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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-myeloma-derived NS0 and

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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 hiruidin 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.

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.

γ-Carboxylation and β-hydroxylation

γ-Carboxylation and β-hydroxylation are PTMs characteristic of a small number of proteins, mainly a subset of proteins involved in blood coagulation [13–15]. These PTMs are undertaken by specific carboxylase and hydroxylase enzymes, with conversion of target glutamate residues in the protein backbone to γ-carboxyglutamate (Glu → Gla) and target aspartate residues to β-hydroxyaspartate (Asp → Hya) (Fig. 1). These modifications are important in facil-

TABLE 1

Biopharmaceuticals approved within the US and/or the EU, 2006–2009 inclusive[a]

Product category	Specific products
Blood factors, anticoagulants, thrombolytics and related	**Atryn (antithrombin)**, Kalbitor (ecallantide), **Recothrom (thrombin, topical, recombinant)** and **Xyntha (antihemophilic factor)**
Antibodies	**Arzerra (ofatumumab)**, Cimzia (certolizumab pegol), **Ilaris (canakinumab)**, Lucentis (ranibizumab), **Removab (catumaxomab)**, **RoActemra (tocilizumab)**, **Vectibix (panitumumab)**, **Simponi (golimumab)**, **Soliris (eculizumab)** and **Stelara (ustekinumab)**
Hormones	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)
EPO and colony-stimulating factors	**Abseamed (erythropoietin alfa)**, **Binocrit (erythropoietin alfa)**, Biograstim (filgrastim), **Biopoin (epoetin theta)**, **Epoetin alfa Hexal (erythropoietin alfa)**, **Eporatio (epoetin theta)**, Filgrastim hexal (filgrastim), Filgrastim ratiopharm (filgrastim), **Mircera (methoxy polyethylene glycol-epoetin beta)**, Ratiograstim (filgrastim), **Retacrit (epoetin zeta)**, **Silapo (epoetin zeta)**, Tevagrastim (filgrastim) and Zarzio (filgrastim)
Interferons	Extavia (interferon beta-1B) and PEGintron/rebetol combo (peginterferon-α)
Additional	**Arcalyst (rilonacept)**, **Elaprase (idursulfase)**, Cervarix (C-terminally truncated major caspid 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)**, **Opgenra (eptotermin alfa)** and Nplate (romiplostim)

[a] Products listed by trade name with INN in brackets. Products produced in mammalian-based systems are listed in bold font.

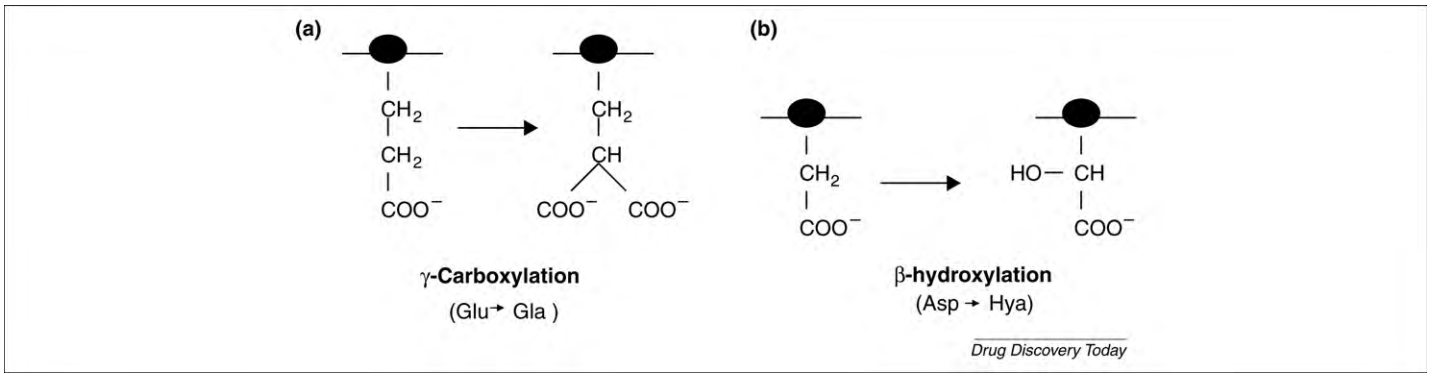

FIGURE 1

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).

itating 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 ($CONH_2$). 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

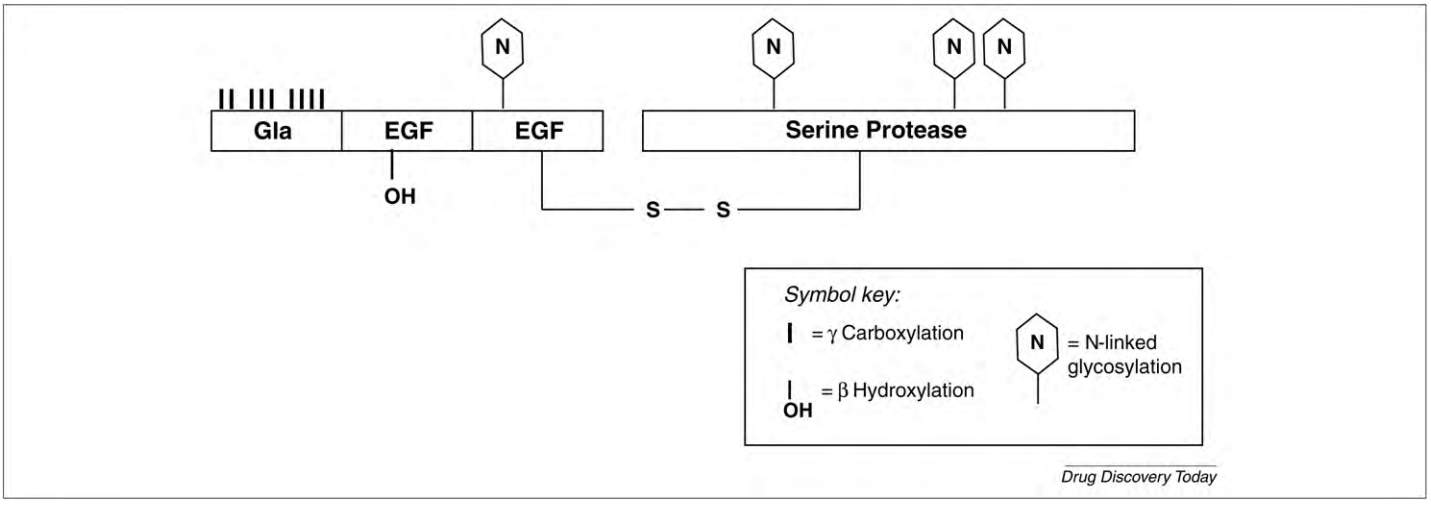

FIGURE 2

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 *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 *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 *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 *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. Knockout 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 glycocomponent 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 glycocomponent of a product during downstream processing.

For example, the product Cerezyme (recombinant glucocerebrosidase) is treated with an exoglucosidase enzyme as part of its downstream processing. Glucosidase treatment removes the terminal sialic-acid residues from the product's glycocomponent, 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 2

The major potential roles/effects of glycosylation in the context of therapeutic proteins[a]

Role or effect	Example
Aids protein folding	The glycocomponent of gonadotrophic hormones such as FSH and LH has been implicated in proper protein folding and assembly (and, indeed, secretion).
Aids protein targeting and/or trafficking	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.
Aids ligand recognition and/or binding	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.
Plays a part in triggering and/or maintaining the biological activity of the protein	Removal of the N-52 (asparagine) linked glycocomponent of gonadotropins actually increases their receptor binding affinity but abolishes their ability to trigger signal transduction upon binding. The glycocomponent of IgG moleules has a central role in triggering antibody effector functions such as antibody-dependant cellular cytotoxicity.
Helps stabilize the protein	α-Galactosidase (trade name Fabrazyme), an enzyme used to treat Fabry's disease, is glycosylated at asparagine residue184. Removal of the sugar results in protein aggregation and precipitation.
Regulates the proteins serum half-life	The sialic-acid content of most glycosylated therapeutic proteins plays a fundamental part in regulating their serum half-life.

[a] Information sourced from references [24–26].

TABLE 3

Glycosylated biopharmaceuticals approved for general medical use in the EU and/or the USA up until December 31, 2009

Product category	Specific products, by tradename with INN in brackets
Blood factors, anticoagulants and thrombolytics	Activase (alteplase), Advate (octocog alfa), Atryn (antithrombin), Benefix (nonacog alfa), Bioclate (antihemophilic factor), Helixate, also known as Kogenate (octocog alfa), Metalyse, also known as TNKase (tenecteplase), Novoseven (eptacog alfa), Recombinate (antihemophilic factor), Refacto (moroctocog alfa), Recothrom (thrombin, topical, recombinant), Xigiris (drotrecogin alfa), Xyntha (antihemophilic factor)
Antibodies	Arzerra (ofatumumab), Avastin (bevacizumab), Bexxar (tositumomab), Erbitux (cetuximab), Herceptin (trastuzumab), Humaspect (votumumab), Humira (adalimumab), Ilaris (canakinumab), Mabcampath, also known as Campath-H1 (alemtuzumab), Mabthera/Rituxan (rituximab), Mylotarg (gemtuzumab ozogamicin), Neutrospec (fanolesomab), Oncoscint (satumomab pendetide), Orthoclone OKT-3 (murumonab-CD3), Prostascint (capromab pendetide), Raptiva (efalizumab), Remicade (infliximab), Removab (catumaxomab), Roactemra (tocilizumab), Simponi (golimumab), Simulect (basiliximab), Soliris (eculizumab), Stelera (ustekinumab), Synagis (palivizumab), Tysabri (natalizumab), Vectibix (panitumumab), Xolair (omalizumab), Zenapax (daclizumab), Zevalin (ibritumomab)
Hormones	Gonal F (follitropin alfa), Luveris (lutropin alfa), Ovitrelle, also known as Ovidrel (choriogonadotropin alfa), Pergoveris (follitropin alfa/lutropin alfa), Puregon/Follistim/Fertavid (follitropin beta), Thyrogen (thyrotropin alfa)
EPO and colony-stimulating factors	Abseamed (erythropoietin alfa), Binocrit (erythropoietin alfa), Biopoin (epoetin theta), Epogen, also known as Procrit (epoetin alfa), Epoietin alfa Hexal (erythropoietin alfa), Eporatio (epoetin theta), Leukine (sargramostim), Mircera (methoxy polyethylene glycol-epoetin beta), Neorecormon (epoetin beta), Nespo, also known as Aranesp (daebepoetin alfa), Retacrit (epoetin zeta), Silapo (epoetin zeta)
Interferons	Avonex (interferon beta-1a), Rebif (interferon beta-1a)
Additional	Aldurazyme (laronidase), Amevive (alefacept), Arcalyst (rilonacept), Cerezyme (imiglucerase), Elaprase (idursulfase), Enbrel (etanercept), Fabrazyme (agalsidase beta), Inductos (dibotermin alfa), Infuse (contains dibotermin alfa), Myozyme (alglucosidase alfa), Naglazyme (galsulfase), Orencia (abatacept), Osigraft also known as OP-1 implant and Opgenra (eptotermin alfa), Pulmozyme (dornase alfa), Regranex (becaplermin), Replagal (agalsidase alfa)

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 enhanced ovulation and embryo developmental rates [34].

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 α-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 glycocomponent are relatively modest. Direct chemical conjugation of an oligosaccharide bearing a terminal MP6 residue onto α-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 glycocomponent 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 glycocomponent, 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 residue to the sugar backbone. These engineered cells, therefore, are capable of generating completely defucosylated antibody with consequent improved cancer-killing ability.

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

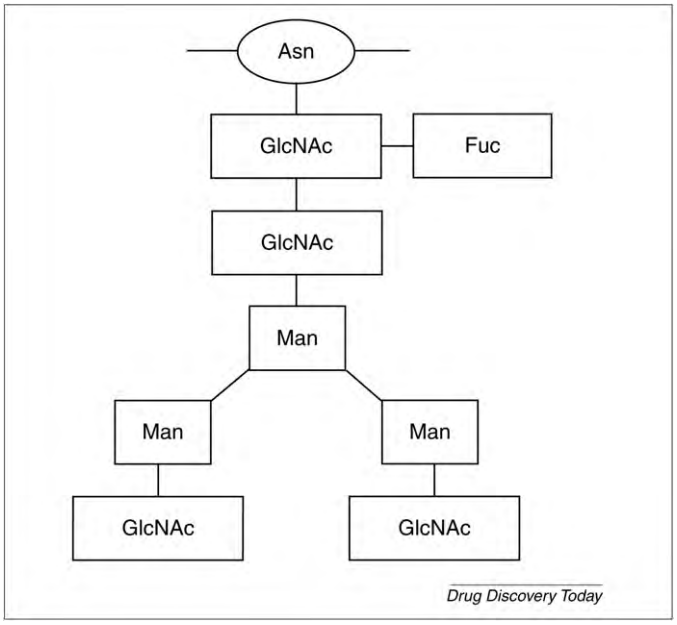

FIGURE 3

Typical diantennary structure of the glycocomponent of IgG produced in CHO cells. The glycocomponent is attached to the antibody's backbone at Asn297 of both heavy chains.

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-xylosyltransferase 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-

TABLE 4

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

Product	Company (year)	Product	Company (year)
Omnitrope (somatropin; rhGH, produced in *E. coli*)	Sandoz (2006)	Filgrastim ratiopharm (filgrastim; rh G-CSF produced in *E. coli*)	Ratiopharm (2008)
Valtropin (somatropin; rhGH produced in *S. cerevisiae*)	Biopartners (2006)	Ratiograstim (filgrastim; rh G-CSF produced in *E. coli*)	Ratiopharm (2008)
Abseamed (recombinant human erythropoietin alfa; rhEPO produced in CHO)	Medice Arzneimittel Putter (2007)	Tevagrastim (filgrastim; rh G-CSF produced in *E. coli*)	Teva (2008)
Binocrit (recombinant human erythropoietin alfa; rhEPO produced in CHO)	Sandoz (2007)	Biopoin *(epoetin theta; rhEPO produced in CHO)*	CT Arzneimittel (2009)
Epoetin alfa Hexal (recombinant human erythropoietin alfa; rhEPO produced in CHO)	Hexal Biotech (2007)	Eporatio *(epoetin theta; rhEPO produced in CHO)*	Ratiopharm (2009)
Retacrit (epoetin zeta; rhEPO produced in CHO)	Hospira enterprises (2007)	Filgrastim hexal (filgrastim; rhG-CSF produced in *E. coli*)	Hexal AG (2009)
Silapo (epoetin zeta; rhEPO produced in CHO)	Stada Arzneimittel (2007)	Zarzio (filgrastim; rhG-CSF produced in *E. coli*)	Sandoz (2009)
Biograstim (filgrastim; rh G-CSF expressed in *E. coli*)	CT Arzneimittel (2008)		

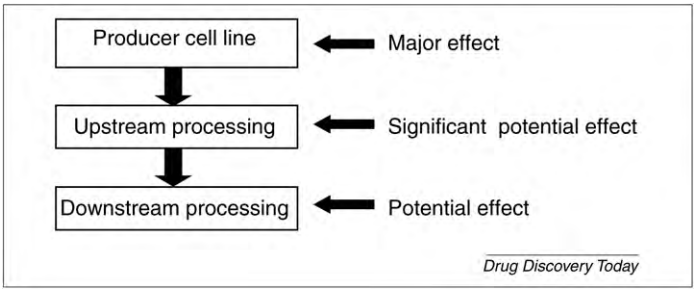

FIGURE 4

An overview of the stages of manufacture of a therapeutic protein which can influence protein PTM detail. Refer to the text for further detail.

icine) and requires the generation of a full quality module, as well as reduced clinical and non-clinical datasets. The quality module must encompass extensive characterization studies to support comparability in terms of product identity, structure and bioactivity and impurity levels. In terms of structure, it is relatively straightforward from a molecular biological standpoint to generate a biosimilar displaying an amino acid sequence identical to its reference product. Undoubtedly however, the presence of a PTM – in particular, a complex one such as glycosylation – can greatly complicate the issue (Fig. 4). Details of the precise producer cell line, as well as the precise details of upstream and downstream processing undertaken to produce the original product, will be highly confidential to its producer company. A biosimilar, therefore, will be produced in a different cell line to that of the reference product. The precise cell line chosen will, through its PTM machinery, obviously dictate the exact PTM detail of the biosimilar product. Likewise, exact conditions characteristic of upstream processing (exact detail of culture media composition and culture format, dissolved oxygen levels and so on) can influence exact PTM detail achieved. Moreover, even downstream processing can affect product PTM profile (e.g. by selectively enriching a particular PTM product variant). Particular attention, therefore, must be paid by biosimilar drug developers to the issue of PTM detail and its characterization, and the developer must satisfy regulatory authorities that any variation in PTM detail observed will not alter the therapeutic characteristics of the biosimilar in comparison to the original product.

The approval of several EPO-based biosimilars (Table 4) confirms in practice the feasibility of developing biosimilar products displaying complex glycocomponents (EPO harbours three N-linked glycosylation sites and one O-linked glycosylation site). Currently, European regulators are updating EPO product-specific guidelines and developing guidelines for biosimilar versions of various other biopharmaceuticals – perhaps most notably monoclonal-antibody-based products. The size, structural and functional complexities of antibody-based products render the development of biosimilar versions particularly challenging [48,49]. Despite this, the European Medicines Agency has already provided scientific advice relating to the development of several biosimilar antibody-based products in development.

Concluding remarks

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

1 Higgins, S. and Hames, B. (1999) *Post Translational Processing, A Practical Approach*. Oxford University Press
2 Kannicht, C. (2002) *Post Translational Modification of Proteins*. Humana Press
3 Walsh, G. (2006) Biopharmaceutical benchmarks, 2006. *Nat. Biotechnol.* 24, 769–776
4 Raider, R. (2007) *Biopharmaceutical Products in the US and European Markets*. Bioplan Associates, Inc.
5 Stadheim, T.A. and Sethuraman, N. (2009) Yeast glycosylation and engineering in the context of therapeutic proteins. In *Post Translational Modification of Protein Biopharmaceuticals* (Walsh, G., ed.), pp. 149–164, Wiley-Blackwell
6 Zuraw, B. *et al.* (2010) Ecallantide. *Nat. Rev. Drug Discov.* 9, 189–190
7 Ledford, H. (2006) The farmyard drug store. *Nature* 443, 16–17
8 Szarewski, A. (2010) HPV vaccine: cervarix. *Expert Opin. Biol. Ther.* 10, 477–487
9 Gemmill, T.R. and Trimble, R. (1999) Overview of N- and O-linked oligosaccharide structures found in various yeast species. *Biochim. Biophys. Acta* 1426, 227–237
10 Gomord, V. *et al.* (2004) Production and glycosylation of plant-made pharmaceuticals: the antibodies as a challenge. *Plant Biotechnol. J.* 2, 83–100
11 Walsh, G., ed. (2009) *Post-translational Modification of Protein Biopharmaceuticals*, Wiley-Blackwell
12 Chance, R.E. and Frank, B.H. (1993) Research, development production and safety of biosynthetic human insulin. *Diabetes Care* 16 (Suppl. 3), 133–142
13 Pipe, S.W. (2008) Recombinant clotting factors. *Thromb. Haemost.* 99, 840–850
14 Hansson, K. and Stenflo, J. (2005) Post-translational modifications in proteins involved in blood coagulation. *J. Thromb. Haemost.* 3, 2633–2648
15 Kaufman, R.J. (1998) Post-translational modifications required for coagulation factor secretion and function. *Thromb. Haemost.* 79, 1068–1079
16 Prigge, S.T. *et al.* (2000) New insights into copper monooxygenases and peptide amidation: structure, mechanism and function. *Cell. Mol. Life Sci.* 57, 1236–1259
17 Bradbury, A.F. and Smyth, D.G. (1991) Peptide amidation. *Trends Biochem. Sci.* 16, 112–115
18 Ray, M.V. *et al.* (1993) Production of recombinant salmon calcitonin by *in vitro* amidation of an *Escherichia coli* produced precursor peptide. *Biotechnology* 11, 64–70
19 Stone, M. *et al.* (2009) Tyrosine sulfation: an increasingly recognised post-translational modification of secreted proteins. *Nat. Biotechnol.* 25, 299–317
20 Moore, K.L. (2003) The biology and enzymology of protein tyrosine-*O*-sulfation. *J. Biol. Chem.* 278, 24243–24246
21 Stone, S.R. and Hofsteenge, J. (1986) Kinetics of the inhibition of thrombin by hirudin. *Biochemistry* 25, 4622–4628
22 El Hajjajii, H. and Collet, J.F. (2009) Disulfide bond formation. In *Post-Translational Modification of Protein Biopharmaceuticals* (Walsh, G., ed.), pp. 277–294, Wiley-Blackwell
23 de Marco, A. (2009) Strategies for successful recombinant expression of disulfide bond-dependent proteins in *Escherichia coli*. *Microb. Cell Fact.* 8, 1–18
24 Chiba, Y. and Akeboshi, H. (2009) New era of glycoscience: intrinsic and extrinsic functions performed by glycans. *Biol. Pharm. Bull.* 32, 786–795
25 Brooks, S. (2009) Protein glycosylation: the basic science. In *Post-Translational Modification of Protein Biopharmaceuticals* (Walsh, G., ed.), pp. 17–50, Wiley-Blackwell

26 Walsh, G. and Jefferis, R. (2006) Post-translational modifications in the context of therapeutic proteins. *Nat. Biotechnol.* 24, 1241–1252

27 Beck, A. *et al.* (2010) Strategies and challenges for the next generation of therapeutic antibodies. *Nat. Rev. Immunol.* 10, 345–352

28 Jefferis, R. (2009) Glycosylation as a strategy to improve antibody-based therapeutics. *Nat. Rev. Drug Discov.* 8, 226–234

29 Vanderschaeghe, D. *et al.* (2010) Glycome profiling using modern glycomics technology: technical aspects and applications. *Biol. Chem.* 391, 149–161

30 Nana, K. *et al.* (2008) Mass spectrometric analysis of carbohydrate heterogeneity for the characterization of glycoprotein-based products. *Trends Glycosci. Glycotechnol.* 20, 97–116

31 Edmunds, T. (2006) β-Glucocerebrosidase, ceredase and cerezyme. In *Directory of Therapeutic Enzymes* (McGrath, B. and Walsh, G., eds), pp. 117–133, CRC Press

32 Sinclair, A.M. and Elliott, S. (2005) Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. *J. Pharm. Sci.* 94, 1626–1635

33 Ceaglio, N. *et al.* (2008) Novel long-lasting interferon alpha derivatives designed by glycoengineering. *Biochimie* 90, 437–449

34 Trousdale, R.K. *et al.* (2009) Efficacy of native and hyperglycosylated follicle-stimulating hormone analogs for promoting fertility in female mice. *Fertil. Steril.* 91, 265–270

35 Zhu, Y. *et al.* (2005) Carbohydrate-remodelled 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

36 Zhu, Y. *et al.* (2009) Glycoengineered acid α-glucosidase with improved efficacy at correcting the metabolic aberrations & motor function deficits in a mouse model of Pompe disease. *Mol. Ther.* 17, 954–963

37 Natsume, A. *et al.* (2009) Improving effector functions of antibodies for cancer treatment: enhancing ADCC and CDC. *Drug Des. Dev. Ther.* 3, 7–16

38 Noboru, T. (2009) Humanization of recombinant glycoproteins expressed in insect cells. *Trends Glycosci. Glycotechnol.* 21, 71–86

39 Yamane-Ohnuki, M. *et al.* (2004) Establishment of a FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependant cellular cytotoxicity. *Biotechnol. Bioeng.* 87, 614–622

40 Hamilton, S.R. and Gerngross, T.U. (2007) Glycosylation engineering in yeast: the advent of fully humanized yeast. *Curr. Opin. Biotechnol.* 18, 387–392

41 Hamilton, S.R. *et al.* (2006) Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science* 313, 1441–1443

42 Karg, S.R. and Kallio, P.T. (2009) The production of biopharmaceuticals in plant systems. *Biotechnol. Adv.* 27, 879–894

43 Koprivova, A. *et al.* (2004) Targeted knockouts of *Physcomitrella* lacking plant-specific immunogenic N-glycans. *Plant Biotechnol. J.* 2, 517–523

44 Nechansky, A. *et al.* (2007) Compensation of endogenous IgG mediated inhibition of antibody-dependant cellular cytotoxicity by glycoengineering of therapeutic antibodies. *Mol. Immunol.* 44, 1815–1817

45 Cox, K.M. *et al.* (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. *Nat. Biotechnol.* 24, 1591–1597

46 Rathore, A.S. (2009) Follow-on protein products, scientific issues, developments and challenges. *Trends Biotechnol.* 27, 698–705

47 Furlani, P. (2008) Biosimilars: a regulatory review. *Drug Inf. J.* 42, 477–485

48 Reichert, J.M. *et al.* (2009) Meeting report: European Medicines Agency workshop on biosimilar monoclonal antibodies: July 2, 2009, London, UK. *MAbs* 1, 394–416

49 Schneider, C.K. and Kalinke, U. (2008) Towards biosimilar monoclonal antibodies. *Nat. Biotechnol.* 26, 985–990