Modifications of therapeutic proteins: challenges and prospects

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Abstract The production of therapeutic proteins is one of the fastest growing sectors of the pharmaceutical industry. However, most proteins used in drug therapy require complex post-translational modifications for efficient secretion, drug efficacy and stability. Common protein modifications include variable glycosylation, misfolding and aggregation, oxidation of methionine, deamidation of asparagine and glutamine, and proteolysis. These modifications not only pose challenges for accurate and consistent bioprocessing, but also may have consequences for the patient in that incorrect modifications or aggregation may lead to an immune response to the protein therapeutic. This review provides examples of analytical and preventative advances that have been devised to meet these challenges, and insights into how further advances can improve the efficiency and safety in manufacturing recombinant proteins.

Keywords Recombinant protein · Cell culture · Aggregation · Folding · Oxidation · Deamidation · Glycosylation · Therapeutics

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
CHO	Chinese hamster ovary
EPO	erythropoietin
ESI-MS	electrospray ionization mass spectrometry
HPLC	high pressure liquid chromatography
MALDI	matrix-assisted laser desorption-ionization
PTM	post-translational modification
UDP	uridine-5'-diphosphate

Introduction

Recombinant proteins, in particular monoclonal antibodies and Fc-fusion proteins (consisting of an active molecule fused to the antibody tail for improved half-life), constitute the fastest growing sector of the biopharmaceutical industry (Chirino and Mire-Sluis 2004). However, these molecules are prone to several types of post-translational modifications (PTMs) that can reduce their efficacy and limit shelf life. In some cases these modifications can also lead to unwanted side-effects, such as triggering an immune reaction against the therapeutic protein (De Groot 2006). The ability to perform complex PTMs is one of the major reasons that the majority of biotherapeutics are manufactured in animal cells. Indeed, only a few biopharmaceutical proteins such as albumin (Recombinant, made by Novozymes) and insulin (e.g. Insulin Lispro made by Lilly and Novo) undergo simple

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modifications such that they can be manufactured using yeast or bacteria (Roach and Woodworth 2002). The most prevalent modifications include variable glycosylation, misfolding and aggregation, oxidation of methionine, deamidation of asparagine and glutamine, and proteolysis (Walsh and Jefferis 2006). Detecting and preventing these modifications has become a major challenge for the biotechnology industry.

Glycosylation

Glycosylation represents the most complex protein PTM, and much research has centred on the measurement and modification of the *N*-glycosylation process. Advances in MALDI and ESI-MS mass spectrometry and novel fluorescent tags for HPLC of released glycans have advanced the field considerably (Sheridan 2007). However, there is still a need for fast and high-throughput assays to detect different glycoforms, such as the Procognia system that uses an array of lectins linked to MALDI mass spectrometry (Koopmann and Blackburn 2003). Another area for improvement is the reliability of in vitro biological assays for therapeutic glycoproteins. For example, poorly sialylated glycoforms of erythropoietin (EPO) actually perform better in vitro (Elliott et al. 2004), but the effect of clearance by the asialoglycoprotein receptor outweighs the EPO receptor binding advantage, with the result that highly sialylated glycoforms are more effective in humans. Indeed, the half-life of the natural EPO molecule has been improved by Amgen by introducing extra *N*-glycosylation sites in its new Aranesp version of EPO, facilitating a longer-acting product (Egrie et al. 2003). Another strategy is to engineer the host CHO cell line with extra glycosyltransferase enzymes (e.g. α 2,6-sialyltransferase is inactivated in CHO cells but present in humans, (Bragonzi et al. 2000)); or β 1, 4-*N*-acetyl-glucosaminyltransferase III to improve IgG antibody-dependent cellular cytotoxicity (ADCC), (Ferrara et al. 2006). Feeding key carbohydrate precursors such as *N*-acetylmannosamine to improve sialylation has also been proposed (Gu and Wang 1998). In addition, the galactose content of IgG can be elevated by a down-stream processing step using a recombinant galactosyltransferase and the precursor UDP-Galactose (Warnock et al. 2005). However, the

cost of these carbohydrate precursors has to be factored into a full-scale industrial process.

Many cell culture parameters can affect the glycosylation pattern of a recombinant glycoprotein such as pH (Muthing et al. 2003), the build up of ammonia primarily as a by-product of glutamine metabolism (Yang and Butler 2002) and the oxygen content of the bioreactor (Serrato et al. 2004). The choice of cell line also greatly influences the glycosylation pattern of the recombinant glycoprotein, since cells differ in their complement of glycosyltransferase enzymes that control the glycosylation process (Baker et al. 2001; Jenkins et al. 1996). In summary, major advances in analytical techniques have paved the way for manipulation of the *N*-glycosylation pathway during bioprocessing, however, methods of controlling the O-glycosylation pathway are still in their infancy.

Protein misfolding, aggregation and methionine oxidation

In contrast to glycosylation, the other PTMs of protein therapeutics are less well studied and understood (Chirino and Mire-Sluis 2004; Harris 2005). Misfolded proteins, in theory, undergo proteolysis in the endosome and the resultant amino acids are recycled in the unfolded protein response (Cudna and Dickson 2003) and the endoplasmic reticulum overload response (Fig. 1). However, in practice cells can become overloaded with misfolded recombinant protein, leading to release of misfolded and aggregated proteins, particularly at high levels of protein expression (Schroder et al. 2002). This can be a particular problem with multimeric proteins such as recombinant IgG (Demeule et al. 2007) and blood clotting proteins such as Factor VIII (Purohit et al. 2006), where protein aggregates trigger an immune response in the patient resulting in inhibitory antibodies to the therapeutic protein (Hermeling et al. 2004). Various strategies are being evaluated to combat aggregation and misfolding that include enhancing chaperone proteins such as BiP (Bertolotti et al. 2000) or modulating the redox potential of the cell (Chakravarthi et al. 2006), however the problem is far from being solved. Protein aggregation problems are not confined to upstream bioprocessing: the techniques used to inactivate viruses during

Fig. 1 The unfolded protein response and subsequent degradation of proteins in the endosome with recycling of amino acids. Following identification of misfolded proteins by specific chaperones, the complexes are actively transported out of the endoplasmic reticulum into the cytosol. Here *N*-glycanase enzymes trim off the *N*-linked sugars and ubiquitin delivers the misfolded protein to the endosome, where proteases degrade the protein and the amino acids are recovered and recycled into other proteins or used in metabolism

downstream processing such as exposure to detergents or extremes of pH can inadvertently damage and aggregate the protein product (Lin et al. 2000). Excipients such as sugars (Cleland et al. 2001) and arginine (Tsumoto et al. 2005) are often used to suppress aggregate formation during protein purification and formulation. Novel fluorescence-based microtitre plate assays (Capelle et al. 2007) and laser light scattering techniques (Ye 2006) will assist in the detection of protein aggregates throughout the upstream, downstream and formulation stages of bioprocessing.

A related problem is the oxidation of methionine residues to methionine sulfoxide, which occurs frequently when cells are transitioned from serum-containing to lean serum-free media. In cases such as α_1 -antitrypsin (used in the treatment of emphysema) oxidation of methionine residues leads to a loss of its critical anti-elastase activity (Taggart et al. 2000). Analytical techniques such as mass spectrometry are now available to identify oxidised methionine residues on recombinant proteins (Houde et al. 2006) although not in high-throughput modes. Formulation excipients can be used to protect the protein from

both methionine oxidation and aggregation (Soenderkaer et al. 2004).

Asparagine and glutamine deamidation

Deamidation of asparagine residue to form aspartic acid and iso-aspartic acid is another cause of protein degradation, particularly during long-term storage (Fig. 2) (Chelius et al. 2005). Most of the deamidated asparagine forms iso-aspartate, which is not a natural amino acid and can potentially be immunogenic. Furthermore, the biological activity of proteins such as IgG₁ and Stem Cell Factor are adversely affected by deamidation (Harris et al. 2001). The non-enzymic deamidation reaction is accelerated at alkaline pH, with surface asparagine residues being more susceptible. Glutamine residues can also be deamidated, but this reaction is one hundred times slower than asparagine deamidation and is rarely detected in recombinant proteins. Although some analytical methods based on HPLC and mass spectrometry have been developed to detect this reaction (Chelius et al. 2005), none is adapted for high-throughput screening. Predicting which asparagine residues will be prone to deamidation in a new therapeutic protein can also be difficult.

Conclusions

The development and manufacturing of protein therapeutic drugs is increasing each year, both in the amount of product required globally and the number of new drugs undergoing clinical trials. In addition, “follow on” and generic protein therapeutics will become more prevalent as the patents associated with the original proteins expire. Despite the success of biotherapeutics there remain significant challenges to be overcome in maintaining product stability and efficacy throughout the production cycle and during long-term storage. Potentially degradative pathways include inappropriate glycosylation, protein aggregation and misfolding, deamidation, oxidation and destruction by proteases. Recent and future advances in protein analysis and preventative strategies should help counteract these degradative pathways and ensure product efficacy, safety and affordability.

Fig. 2 Non-enzymic deamidation of asparagine to aspartic acid (30%) and iso-aspartic acid (70%), via a succinimide intermediate

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