp) can integrate the long-acting and the rapid-acting features of IDeg and IAsp, respectively, and it may be a promising alternative, especially for treatment of diabetes type 2. This combination has the additional benefit of targeting postprandial glucose, which is proposed to be related to cardiovascular disease [45].

## CONCLUSION

Conjugative PTMs can be engineered onto the structure of therapeutic proteins by biotechnology industries at the end of downstream processing or right after they are synthesized by a chosen cell line. Even though eukaryotic cells are able to produce therapeutic proteins with complex folding, multiple subunits, and consistent profile of PTMs (which is essential for the safety and efficacy of biopharmaceuticals), prokaryotic cell lines grow faster in relatively inexpensive culture medium, reaching high cell densities and making the production more efficient and economic. Furthermore, prokaryotic cells are easier to be genetically engineered through recombinant DNA technology and can have their behavior and product yield better controlled and predicted during the bioprocess. Therefore, conjugative PTMs are advantageous for industrial production since therapeutic proteins can also be pharmacologically improved by them even when produced by prokaryotic cell lines, resulting in an efficient and high yield manufacture of biopharmaceuticals. Other potential uses for conjugative PTMs could be explored such as helping complex folding, preventing formation of inclusion bodies in production systems with prokaryotic cells, and assisting protein isolation and purification. Also, conjugative PTMs could be used to decrease or reverse tissue cross-reactivity (TCR) and exaggerated on-target effects of therapeutic proteins by promoting steric hindrance between the specific sites of the therapeutic protein and the off-target receptor responsible for the TCR, and by minimizing the therapeutic protein's affinity for the receptor responsible for the exaggerated on-target effect. Whereas numerous PTMs still need to have their functions and mechanisms of action uncovered and developed to new perspectives, the diversity of PTMs can be expanded by new conjugative molecules. They may be chosen from a pool of molecules that do not naturally participate in cellular mechanisms but have the potential to alter the protein's structure, interaction, and pharmacological activity. Those PTMs need to be ideally chosen for efficient and economic production as well as for suitability to isolation and purification strategies. Moreover, it is essential that they do not form harmful side products in the organism and produce side effects, toxicity, immunogenicity, or other detriments such as the cellular vacuolation that seems to be caused by PEGylated proteins. As can be seen, exploring the potentials of conjugative PTMs can bring many benefits, which may result in the development of new generations of biopharmaceuticals.

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