Post-translational modifications of protein biopharmaceuticals

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The majority of therapeutic proteins display one or more post-translational modifications (PTMs). These modifications normally influence the biochemical and therapeutic properties of such proteins. Choosing an expression system capable of generating an appropriate product PTM profile remains one of the most crucial decisions a drug developer must make. This review considers the PTMs most often associated with therapeutic proteins and focuses upon more recent advances in engineering PTMs with the aim of improving the application-relevant functional characteristics of these drugs.

PTMs in context

The majority of proteins derived from eukaryotes undergo covalent modification either during or, more commonly, after their ribosomal synthesis. This gives rise to the concept of co-translational and post-translational modifications, although both modifications are often referred to simply as post-translational modifications (PTMs). Several hundred PTMs have been characterized to date [1,2], and these modifications invariably influence some structural aspect or functional role of the affected protein. Most PTMs are introduced into target proteins via specific enzymatic steps and/or pathways. Some such modifications (e.g. glycosylation) are common, whereas others (e.g. AMPylation) occur only rarely. Some are predominantly or exclusively associated with intracellular proteins (e.g. phosphorylation and ADP ribosylation), and others are mainly characteristic of extracellular proteins (e.g. glycosylation, disulfide linkage formation and carboxylation). Only a subset of PTMs are generally associated with therapeutic proteins, and those PTMs form the main focus of this review.

The biopharmaceutical sector

It is now 28 years since the approval of the first biopharmaceutical for general medical use (‘humulin’, a recombinant human insulin produced in Escherichia coli, initially approved in 1982). The pharmaceutical biotechnology industry has matured rapidly in the intervening years. Today, there are more than 220 such products in general medical use. Typically, 8–12 new products gain approval annually, which represents approximately 25% of all genuinely new molecular entities coming on the market [3]. Global market value estimates vary depending upon source and exactly how you define a biopharmaceutical, but generally estimates of approximately $70–80 billion predominate [4].

The term ‘biopharmaceutical’ normally encompasses recombinant therapeutic proteins (including antibodies), nucleic-acid-based products and, indeed, engineered cell or tissue-based products. Proteins currently predominate, however, and this profile is unlikely to change considerably in the near to intermediate future. The vast majority of these products are recombinant or engineered versions of native eukaryotic extracellular proteins, and as such the majority are subject to PTM.

Expression systems and their influence upon PTMs

At a research level, numerous prokaryotic- and eukaryotic-based expression systems have been developed to facilitate the production of recombinant proteins. For various technical, economic and regulatory reasons, only a subset of these expression systems find use in the production of parenteral proteins. Systems currently used are based upon recombinant E. coli, yeast and mammalian cell lines, along with insect cell lines and transgenic animals [4].

In the region of 50% of all products approved thus far are expressed in E. coli, and the majority of the remainder is produced in mammalian cell lines – mainly Chinese hamster ovary (CHO), baby hamster kidney, and the murine-mesoblast-derived NS0 and...
SP2/0 cell lines. A smaller number of products are produced in yeast-based systems. Novo Nordisk’s insulins, several recombinant hepatitis B surface antigens and recombinant hirudin are all produced in engineered Saccharomyces cerevisiae. The first ever biosimilar approved (Valtropin, a recombinant hGH (human growth hormone)) is also produced in this system. Moreover, last year witnessed the approval of the first product (Kalbitor, a recombinant human plasma kallikrein inhibitor used to treat hereditary angioedema) produced in an alternative yeast system, that of Pichia pastoris [5,6].

In addition, the past few years have witnessed the approval of the first (and thus far the only) products produced in transgenic animals or in an insect cell line. Atryn is a recombinant human antithrombin produced in the milk of transgenic goats [7]. It is approved for the treatment of hereditary antithrombin deficiency and came to the market in 2006. Cervarix, the cervical cancer subunit vaccine approved for general medical use in 2007, is produced in a baculovirus-based system [8].

Choosing an appropriate expression system is one of the most fundamental decisions a drug developer must make in the context of therapeutic proteins. Although issues such as patenting status and economics influence the choice, the fundamental scientific determinant of which potential expression systems are most appropriate or, indeed, might be inappropriate is invariably the PTM requirements of the protein in question.

Thus, for example, the expression of a normally glycosylated protein in E. coli will result in the production of an aglycosylated moiety. Expression in a yeast-based system, however, invariably results in the attachment of sugar side chains high in mannose content, which will negatively influence product’s serum half-life in humans [9]. This renders native yeast-based systems unsuited to the production of glycosylated product. Production in plant-based systems generally results in hyperglycosylated proteins containing xylose and fucose moieties that are immunogenic in humans [10]. The use of CHO or other mammalian cell lines, therefore, is largely dictated by the latter’s ability to undertake appropriate PTMs, generating product with acceptable therapeutic properties. Table 1, for example, summarizes the biopharmaceuticals approved for general medical use from 2006 to 2009 inclusive. Forty-six products are listed. Of these, 25 are produced in mammalian-based systems, as opposed to technically more straightforward and economically more attractive microbial-based systems. The major determinant driving the manufacture of the latter 25 products in mammalian systems is their stringent PTM requirements, most notably but not exclusively glycosylation.

Table 1, for example, summarizes the biopharmaceuticals approved for general medical use from 2006 to 2009 inclusive.

<table>
<thead>
<tr>
<th>Product category</th>
<th>Specific products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood factors, anticoagulants, thrombolytics and related</td>
<td>Atryn (antithrombin), Kalbitor (ecallantide), Recothrom (thrombin, topical, recombinant) and Xyntha (antithrombin)</td>
</tr>
<tr>
<td>Antibodies</td>
<td>Azerrra (ofatumumab), Cimzia (certolizumab pegol), Ilaris (canakinumab), Lucentis (ranibizumab), Removab (catumaxomab), RoActemra (tocilizumab), Vectibix (panitumumab), Simponi (golimumab), Soliris (eculizumab) and Stelara (ustekinumab)</td>
</tr>
<tr>
<td>Hormones</td>
<td>Accretropin (somatropin), Exubera (insulin), Fertavid (follitropin beta), NovoLog mix (insulin aspart mix), Omnitrope (somatropin), Pergoveris (a combination of follitropin alfa and lutropin alfa), Preotach (parathyroid hormone), Valtropin (somatropin) and Victoza (liraglutide)</td>
</tr>
<tr>
<td>EPO and colony-stimulating factors</td>
<td>Abseamed (erythropoietin alfa), Binocrit (erythropoietin alfa), Biogristim (filgrastim), Biopin (epoetin theta), Epoetin alfa Hexal (erythropoietin alfa), Eporatix (epoetin alfa), Filgrastim (filgrastim), Filgrastim ratiopharm (filgrastim), Mircera (methoxy polyethylene glycol-epoetin beta), Prepar (filgrastim), Retacrit (epoetin zeta), Silapo (epoetin zeta), Tevagristim (filgrastim) and Zarzio (filgrastim)</td>
</tr>
<tr>
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<td>Extavia (interferon beta-1B) and PegIntron/Rebetol combo (peginterferon-α)</td>
</tr>
<tr>
<td>Additional</td>
<td>Arcalyt (rilonacept), Elaprase (idursulfase), Cervarix (C-terminally truncated major caspese L 1 proteins from human papillomavirus types 16 and 18), Gardasil, also known as Silgard (human papillomavirus vaccine, type 6, 11, 16, 18, recombinant), Myozyme (alglucosidase alfa), Opendra (eptotermin alfa) and Nplate (romiplostim)</td>
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PTMs associated with therapeutic proteins

The PTMs most commonly associated with currently licensed therapeutic proteins include carboxylation and hydroxylation, amidation and sulfation, disulfide bond formation and proteolytic processing, as well as glycosylation [11]. These are also reflective of PTMs associated with proteins in the developmental pipeline.

Several of these PTMs (e.g. proteolytic processing and disulfide bond formation) are characteristics of many biopharmaceuticals but tend not to be problematic in terms of product manufacture. If necessary, such modifications can sometimes be introduced as part of downstream processing. For example, several recombinant insulins are expressed in zymogen form, with a specific proteolysis step included during downstream processing, thereby generating the biologically active, mature product [12]. Other PTMs are somewhat more restricted in their occurrence. Included in this category are γ-carboxylation and β-hydroxylation, as well as amidation and sulfation. Glycosylation represents both the most widespread and the most complex of all PTMs and is the PTM most prominently associated with biopharmaceutical products.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Biopharmaceuticals approved within the US and/or the EU, 2006–2009 inclusive a</th>
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a Products listed by trade name with INN in brackets. Products produced in mammalian-based systems are listed in bold font.
ilitating calcium binding, which is important – and in some cases essential – to the functioning of several blood factors (notably factors VII, IX and X) and related proteins. One such related protein is that of the antithrombotic molecule, activated protein C. A recombinant form of activated protein C used to treat severe sepsis is marketed under the trade name Xigris. The native molecule displays nine carboxylation sites within its so-called Gla domain (consisting of the N-terminal 30 amino acid residues) and one hydroxylation site at aspartate residue 71 (Fig. 2). Full carboxylation and hydroxylation are required to sustain biological activity. Such a stringent PTM requirement could not be met in full by CHO cells, rendering necessary its production in an engineered human cell line (HEK 293). Activated protein C also illustrates the point that many proteins exhibit multiple different PTMs. In addition to carboxylation and hydroxylation requirements, it also harbours four potential glycosylation sites and one disulfide linkage and is proteolytically processed (Fig. 2).

**Amidation and sulfation**

Amidation refers to the replacement of a protein’s C-terminal carboxyl group with an amide functional group (CONH₂). It is a PTM characteristic of many bioactive peptides, although far less frequently associated with polypeptides [16,17]. In many cases, its exact biological role or roles remain(s) to be fully elucidated; however, it often contributes to peptide stability and/or activity. Amidation is characteristic of only one biopharmaceutical approved to date – that of recombinant salmon calcitonin [18]. Calcitonin regulates serum calcium and phosphate levels and, interestingly, the salmon-derived hormone is some 100 times more potent than endogenous calcitonin in humans. It is indicated for the treatment of Paget’s disease and hypercalcaemia of malignancy. Produced in E. coli, the product initially lacks this PTM and, therefore, must be amidated subsequently as part of downstream processing, which is undertaken using a recombinant α-amidating enzyme.

Sulfation is yet another PTM characteristic of a limited number of biopharmaceuticals [19,20]. This modification often influences protein–protein interaction, but the absence of sulfation generally reduces rather than abolishes activity. In the context of biopharmaceuticals, native hirudin (a 65 amino acid leech-derived anticoagulant) and blood factors VIII and IX are usually sulfated. Neither of the recombinant forms of hirudin approved for general

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**FIGURE 1**

Protein γ-Carboxylation and β-hydroxylation. (a) The γ-carboxylation of glutamate residues (Glu) yields γ-carboxyglutamate (Gla). (b) The β-hydroxylation of aspartate residues (Asp) yields β-hydroxyaspartate (Hya).

**FIGURE 2**

Schematic of activated protein C, a therapeutic protein displaying multiple PTMs. The protein comprises four domains (Gla, two EGFs and a serine protease domain). The Gla domain houses nine γ-carboxylation sites (all within the molecule’s first 30 amino acid residues). The first EGF domain houses a single β-hydroxylation site (Asp71). Additional PTMs undertaken include proteolytic processing, disulfide bond formation and the attachment of four N-linked carbohydrate side chains. Reproduced, with permission, from Ref. [11].
medical use (trade names Revasc and Refludan, both of which are produced in \textit{S. cerevisiae}) are sulfated, and yet they are therapeutically effective. Studies, however, have shown that sulfated hirudin (at Tyr63) displays tenfold tighter affinity for thrombin than does unsulfated analogues [21].

**Disulfide linkages**

Disulfide linkages are characteristic of a wide range of extracellular proteins, including many biopharmaceuticals (e.g. recombinant insulins, antibodies, blood factors and interferons) [22]. This covalent linkage helps stabilize and maintain tertiary structure and, indeed, quaternary structure in the context of many multi-subunit proteins, including antibodies. Because their formation is in effect an oxidation process, disulfide linkages are not characteristic of cytoplasmic proteins as both eukaryotic and prokaryotic cells maintain a reduced cytoplasm. In eukaryotes, disulfide linkages are formed in the endoplasmic reticulum, which displays an oxidizing environment; hence, the expression of disulfide-containing proteins in eukaryotic systems is generally not problematic.

In the case of prokaryotes, disulfide linkages are invariably introduced into extracellular proteins within the periplasm as part of protein export. The periplasm naturally houses several disulfide-forming and -rearranging enzymes (the Dsb family of proteins), as well as more general chaperones and foldases that assist in appropriate protein folding. However, high-level intracellular expression of recombinant proteins in prokaryotes such as \textit{E. coli} generally results in inclusion body formation. Downstream processing in such cases generally entails initial inclusion body isolation, followed by solubilization to a level of monodispersity using chaotrophic agents, with subsequent oxidative refolding. This approach might or might not facilitate appropriate disulfide bond formation.

More recent research in this area has focussed upon developing engineered prokaryotic cell lines with enhanced disulfide bond forming capacity [23]. Several approaches have been pursued, including: (i) overexpression of Dsb proteins, either alone or in combination with chaperones and foldases, in the \textit{E. coli} periplasm; (ii) optimization of leader sequences, thereby ensuring more efficient protein translocation into the periplasm (leader sequences can be attached to proteins to prompt their extracellular export from \textit{E. coli}, but the translocation process is often a bottleneck, particularly if high-level expression is achieved); and (iii) the facilitation of cytoplasmic-based disulfide bond formation. Knock-out cell lines lacking thioredoxin reductase activity have been developed, which display a slightly oxidizing intracellular environment. In addition, some of these lines have been further modified by the introduction of Dsb activity, further aiding intracellular disulfide bond formation.

**Protein glycosylation and glycoengineering**

Glycosylation is by far the most common and complex PTM associated with therapeutic proteins, and this PTM can have several potential roles and/or effects upon proteins [24–26] (Table 2). Table 3 lists glycosylated therapeutic proteins approved for general medical use. These represent almost 40% of all approved protein biopharmaceuticals. The single most notable category is antibodies, in which 29 products are listed.

A notable technical trend within the biopharma sector relates to engineering PTMs, in particular glycosylation, to tailor or in some way improve the therapeutic product [27,28]. The past five to ten years has witnessed very notable advances in the context of understanding the glyco component and how its structure and function are linked. Contributing to this have been advances in a whole range of supporting techniques and technologies, including analytical methodologies (in particular mass spectrometry) and advances in genomics, bioinformatics and proteomics [29,30].

**Approaches to glycoengineering**

Approaches to glycoengineering can vary. Earlier approaches included the in vitro modification of the native glyco component of a product during downstream processing.

For example, the product Cerezyme (recombinant glucocerebrosidase) is treated with an exoglycosidase enzyme as part of its downstream processing. Glucosidase treatment removes the termin al sialic-acid residues from the product’s glyco component, exposing mannose residues underneath. This, in turn, facilitates macrophage-specific cellular uptake (the target cell type) owing to the presence of a mannose receptor on the latter’s surface [31].

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>The major potential roles/effects of glycosylation in the context of therapeutic proteins*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Role or effect</td>
<td>Example</td>
</tr>
<tr>
<td>Aids protein folding</td>
<td>The glyco component of gonadotrophic hormones such as FSH and LH has been implicated in proper protein folding and assembly (and, indeed, secretion).</td>
</tr>
<tr>
<td>Aids protein targeting and/or trafficking</td>
<td>The removal of two or more of EPO’s three N-linked glycosylation sites results in a product that is very poorly secreted from the producer cell.</td>
</tr>
<tr>
<td>Aids ligand recognition and/or binding</td>
<td>The terminal mannose residues on the therapeutic enzyme glucocerebrosidase (trade name Cerezyme) facilitate binding to cell surface mannose receptors, thus facilitating cellular uptake of product.</td>
</tr>
<tr>
<td>Plays a part in triggering and/or maintaining the biological activity of the protein</td>
<td>Removal of the N-52 (aspartagine) linked glyco component of gonadotropins actually increases their receptor binding affinity but abolishes their ability to trigger signal transduction upon binding. The glyco component of IgG molecules has a central role in triggering antibody effector functions such as antibody-dependent cellular cytotoxicity.</td>
</tr>
<tr>
<td>Helps stabilize the protein</td>
<td>α-Galactosidase (trade name Fabrazyme), an enzyme used to treat Fabry’s disease, is glycosylated at asparagine residue 184. Removal of the sugar results in protein aggregation and precipitation.</td>
</tr>
<tr>
<td>Regulates the proteins serum half-life</td>
<td>The sialic-acid content of most glycosylated therapeutic proteins plays a fundamental part in regulating their serum half-life.</td>
</tr>
</tbody>
</table>

*Information sourced from references [24–26].
An alternative engineering approach entails the introduction of additional glycosylation consensus sites into the protein, thereby promoting the biosynthesis of hyperglycosylated product variants. This is exemplified by the EPO analogue Aranesp (trade name), which contains five N-linked glycosylation sites as opposed to the native molecule’s three. The resultant product displays a notably extended half-life, a consequence of the additional carbohydrate present [32].

More recently, it has been established that the introduction of four additional N-glycosylation sites into recombinant interferon-α increased its plasma half-life 20–25-fold [33]. It has also been reported that a hyperglycosylated variant of follicle-stimulating hormone also containing four additional sugar side chains notably enhanced the therapeutic properties of this molecule in terms of extended half-life, a consequence of the additional carbohydrate present [32].

An alternative approach to glycoengineering entails the chemical conjugation of synthetic oligosaccharides directly to a preformed protein backbone. For example, a recombinant form of human lysosomal acid a-glucosidase (trade name Myozyme), is used to treat Pompe disease, a rare genetic condition characterized by a deficiency of that activity. Product administration of this glycosylated enzyme is followed by cellular uptake, probably via binding to cell surface mannose-6-phosphate (MP6) receptors, with subsequent lysosomal delivery; however, the levels of MP6 associated with the enzyme’s glyco component are relatively modest. Direct chemical conjugation of an oligosaccharide bearing a terminal MP6 residue onto a-glucosidase, however, increases binding to the MP6 receptor and cellular uptake [35]. Further studies have indicated that the MP6 engineering of the enzyme correlates with notable therapeutic improvements in Pompe disease mouse models [36].

An alternative approach to glycoengineering entails focussing not upon the product itself (at least not directly) but upon the producer cell line and, more particularly, upon the PTM capacity of that cell line [24,37,38]. This approach is at the cutting edge of PTM engineering and entails the inactivation of ‘problematic’ and/or the introduction of desired glycosylation enzymes. This approach is complex and challenging because protein glycosylation is undertaken via a multi-enzyme pathway often involving up to two or three dozen glycosyltransferase and glucosidase enzymes spanning the endoplasmic reticulum and the golgi.

A specific example of this approach is exemplified by the engineering of antibodies (IgGs) produced in CHO cell lines [39]. IgGs are invariably glycosylated at asparagine residue 297 of the antibody’s heavy chain. This glyco component plays a central part in triggering antibody-dependent cellular cytotoxicity (ADCC), which seems to be the principle mechanism by which several approved antibody-based products mediate the destruction of cancer cells. Figure 3 illustrates the typical diantennary structure of the IgG glyco component, a structure normally containing a fucose sugar residue.

Removal of this fucose residue enhances antibody ADCC activity up to 100-fold. Consequently, a CHO knockout cell line has been generated that is devoid of the FUT8 gene – the gene coding for the fucosyltransferase enzyme that normally attaches this fucose sugar residue.

Glycoengineering in yeast and plants

Expression of glycoproteins in wild-type yeasts usually results in the attachment of sugar side chains high in mannose content and...
very largely devoid of sialic-acid caps [9]. This negatively influences product serum half-life and, hence, yeasts have not been traditionally used to produce glycosylated product. Notable advances have been recorded over the past decade in terms of engineering the yeast’s glycosylation machinery to render possible the production of glycosylated therapeutic proteins with acceptable glycoprofiles [24,40]. For example, the elimination of an α-1,6-mannosyltransferase enzyme (OCH1 gene product) helps eliminate the hypermannosylation usually characteristic of yeast. Another notable advance was the introduction of α1,2 mannosidase genes, generating yeast cells capable of producing core ‘mammalian-like’ sugar chains. Subsequent engineering has yielded yeast strains capable of generating complex sialic-acid-capped glycoproteins, a characteristic crucial to ensuring adequate serum half-life of many proteins [41].

Glycoengineering of plant-based systems has also recorded notable advances over the past several years [42]. Glycoprotein production in native plant-based systems generally results in the formation of hyperglycosylated product containing xylose and fucose moieties that are immunogenic in humans [42]. Moreover, the sugar side chains present are usually not sialic-acid capped. Glycoengineering of plant-based glycosylation enzymes has led, for example, to the development of a glycoengineered knockout moss (Physcomitrella patens) system that lacks core fucose and xylose transferase activity, and antibodies produced therein display enhanced ADCC activity [43,44]. An alternative bioengineered system is based on duckweed (Lemna minor) [45]. In this system, the expression of endogenous α-1,3-Fucosyltransferase and β-1,2-xyllosyltransferase is inhibited by RNAi technology, and it has been shown that antibodies expressed therein are devoid of the offending xylose and fucose residues. Moreover, it has been reported that compared to CHO-produced antibodies, such engineered antibodies have a more homogeneous glycocomponent and higher ADCC capacity.

**Biosimilars and the importance of PTMs**

The loss of patent protection from most first-generation biopharmaceuticals over the past decade has provided scope for the emergence of generic versions of such products. However, the structural complexity of proteins, compounded by the inherent complexity of biological manufacturing processes, rendered the production of generic product identical to the originator technically challenging or impossible. The concept of biosimilarity (product displaying very substantially similar characteristics) thus emerged [46,47]. By the end of 2009, 15 biosimilars, based upon three product types (growth hormone, erythropoietin and granulocyte-colony-stimulating factor), had been approved within Europe (Table 4). Approval of a biosimilar product entails its comparison to a medicine already approved (the reference med-

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**TABLE 4**

Biosimilar products approved for general medical use within the EU (2006–2009 inclusive)

<table>
<thead>
<tr>
<th>Product</th>
<th>Company (year)</th>
<th>Product</th>
<th>Company (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biograstim (filgrastim; rh G-CSF expressed in E. coli)</td>
<td>CT Arzneimittel (2008)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
An overview of the stages of manufacture of a therapeutic protein which can influence protein PTM detail. Refer to the text for further detail.

PTMs, their function, their characterization and their engineering have become a core and increasingly important focus of investigation within the biopharmaceuticals sector. Advances recorded within the past 10–15 years in terms of understanding the relationship between PTM structure and function have enabled the rational engineering of PTMs to enhance specific functional or therapeutic characteristics of many biopharmaceuticals. In parallel, advances in the elucidation of PTM pathways within a range of eukaryotic cell types have facilitated the rational engineering of yeast and other expression systems, enabling these systems to produce recombinant proteins displaying PTM profiles appropriate for human use. These advances will, without doubt, culminate in the continued development and approval of many next-generation biopharmaceuticals displaying enhanced therapeutic properties.

**References**

35 Zhu, Y. et al. (2005) Carbohydrate-remodeled acid α-glucosidase with higher affinity for the cation-independent mannose 6 phosphate receptor demonstrates improved delivery to muscles of Pompe mice. Biochem. J. 389, 619–628