A Matter of Cell Line Development

Hans Baumeister and Steffen Goletz at Glycotope review the processes and developments involved in the glycooptimisation of biotherapeutics

At the time that the first therapeutic proteins were developed, glycosylation was largely ignored, and glycoproteins were most likely to be excluded from the list of drug candidates. Today, glycosylation is accepted as part of the therapeutic protein, and companies developing such biotherapeutics focus on analysing and understanding the structure of glycosylation to keep the glycosylation consistent from batch to batch. Since it was found that the degree of sialylation affects the *in vivo* bioavailability of erythropoietin, the first 'glycoengineered' product came on the market (Aranesp®) (1). This product has two additional glycosylation sites integrated into the protein to increase the degree of sialylation and elongate the bioavailability. There are now many examples demonstrating that glycosylation does not only affect the bioavailability of biopharmaceuticals, but strongly influences the activity, immunogenicity, antigenicity, solubility and stability of proteins too (2,3). Therefore the aim of the industry is to improve biopharmaceuticals by optimising and humanising their glycosylation.

There are at least four approaches to optimise glycosylation:

- The glycosylation is modified enzymatically after production
- The cell culture and production conditions are controlled in order to achieve a more reproducible and homogenous glycosylation
- The protein is engineered to add or eliminate sites for attachment of glycans
- A novel cell line is engineered for production of biotherapeutics with a modified glycosylation profile

In this article we will focus on the development of novel glycoengineered cell lines for production of glycooptimised biotherapeutics.

There are a small number of companies that deal with the glycoengineering of cell lines. The two companies Glycart, which was acquired by Roche in 2005, and Kyowa (BioWa in the USA), which has been part of Kirin Holding since 2008, developed genetically modified Chinese hamster ovary (CHO) cell lines to modify glycosylation and produce higher active therapeutic antibodies. Other companies, such as GlycoFi (now part of MSD), Biolex or Greenovation, focus on glycoengineering plant or yeast cell systems to combine the advantages of a very high-yield and cheap production system with a glycosylation more similar to a human glycosylation than the original cell system. Glycotope decided to develop high-yield human cell lines for production of therapeutic glycoproteins and now has a toolbox of glycoengineered human cell lines available, all of leukemic origin.

FULLY HUMAN GLYCOSYLATION

One important aspect of modern cell line development is to achieve fully human post-translational modifications (PTMs) of biotherapeutics (4). Currently, human biotherapeutics are produced predominantly in *E coli*, yeast or cell lines derived from insects (SF9), mice (SP2/0) or CHO. While a protein produced in different systems of bacterial or mammalian origin has at least the same primary structure, the PTM of this protein, and most importantly the glycosylation, differs from organism to organism and even from cell type to cell type within one organism. Mammalian cell lines of rodent origin (such as SP2/0, CHO or BHK) are able to confer a glycosylation that has some similarity to a human glycosylation. However, some human components are missing (such as the 2,6-linked sialylation) and a number of non-human components have been found to significantly increase the likelihood of immunogenic reactions, such as terminal sialic acids that do not exist in human cells (NeuGc, for example) or terminal galactose linked to another galactose in a way that is absent from human cells (Gal-Gal structures). The latter has been found to be the reason for the clinically observed severe hypersensitivity reactions in about 33 per cent of the patients treated with Erbitux® (5).

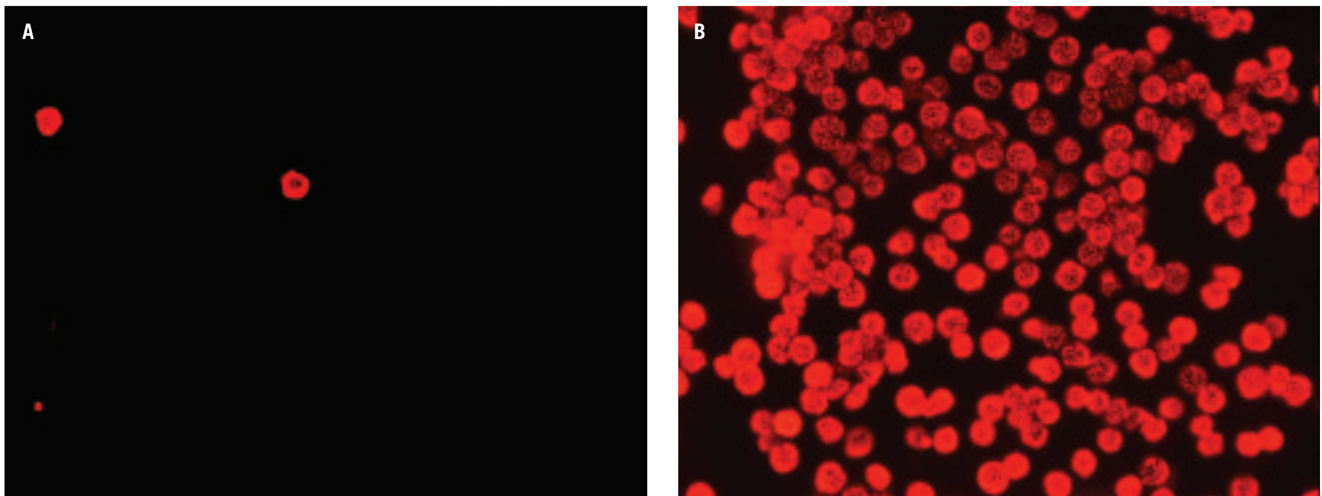
For this reason, human cell lines providing a human glycosylation have attracted increasing amounts of attention over the past years. But it is not only about fully human glycosylation: as mentioned above, the glycosylation profile varies from cell type to cell type within one organism. It is therefore not surprising that a glycoprotein produced in different human cell lines is not glycosylated in the same way and that identifying the optimal glycosylation can result in proteins with largely higher activity, longer serum half-life and improved stability as compared to others. To screen for and produce proteins that are glycosylated in the optimal way, human cell lines can be engineered to achieve a set of cell lines with different glycosylation profiles.

GLYCOENGINEERING OF CELL LINES

There are several ways to engineer a given cell line in order to modify its glycosylation profile. The conventional technique is to generate a genetically modified organism (GMO) by introducing or eliminating a gene relevant for a desired glycosylation profile. For example, to engineer plant cells that are unable to glycosylate with xylose, a monosaccharide that is common in glycans attached to asparagines (N-glycans) in plants, but absent from human N-glycans, researchers knocked-out the relevant gene. In one particular large research programme, all genes that are involved in the yeast-specific synthesis of N-glycans were knocked-out and the relevant human genes were knocked-in. However, a similar approach proved impossible for O-glycans, since the yeast cells did not survive. In other projects, CHO cells were glycoengineered to synthesise N-glycans with a reduced degree or almost no 1,6-fucosylation, which is important for improving the activity of antibodies. While one company directly knocked-out the relevant gene, resulting in almost no 1,6-fucosylation, another applied a more indirect strategy by introducing the gntIII gene, which results in the generation of N-glycans with a bisecting N-acetylglucosamine (GlcNAc) and in consequence to a somewhat reduced level of 1,6-fucosylation.

An alternative approach to glycoengineering consists of two major steps:

Figure 1: Cells stained for a desialylated key glycan analysed before (A) and after (B) glycoengineering (both panels show a similar cell number)



- A number of human cell lines were analysed by profiling them for the presence of important key structures in the glycosylation, for key enzymes of important glycosylation pathways, and for excellent biotechnological features to allow the glycoengineered cells to be used for production of the glycooptimised biotherapeutics.
- A chosen human cell line is glycoengineered for the desired new glycoprofile (the presence or absence of a certain glycostructure) while avoiding the generation of a GMO. Hence, regulatory hurdles are lower for one-time GMO cells expressing recombinantly the protein of interest than for two-times GMO cells. All the resulting cell lines exhibit a very stable phenotype. An example is given in Figure 1.

Using this approach, a set of human glycoengineered cell lines have been generated with very similar genetic backgrounds but different glycosylation profiles addressing the degree of 1,6-fucosylation, 2,3- and 2,6-sialylation, galactosylation, the presence of bisecting GlcNAc and the complexity of the antennary structures. To glycooptimise a protein, the first step is to express it in a set of glycoengineered cell lines and produce different glycoforms of that protein, for example: with or without 1,6-fucosylation, with or without 2,3- and 2,6-sialylation, with a high degree of galactosylation (important for CDC activity of antibodies) and in the presence of bisecting GlcNAc (see Figure 2). Since the degree of sialylation is critical for the bioactivity of a glycoprotein there is a special focus on sialylation among the glycoengineered human cell lines. With two of the glycoengineered cell lines, it is now possible to produce a given protein with a very high degree of sialylation and at any lower level of sialylation between zero per cent and the natural possible maximum by means of medium supplementation (metabolic engineering) and in-process control.

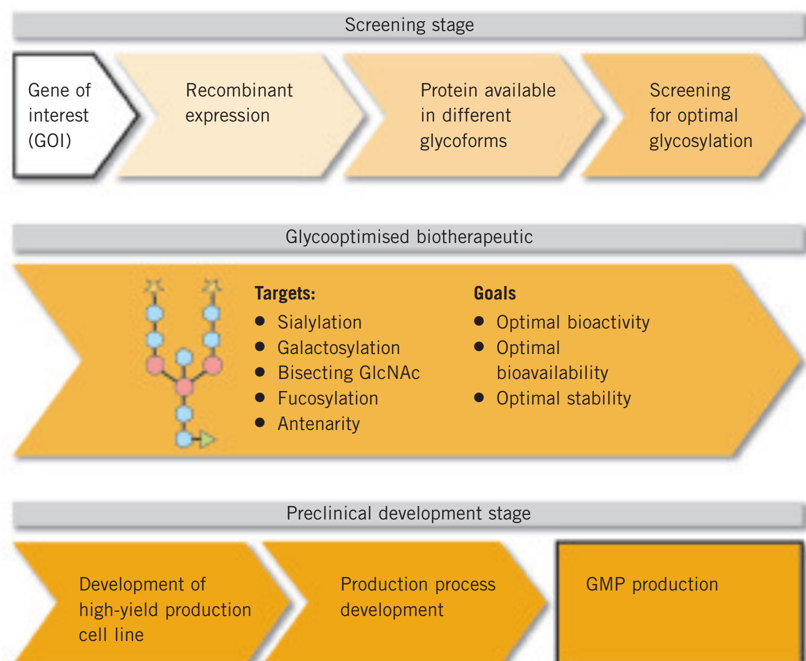
An additional approach of glycooptimisation, beyond the scope of this article, is the introduction or elimination of glycosylation sites within one given protein.

Hence, a glycoprotein expressed in these glycoengineered cell lines will be available in various glycoforms, and screening in suitable bioassays allows the identification of the particular glycosylation pattern that confers optimal product characteristics to the product. In the following, successful glycooptimisation of two biotherapeutic products is described in more detail.

GLYCOOPTIMISATION OF ANTIBODIES

Antibodies are currently a highly demanded target for glycooptimisation because of the remarkable economic success therapeutic antibodies have enjoyed in recent years and the observations that glycosylation can affect the activity of an antibody *in vitro* and *in vivo*. Antibodies have two N-linked glycans attached to the Fc part of IgG molecules. In addition, about 30 per cent of the antibodies isolated from human serum have an additional glycosylation site within the Fab fragment. What can be achieved by glycooptimisation? Figure 3 gives an

Figure 2: Glycooptimisation process



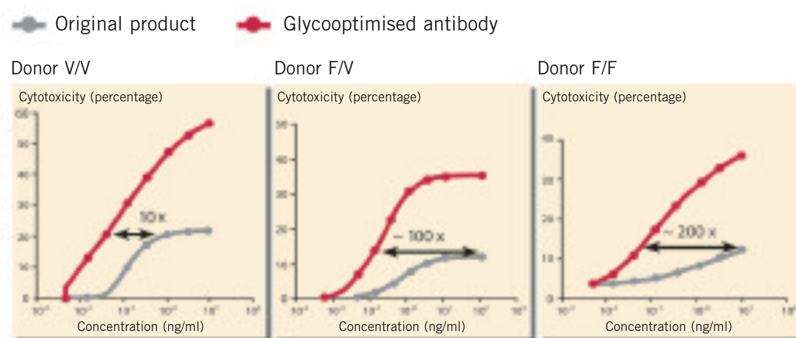
example of an antibody that mediates tumour cell killing by activating natural killer (NK) cells and the antibody dependent cell mediated cytotoxicity (ADCC).

By producing this antibody in the human 1,6-fucosylation-negative cell line, the anti-tumour ADCC activity of that glycooptimised antibody is between 10 and 200 times higher compared with the original antibody (see Figure 3). Hence, for the glycooptimised antibody, amounts that are up to 200 times less compared to the original are sufficient to achieve the same levels of cytotoxicity. Also, at a given antibody concentration, the percentage of killed tumour cells was consistently higher (up to four times).

This effect is caused by much better binding and activation of the FcγRIIIα receptor by the glycooptimised antibody (6). The FcγRIIIα receptor is located on NK cells and is responsible for mediating the ADCC activity on the cellular site. A polymorphism of this receptor in the population causes the high degree of deviation of 10 to 200 times observed with the glycooptimised antibody (see Figure 3). The donor cells of the F/V and F/F phenotypes respond to the glycooptimised antibody 100 to 200 times better than to the original antibody, while donor cells of the V/V phenotype are less responsive to improvements by glycooptimisation (10-fold). This large increase of bioactivity with cells of V/F and F/F donors is largely explained by the fact that the original antibody is almost inactive with these cells. The original antibody is most active with cells of the V/V phenotype, which explains the 10-fold increase of ADCC activity when the glycooptimised antibody is applied. However, among patients in Europe and Asia who are treated with that antibody, only four to 25 per cent are of the V/V phenotype (see Figure 3).

It is not only about 1,6-fucosylation: the degree of sialylation was also increased, resulting in a glycooptimised antibody with significantly elongated bioavailability *in vivo* (mice and monkeys, data not shown). Based on the fully human glycosylation, no immunogenic reaction against non-human glycans is expected to occur in the clinic.

Figure 3: Glycooptimisation of an antibody



Glycooptimisation results in:

- **10- to 200-fold higher ADCC** anti-tumour activity
- Extensive **broadening of the number of patients suitable for successful therapy** (due to FcγIIIα-receptor polymorphism, see table below)

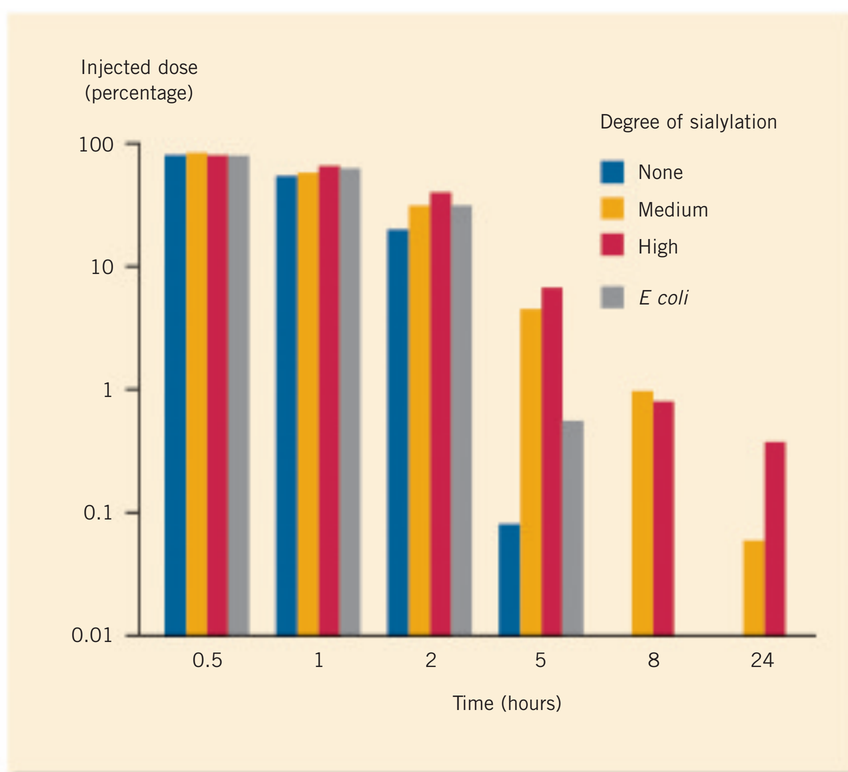
Distribution of FcγIIIα-receptor polymorphism (V/V, V/F, and F/F)

Receptor type	V/V	V/F	F/F
Europe (percentage)	8-27	48-60	33-49
Asia (percentage)	4-11	35-47	43-54
ADCC increase	10x	100x	200x

GLYCOOPTIMISATION OF NON-ANTIBODY GLYCOPROTEINS

It is not only antibodies that can be successfully glycooptimised, but also therapeutic proteins such as growth factors, glycoprotein hormones, cytokines, certain enzymes, blood factors and thrombolytica, which are all, by nature, glycosylated and good candidates for glycooptimisation. A growth factor was chosen for glycooptimisation which is marketed by two pharmaceutical companies that currently produce this protein in *E coli* and yeast, which either do not glycosylate at all or glycosylate in a manner lacking any sialylation. This growth factor was expressed in a cell line that allows production in various degrees of sialylation by metabolic engineering. Five variants with increasing degrees of sialylation were analysed in a

Figure 4: Improvement of the bioavailability (shown in percentage of injected dose) of a growth factor



mouse model to determine the bioavailability and in *in vitro* assays to determine the bioactivity, and then compared with one commercial product. The degree of sialylation clearly had a strong impact on the activity, which reached a high – but interestingly not the highest – sialylation degree (7). Comparison of increasing concentrations of the optimally sialylated product with the commercially available yeast product revealed that 0.01ng/ml of the glycooptimised growth factor were as active as 5ng/ml of the yeast product. Therefore, the glycooptimised product was about 500 times more active than the commercial product. The glycooptimised growth factor's level of maximal activity was found to be three times higher than those of the yeast products. When comparing the *in vivo* half-life, the optimised form with a high degree of sialylation was detectable for the longest time by far after injection in mice (see Figure 4, page 57). Interestingly, the unsialylated (but nevertheless galactosylated) glycoform exhibited a half-life even shorter than that of the *E coli* product, which is not at all glycosylated.

CHOOSING GLYCOENGINEERED CELL LINES FOR PRODUCTION OF THERAPEUTIC PROTEINS

CHO cells are the standard mammalian cells for production of biotherapeutics. Therefore novel mammalian cell lines need to at least to match CHO cells in productivity (1 to 4g/L in case of antibodies) and biotechnical characteristics that allow use in a production process. Human cells have already been described for the production of biotherapeutics. Crucell developed the retina-derived Per.C6 cell line which was immortalised using a recombinant adenoviral technology and a perfusion-based XD process with very high cell titres of more than 10^8 cells/ml and good antibody yields.

The glycoengineered cell lines of leukemic origin are, by nature, suspension cells (in contrast to Per.C6 and CHO cells) and are therefore favourable due to their excellent biotechnical characteristics, such as the absence of any viral particles, genetic stability and high shear force resistance. These cell lines produce around 40pg of antibody per cell per day under serum-free conditions, with cell doubling-times of 14 to 24 hours.

Instead of a standard-fed batch process used with CHO cells, leukemic cells are well suited for a perfusion process, which offers significant advantages in order to achieve high productivity with consistent final product quality. With perfusion processes, the product is continuously harvested by withdrawal of the cell culture medium and the addition of fresh medium. Consequently, the product is constantly removed from the process and is not subject to any metabolism that occurs at 37°C in the fed batch process for about 14 days. Consistently, the products of three independent GMP perfusion runs with a harvest of 3,000L each revealed identical glycosylation patterns. The established perfusion process is characterised by relatively high cell titres of 2 x 10^7 viable cells/ml and productivities of 2 to 5g/L bioreactor volume in processes lasting 20 days.

In 2009, the German and Italian regulatory authorities approved the use of the first leukemic cell line for production of biotherapeutics.

CONCLUSION

Glycosylation has increasingly shifted into the focus of drug development. Its influence on a protein's characteristics has not only been acknowledged by regulatory requirements; there is an increasing body of evidence indicating that it is highly critical to take into account the glycosylation of biotherapeutics and to aim for optimisation in order to achieve higher bioactivity, half-life and lower immunogenicity – properties that are essential for therapeutic application. With these improvements, glycooptimised biotherapeutics have a high likelihood of receiving new IP protection. In the near future, it is expected that a number of novel biotherapeutics will be developed whose therapeutic or economic benefit rests upon proper glycosylation.

About the authors

Hans Baumeister was trained in Biochemistry and Molecular Biology at the Universities of Berlin and Hamburg, 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 (such as the Roche Institute of Molecular Biology in Nutley, New Jersey). 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. Email: hans.baumeister@glycotope.com

Steffen Goletz, CEO, CSO and co-founder of Glycotope, studied Biology, Biochemistry and Molecular Biology at the Universities of Heidelberg, Kaiserslautern, Manchester 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), and the German Cancer Research Centre in Heidelberg. During his research, Steffen has focused on glycobiology, tumour immunology, antibody engineering and cellular engineering.

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