



Novel Glycosylation Technologies for the Development of Biosimilars and Biobetters

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Glycosylation has moved centre-stage in the development of biopharmaceutical products and, in the near future, it can be expected that the therapeutic and commercial success of many products will depend on correct glycosylation patterns.

In recent years, therapeutic proteins have enjoyed a remarkable success. The five therapeutic antibodies – Rituxan, Remicade, Avastin, Herceptin and Humira – alone have brought in a combined revenue of \$4 billion, while the entire market segment has reached a value of more than \$30 billion. This commercial success has spurred the development of new biological entities (NBEs) and enhanced the development of existing products as second- and third-generation versions, while loss of patent protection for the first generation is creating opportunities for generic or biosimilar competition.

Post-translational modifications – and in particular glycosylation – play a decisive role in ensuring proper protein function. Glycosylation has been found to occur in a large variety of protein classes, including – but not limited to – antibodies, protein hormones, growth factors, cytokines and vaccines. While antibodies of the IgG class possess a single glycosylation site in the Fc-fragment, and in some cases a second glycosylation site in the Fab fragment, other proteins carry an abundance of glycosylation sites, virtually shielding the peptide backbone from the extra-cellular environment.

Glycosylation has taken on an increasingly important role in the development of both NBEs and biosimilars for two reasons:

- 1) Glycosylation has a large influence on a protein's functional characteristics. Full activation of a cell by growth factors, antibody-dependent cell-cytotoxicity (ADCC), half-life, stability and folding – to name a few – have all been found to be highly dependent on glycosylation patterns.
- 2) Glycosylation is a highly complex process, involving a network of several hundred enzymes

and transporters, and – in contrast to the protein's genetically encoded amino acid sequence – varies with the capabilities of the cell line and even the cell clone used for production. Furthermore, design of the production process and conditions during fermentation can alter the outcome, posing a major challenge to manufacturers of biosimilars that are required to reproduce the glycosylation of the original product.

CHOICE OF CELL LINE

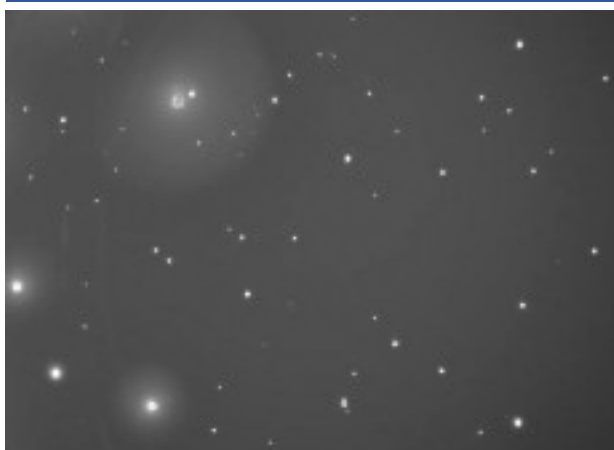
One of the most influential decisions during the development of a glycosylated therapeutic protein has to be made during the very early stages – namely the choice of an appropriate cell line for production. Current production processes usually use microbial systems, yeast or cell lines derived from insects (SF9), mice (SP20) or hamster (CHO). As a consequence of being a highly complex process, a fully human glycosylation pattern is not achieved with any of these systems, although the mammalian ones exhibit the greatest similarities to a human system. Nevertheless, the non-human components have been found to significantly increase the likelihood of immunogenic reactions, which in some cases can lead to severe clinical problems. For this particular reason, human cell lines providing a human glycosylation pattern – such as those of Crucell (Per.C6), Cevic (CAP) and Glycotope (GlycoExpress) – have attracted increasing amounts of attention over the past years. The GlycoExpress cell lines recently received approval from the German regulatory authority for use in the production of a glyco-optimised antibody that is currently undergoing clinical trials.

The chosen cell line is transfected to express the protein of interest recombinantly. Single cells have to be cloned and expanded to generate a cell clone with high

Table 1: Overview of the glycotechologies available for development of biosimilars and 'biobetters'	
GlycoProfiling	Characterisation of the cellular glycosylation machinery (mRNA, enzymatic) and analysis of key structures on cell colonies, cell lines and recombinant products
GlycoAnalytics	Analysing the structure of N- and O-glycans attached to recombinant proteins in detail
GlycoAccess	The preclinical development of glycosylated biotherapeutics including <i>in vitro</i> and <i>in vivo</i> bioassays
GlycoEngineering of cells	Engineering of cell lines with a novel and stable glycosylation profile without necessarily generating a genetically modified organism for biopharmaceutical production
GlycoProtein engineering	Recombinant protein and antibody engineering and optimisation, such as addition/omission of glycosylation sites, humanisation and chimaerisation
Glyco-optimisation	Human screening and production platform for fully human and optimised glycosylation of biopharmaceuticals based on GlycoExpress
GlycoExpress	Toolbox of human, glyco-engineered cell lines for glyco-optimisation
GlycoBodies	Generation of monoclonal antibodies specifically recognising carbohydrates and carbohydrate-protein mixed epitopes

productivity. In the case of a biosimilar, regulatory approval now requires an extensive programme of bioequivalence studies to be undertaken to characterise the product in terms of its biochemical properties, safety and activity. As a consequence, glycosylated biosimilars need to be equipped with a similar pattern of glycosylation. For example, the degree of sialylation should not deviate by more than 20 per cent from that of the original product. Hence, the chosen cell clone with high productivity has to be able to provide post-translational modifications as closely related to the originator's cell line as possible. However, since glycosylation differs within clones, during the

Figure 1: Visualised GlycoExpress cell colonies



Images: Glycotope

bioequivalence study it is often realised that the product carries different carbohydrates, usually resulting in a hyposialylation; this requires the screening process to be repeated in order to identify a new cell clone that is able to provide the equivalent glycosylation.

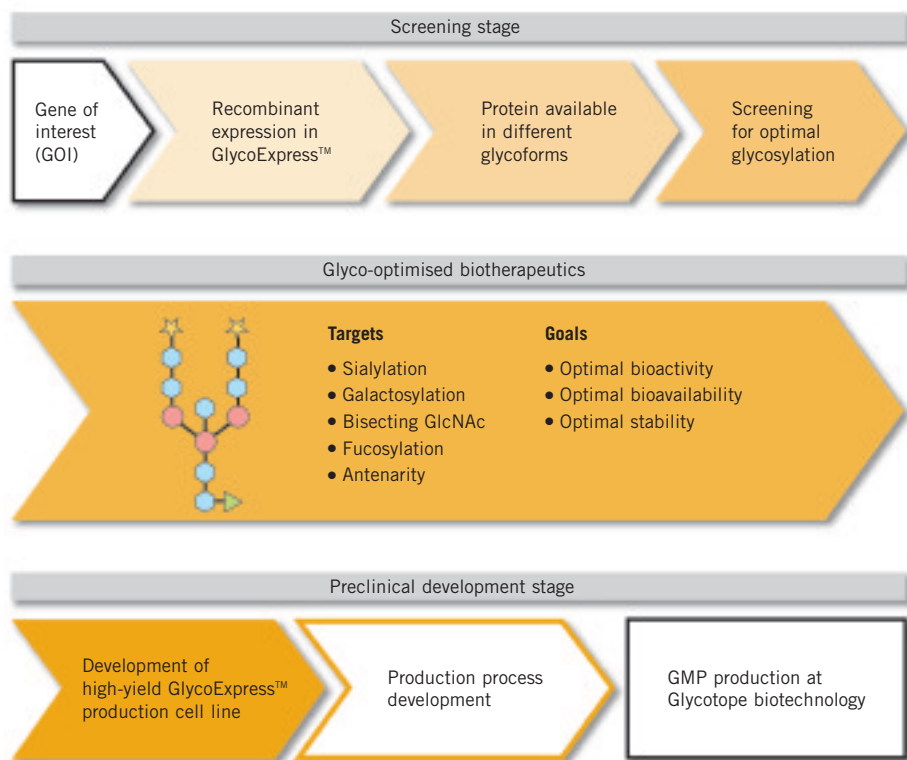
GLYCOSYLATION ANALYSIS

Among companies that analyse the glycosylation of proteins, Glycotope has established novel technologies for the development and production of glycosylated biotherapeutics, applicable to both biosimilars and NBEs or second-generation biobetters (see Table 1). These include:

- ◆ A number of techniques for analysing and profiling the glycosylation of cell colonies, cell lines and recombinant products allowing: a) the identification of key carbohydrate structures already at early stages of development (glycoprofiling); b) analysis of the cellular glycosylation machinery; and c) in-depth analysis of glycosylation structures (glyco-analytics)
- ◆ Engineering of cell lines to generate new cell lines with a modified glycosylation profile (glyco-engineering)
- ◆ Optimisation of biotherapeutics using the GlycoExpress platform technology in order to achieve a favourable glycosylation pattern (glyco-optimisation)

A novel glycoprofiling technique was developed to detect key glyco-structures at an early stage of cell cloning, hence avoiding the time-consuming 'back and forth' of cell cloning, expansion and product characterisation. Already, either the cell itself or the recombinant product can be analysed at the level of colonies with just a few hundred cells. The desired glycosylation phenotype is visualised on the cell colonies (see Figure 1), and the positive cell colonies are then selected for cell expansion and validation of their phenotypic stability. Beyond the glycosylation profile, the productivity of the cell colonies can be analysed to select cell clones with high productivities. The system is based on a semi-automatic detection and fully-automatic selection of cell colonies, allowing the screening of approximately 4,000 cell colonies in one screening round. In summary, this screening system allows cell clones to be selected

Figure 2: The glyco-optimisation process



In contrast to biosimilars, second-generation ‘biobetter’ biotherapeutics should show an improvement compared with their originals – mostly in terms of their bioactivities, bioavailability and immunogenicity. Beyond the fully human post-translational modification of a protein, which reduces the immunogenicity due to the absence of any non-human glycosylation, GlycoExpress technology can be used to glyco-optimize the protein. This is achieved by simultaneous expression of the protein in a set of human glyco-engineered cell lines, which exhibit different glycosylation profiles. Hence, the expressed glycoprotein will be available in various glycoforms, and suitable bioassays can then be used to allow identification of the particular glycosylation pattern that confers optimal product characteristics on that product. The key glycosylation parameters that can be addressed are fucosylation, sialylation,

galactosylation, the antenarity and the bisecting N-acetylglucosamine (see Figure 2). In addition to a set of cell lines with different glycosylation profiles, it is possible to control the degree of sialylation or fucosylation up to the naturally possible maximum via medium supplementation.

for their optimal glycosylation profile and productivity – thereby saving large amounts of time later when the recombinant product needs to be glycosylated in a specific fashion, as in the case of a biosimilar product.

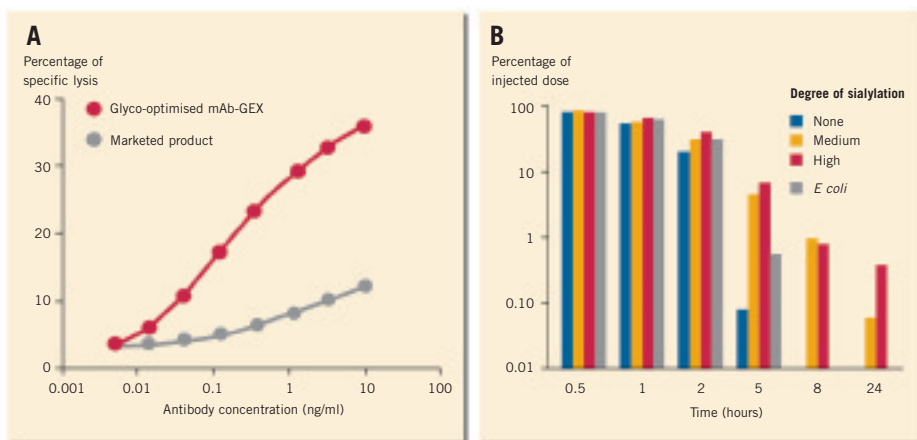
In addition to glycoprofiling of a number of key structures on the cell surface, there are a number of techniques available to analyse the glycosylation machinery of a cell line in detail – such as the presence/absence of glycosyltransferases, glycosidases, transporters and enzymes that are involved in the monosaccharide metabolism, and the activity of certain enzymes. This glycoprofiling is basically the first step in the modification of cell lines in order to achieve a novel glycosylation profile of both the cell line itself and the recombinant product expressed in that cell line (glyco-engineering).

Glycosylation of recombinant proteins is complex and may need to be addressed by a number of methods depending on the degree of information that is necessary. A handful of companies are able to offer a broad range of approaches to analyse glycosylation by methods such as mass spectrometry, HPLC or CE. Currently, there are efforts being made to establish techniques for a higher throughput of glyco-analysis.

GLYCO-OPTIMISATION

Various projects and case studies have shown that the function of therapeutic proteins can be significantly enhanced via glyco-optimisation. A strong emphasis has been put on monoclonal antibodies for the treatment of cancer. One of their main modes of action – ADCC-activity – can be improved for a number of antibodies up to 200-fold. An example is given in Figure 3A (page 58). In addition, the limitations that are set by a polymorphism in the FcγIIIa-receptor, responsible for mediating ADCC, could be overcome. While this polymorphism renders antibodies of the first generation virtually ineffective in nearly two thirds of the patient population, with the glyco-optimised version a strong ADCC-response across all receptor-subtypes can be restored. Finally, a significant elongation of the serum half-life of antibodies can be achieved by glyco-optimisation (results not shown).

Figure 3: Improvement in the ADCC activity of an mAb (A), and the bioavailability of a growth factor (shown as a percentage of injected dose) (B)



Glyco-optimisation is also suitable for other glycosylated biotherapeutics such as cytokines, glycoprotein hormones, growth factors, blood factors and enzymes. In different case studies, it has been demonstrated that the degree of sialylation can affect the half-life and activity of such products. Figure 3B gives an example of a growth factor produced with various degrees of sialylation. A comparison of the half-life in mice of these glycoforms with that of the commercially available product from *E. coli*, shows that as expected those with a high degree of sialylation were detectable for the longest time after injection. Furthermore, non-sialylated glycoforms exhibited a half-life time even shorter than that of the non-glycosylated *E. coli* product (see Figure 3B).

Importantly, GlycoExpress technology offers excellent biotechnical characteristics that facilitate the integration of glyco-optimisation into the development of high-yield expression clones; current productivity amounts to around 40pg antibody/cell/day under serum-free conditions with division-times of 14 to 24 hours – a high stability of productivity even in the absence of an active selection and high sheer-force resistance.

These conditions formed a solid basis for a GMP-compliant production process for glyco-optimised antibodies to be established. Instead of a standard fed-batch process, GlycoTope opted for a perfusion process, as this offers significant advantages in terms of achieving high productivity with highly consistent final product quality at lower costs for downstream processing. Continuous harvesting of the culture requires the constant addition of media and thus significantly larger amounts of media; however, as the product is rapidly removed and stored, it is not subject to any metabolism that would occur at 37°C in a fed-batch bioreactor. The products of three independent GMP perfusion runs with a harvest of 3,000 litres each revealed identical glycosylation patterns for all three batches; this was one reason for the German authorities' rapid approval of the clinical application of the first glyco-optimised antibody.

CONCLUSION

Glycosylation has moved centre-stage in the development of biopharmaceutical drug products. Its influence on a protein's characteristics has been acknowledged by regulatory requirements for the development of biosimilars that demand a high level of glycosylation-based similarity to the original product. There is an ever-increasing number of studies demonstrating that it is critical to examine the glycosylation of biotherapeutics and for it to be optimised in order to achieve higher bioactivity, wider patient coverage, longer half-life and lower immunogenicity – properties that are essential for therapeutic applications. With these improvements, glyco-optimised biotherapeutics will most probably also receive new intellectual property protection. Hence, it is expected that in the near future a number of novel biotherapeutics will be developed for which the therapeutic and commercial benefits will rest upon proper glycosylation.



Hans Baumeister was trained in Biochemistry and Molecular Biology at the Universities of Berlin and Hamburg (Germany) and holds a PhD in Biochemistry. During his scientific career, he worked in a number of renowned research institutes in Germany (for example, the German Institute of Human Nutrition in Potsdam) and in the US (the Roche Institute of Molecular Biology in Nutley, NJ). At the start-up of GlycoTope GmbH in 2001, he was Head of the GlycoEngineering Group where he developed the GlycoExpress technology. In 2004, Hans changed to the Business Development Group and in 2007, was appointed Chief Operating Officer and Head of the Service Unit. Email: hans.baumeister@glycotope.com

Steffen Goletz, CEO, CSO and co-founder of GlycoTope, studied biology, biochemistry and molecular biology at the universities in Heidelberg, Kaiserslautern, Manchester (UK) and Berlin and holds a PhD in biochemistry. During his scientific career he worked for renowned research institutions including the Max Delbrueck Centre for Molecular Medicine (Berlin), the MRC Centre for Protein Engineering (Cambridge, UK), and the German Cancer Research Centre in Heidelberg. During his research, Steffen has focused on glycobiology, tumour immunology, antibody engineering and cellular engineering. As CSO, Steffen is responsible for the development of GlycoTope's product pipeline of glyco-optimised biotherapeutics, with the lead product recently approved for clinical trials.