

A novel technology for the production of highly active therapeutic proteins

by Dr. Hans Baumeister and Dr. Steffen Goletz

GlycoExpress is a novel expression platform which allows the generation of proteins with full human glycosylation and optimised sialylation. The technology is based on a glycoengineered human cell line and enables the production of large amounts of improved recombinant proteins for clinical applications. These applications include biologicals with higher activity, optimised pharmacokinetics, and decreased antigenicity and immunogenicity.

In recent years, the so-called glycobiological aspects of biopharmaceuticals have been attracting increased attention and investment. From the regulatory point of view, the regulatory bodies nowadays expect a detailed analysis of the carbohydrates attached to the therapeutic protein and require data on the stability of the glycosylation and whether this stability can resist any changes in the production process. From the product point of view, the importance of glycosylation is increasingly being appreciated. There are more and more examples of product improvements resulting from appropriate glycosylation, thus increasing market potential. GlycoEngineering technology [figure 1] allows the generation of cellular systems that produce highly stable proteins that are optimally glycosylated. Expensive *in vitro* glycosylation of the cellular product is no longer necessary.

The global market for therapeutic biologicals is estimated as being in excess of \$12 billion with a grow rate of 10 per cent per annum. In the last 20 years more than 70 therapeutic proteins have been approved for medical use and around 120 are estimated to be currently undergoing clinical trials [figure 2]. Many therapeutic proteins will soon lose patent protection, thus stimulating the development of biogenerics and follow-on biologicals. In the future it is likely that increased effort will be directed to the development of improved (longer-lasting, safer and more efficacious) biogenerics.

In vivo, most therapeutically-active proteins are glycoproteins with either single, short carbohydrates or large carbohydrate moieties attached to the protein. Most therapeutic proteins produced by recombinant means are however either not glycosylated at all, have inappropriate glycosyl residues, or are simply different from the original human one. This is despite the fact that there are now many

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Figure 1. Overview of GlycoEngineering technology to generate a desired glycoprofile of a cell line and its products. For more details of GlycoEngineering technology see figure 5. Genetic modifications refer to the introduction or knock-out of specific genes, while GlycoEngineering technology itself does not generate genetic modified organisms.

examples showing clearly that elimination or modification of the naturally-occurring carbohydrates has a significant negative effect on overall bioactivity. Vital properties such as stability, serum-half-life, receptor binding and/or cell activation can all be affected. In addition molecules with different glycosyl residues from those in the normal human form have the potential of inducing undesired immune reactions when used in patients.

This combination of achieving higher bioactivity when the molecule is optimally glycosylated, with the risk of immune reactions if the glycosylation is inappropriate is the reason for more and more attention being focussed on glycosylation both by the pharmaceutical companies developing and manufacturing the product and the regulatory bodies. Glycoengineering technology has the potential to facilitate screening for biologicals with higher bioactivity and fewer side effects. These improved biotherapeutics will in addition be sufficiently different from the original forms that they will qualify for new and extended patent protection.

Current methods for the production of therapeutic proteins

There are two main approaches that are currently used for the production of therapeutic proteins. The first generation of therapeutic

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Figure 2. Therapeutic proteins approved or in clinical trials, by product category (cytokines include interferons, interleukins, colony stimulating factors, and tumour necrosis factors).

proteins was, and still is, produced mainly in bacteria (*Escherichia coli*) and in yeast cells (*Saccharomyces cerevisiae*). The advantage of this method is that the production process is relatively easy and cheap and gives very high yields of recombinant proteins. In addition to the difficulty of downstream purification while maintaining optimal molecular conformation, the major drawback of the approach is that in bacteria and yeast there is either no glycosylation at all or whatever glycosylation there is is significantly different from that achieved in mammalian cells. Despite these drawbacks, bacterial and yeast-

based systems are still in use for the production of several proteins such as insulin and some cytokines. The reduced bioactivities of non- or very differentially glycosylated proteins must however always be carefully considered when using bacterial or yeast-based production systems.

The second frequently-chosen expression system is Chinese hamster ovary (CHO) cells. Here the advantage is that protein products are glycosylated in the mammalian manner and the final protein yield is acceptable. The main reason for using CHO is that these cells are widely established as a production cell line for therapeutic proteins but, as is often the case with established systems, this is not necessarily the best system. Whilst CHO cells are capable of glycosylation in the complex manner of mammalian cells, the glycosylation process itself involves more than 200 enzymes and transporters, and differs from species to species and even from cell type to cell type in the same organism. Thus, although produced in a mammalian system, glycoproteins from CHO cells are not of human origin and are thus glycosylated in a different manner from the human molecules. It has been established that there are several significant differences between CHO and human cells from the point of view of glycosylation. Thus, it is well known that CHO cells sialylate at a lower degree and lack a complete sialylation pathway. In addition, CHO cells incorporate into the protein not only the common sialic acid NeuAc but

also another sialic acid, NeuGc which is not found at all in glycoproteins from human cells. CHO cells also contain terminally bound α 1-3 galactose which is absent in human cells. All these differences have a tremendous impact on the efficiency of most therapeutic proteins, affecting serum-half-life, immunogenicity,

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Figure 3. A human cell line glycoengineered for expression of a sialic acid free glycotope. Cells were visualised by immunocytochemical staining for the sialic acid free Thomsen-Friedenreich disaccharide. The left and right panel shows cells before and after GlycoEngineering, respectively, at comparable densities.

1/3 Vertical
58 x 254 mm

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Figure 4. GlycoExpress: an integrated human expression system to screen for the optimal sialylation of protein targets and transfer to production scale.

and bioactivity, thus having a significant effect on dosages and side-effects.

For example, a low level of sialylation of therapeutic proteins leads to a short serum-half-life. Non-human types of glycosylation (e.g. NeuGc and α 1-3 bound galactose) are antigenic in humans, leading to both a decrease in the therapeutic efficiency of the administered product and to the development of the symptoms of autoimmunity. Recombinant antibodies produced in CHO cells also are less efficient because of the specific changes in the glycosylation pattern.

The importance of sialylation

Sialic acids are a family of terminal monosac-

charides present on N- and O-glycans that are essential for the prolonged serum half-life of glycoproteins. Glycoproteins lacking sialic acids are recognised by specific receptors in the liver and are removed from the circulation. It is less well known that sialylation also affects the bioactivity of therapeutic protein. One example is follicle-stimulating hormone (FSH) used for the treatment of human infertility. In response to a physiological need, FSH is released by the hypophysis either in a highly-active form or in a longer-lasting form with lower physiological activity. The molecular difference between the two forms is solely the degree of sialylation.

The recently developed GlycoExpress technology can aid in identifying the optimal

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Figure 5. The GlycoProfiling and cellular GlycoEngineering technology in more detail.

degree of sialylation for therapeutic proteins. Results obtained at the laboratory scale can then be extrapolated to large scale production of therapeutic proteins.

GlycoExpress, a platform for the production of proteins with full human glycosylation and optimal sialylation

A particular technology for Glycoengineering has been developed at GlycoTope in order to generate new cell lines with defined types of glycosylation. Using this technology a human cell line, GlycoExpress, has been engineered [figure 3] that is capable of producing glycoproteins with full human glycosylation and optimal sialylation. This was achieved by using the technology to generate a cell line with a specific genetic defect. This virus-free cell line has optimal biotechnological properties. It can grow in suspension with a very high proliferation rate and is easily cloned. In addition, molecular biological modification is possible, serum is not needed for growth and large amounts of glycoproteins are produced. The cell line produces proteins with full human glycosylation and a defined degree of sialylation that can range from virtually 0% to 100% depending on the supplements added to the medium [figure 4]. Thus the system is ideal for determining the optimally sialylated form of a therapeutic protein, and consequently the optimal *in vivo* half-life, activity, immunogenicity and stability of the protein. Once established, the optimal sialylation conditions can then be relatively easily transferred to a production process.

This 2-step strategy has been used in practice to express a human growth factor. The molecule produced by the system was found to be several hundred times more active, compared to the commercial equivalent. This increase in activity was achieved at a high (but not the highest possible) degree of sialylation thus guaranteeing a good half-life.

The GlycoEngineering technology

The Glycoengineering technology comprises several packages that interact with and depend on each other [figure 5]. The cellular glycosylation mechanism is first analysed by glycoprofiling in order to determine which cell line could be engineered to generate the desired glycosylation. To facilitate such glycoprofiling an oligonucleotide array (GlycoProfiler) specific for enzymes and other proteins of the glycosylation pathway has been developed and validated by RT-PCR. Additionally, selected glycosyltransferase activities are determined and the presence of carbohydrates and glycoproteins on the cell surface is analysed. On the basis of this information, a selected cell line is then glycoengineered. Random mutations are induced and the cells with the desired phenotype are selected by using carbohydrate-specific antibodies or lectins, followed by cloning for stable expression of the desired phenotype. The glycosylation profile of the engineered cell line is fine-tuned by metabolic engineering or optionally by recombinant expression or down-regulation of glycosyltransferases. Glycosylation of the produced protein is analysed biochemically (GlycoAnalytics). The GlycoProfiling package can also be used for the characterisation of existing production cell lines and for quality control during the glycoprotein production process.

Summary

The GlycoEngineering technology and GlycoExpress systems are particularly suited for the production of the new generations of improved therapeutic proteins, such as Erythropoietin, G-CSF, GM-CSF and FSH. In addition, from an intellectual property point of view, new patent protection of the product and the process will be possible. Of special interest is a new expression system for the production of recombinant functional antibodies mediating cytotoxicity. Yet another application of GlycoEngineering is the generation of new cell lines expressing carbohydrate antigens that are highly specific for tumour cells. Such cell lines have been successfully developed at GLYCOTOPE for use both as cellular vaccines against cancer as well as the generation of therapeutic antibodies against the tumour antigens.

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