Beilstein-Institut

Glyco-Bioinformatics – *Bits 'n' Bytes of Sugars*
October 4th – 8th, 2009, Potsdam, Germany

GlycoOptimization for Fully Human and Largely Improved Biopharmaceutical Antibodies and Proteins

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Received: 28th May 2010 / Published: 10th December 2010

Abstract

The vast majority of therapeutic proteins are, by nature, glycosylated. In the last years the attached glycans attracts more and more attention since an increasing number of data are available showing that glycosylation greatly affects the biopharmaceutical characteristics of the product. This article focuses on the novel GlycoExpress technology that allows to screen for the optimal glycosylation of any protein and to produce the glycooptimized biopharmaceutical for clinical use. GlycoExpress is a toolbox of human, glyco-engineered cell lines to express any protein with fully human glycosylation and in a variety of differentially glycosylated isoforms. In a number of *in vitro* and *in vivo* assays the optimally glycosylated protein is identified and the corresponding cell line is further developed for GMP-production of that glycooptimized biopharmaceutical.

Twelve proteins have been successfully glycooptimized so far with four antibodies and one protein hormone in late preclinical/early clinical development. Glycooptimization improved these biologics up to several hundred folds in respect to activity, bioavailability, immunogenicity and/or patient coverage. And it is expected that in the near future a number of novel biotherapeutics will be developed whose therapeutic or economic benefit rests upon a fully human and optimized glycosylation.

Introduction

Therapeutic proteins, such as antibodies, growth factors, protein hormones, cytokines, therapeutic enzymes, thrombolytic and blood coagulation factors, are produced by biotechnological techniques in genetically modified organisms (GMO) and assure the pharmaceutical and biotech industry excellent growth rates since many years. The vast majority of these therapeutic proteins are by nature glycosylated meaning that a number of different monosaccharides are attached to the amino acid sequence of the protein to form mainly species specific N-linked and O-linked glycans which contribute about 40% to the protein mass of for example the well-known erythropoietin (EPO) with tremendous impact on the potency of a protein.

The potential to improve these biopharmaceuticals by targeting its glycosylation is large. Therapeutically important characteristics of a protein, *e.g.* its activity, serum half-life, antigenicity and immunogenicity, its stability, solubility and even the productivity in a heterologous cell line, can be affected by the presence and/or the nature of the attached glycans [1, 2]. There is a wide range of mechanisms how the attached glycans contribute to the characteristics of a biopharmaceutical. To give some examples, glycans are synthesized by a portfolio of often species specific enzymes which results in a species specific glycosylation of the recombinant protein. In consequence biopharmaceuticals glycosylated in a non-human way bear the risk of immunogenic reactions as observed in several cases and in suboptimal performance in humans (for more details see below). The complex nature of hundreds of enzymes and transporters provided to assemble the glycan in a sequence of enzymatic reactions taking place after translation of the protein in certain compartments of the cell (ER, Golgi) explains why the productivity of a protein can be affected by its glycosylation. Negative charges introduced by the terminal monosaccharide sialic acid but also neutral monosaccharides influence intra- and intermolecular interactions, with the consequence that a protein is more or less stabilized or soluble or the binding characteristics of a ligand to its receptor or an enzyme to its substrate are modified. Glycans with terminal monosaccharide galactose and mannose-rich glycans are recognized for example by specific receptors in the liver that mediate a high clearance rate from circulation in the body. Certain therapeutic antibodies are much more active when glycosylated optimally and even the number of patients that is successfully treated (patient coverage) with that antibody depends on the type of glycosylation attached to the antibody due to a genetic receptor polymorphism in the population (for more details see below).

Since 2001 Glycotope GmbH develops and establishes novel technologies and products in the field of glycomics to improve biopharmaceutical proteins and combines today all the expertise to develop glycosylated biotherapeutics from the gene and the GMP production of the drug to clinical development in phase I and II trials. One focus was always to provide novel cell lines for production of improved biopharmaceuticals that are of human origin and allow the optimization of glycosylation.

Development of the GlycoExpress Technology for Glycooptimization of Therapeutic Proteins

There are several approaches to modify and optimize glycosylation:

1. The cell culture and production conditions are controlled to achieve a more reproducible and homogenous glycosylation.
2. The glycosylation is modified after production *in vitro* enzymatically.
3. The protein is glyco-engineered on the genetic level to add or eliminate sites for attachment of glycans.
4. A novel cell line is glyco-engineered for production of biotherapeutics with a modified glycosylation profile.

To optimize the glycosylation of a given protein at Glycotope all these approaches, except *in vitro* enzymatic modifications which are less suitable for pharmaceutical production, are used and if necessary combined to achieve a successful glycooptimization. However, in this article we will focus on the novel toolbox of human glyco-engineered cell lines (GlycoExpress), the successful glycooptimization using the GlycoExpress toolbox and the production of glycooptimized biopharmaceutical proteins in GlycoExpress cell lines.

Fully Human Glycosylation

One important aspect of glycooptimization is to achieve a fully human glycosylation of biopharmaceuticals [3]. Currently, human biopharmaceuticals are produced predominantly in *E. coli*, yeast or cell lines derived from insects (SF9), mice (SP2/0) or hamster (CHO). While a protein produced in different systems of bacterial or mammalian origin has at least the same primary structure, all post-translational modifications (PTMs) of this protein, most importantly the glycosylation, differ from organism to organism and even from cell type to cell type within one organism [4]. The mouse- and hamster-derived cell lines (such as SP2/0, CHO or BHK) used in industry and academia for production of glycosylated therapeutics are able to confer a glycosylation that has some similarity to a human glycosylation. However, important components found with human cells are missing (*e.g.* the 2,6-linked sialylation and the bisecting *N*-acetylglucosamine (GlcNAc)) 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 (*e.g.* NeuGc [5]) or terminal galactose linked to another galactose in a way that is absent from human cells (Gallili-Epitope). The latter has been found to be the major reason for the clinically observed severe hypersensitivity reactions in 33% of 72 patients in southern states of the USA treated with Erbitux® [6].

For this reason, Glycotope based its GlycoExpress technology on human cell lines providing human biopharmaceuticals with human PTMs and especially a fully human glycosylation. However, as mentioned above the glycosylation profile varies from cell type to cell type within one organism. It's 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 improved therapeutic potency as compared to others. To screen for and produce proteins that are glycosylated in the optimal way, Glycotope glyco-engineered its human cell lines to achieve a set of cell lines with different glycosylation profiles.

Glyco-engineering of Cell lines to Achieve an Optimal Glycosylation

Since the glycosylation is a very complex process within the cell, the first step in glyco-engineering needs to be to understand and characterize the cell line specific glycosylation machinery. Therefore in a research program a large number of human cell lines was analysed by profiling them for the presence of important carbohydrate structures on the cell surface and for the presence of key enzymes of important glycosylation pathways on the mRNA and enzymatic level. Beyond this glycoprofiling it was important that a cell line chosen for glyco-engineering had excellent biotechnological features to allow the glyco-engineered cells to be used for production of the glycooptimized biotherapeutics.

For glyco-engineering of a chosen cell line several techniques are available:

1. Random and spontaneous or induced mutagenesis with phenotypic selection;
2. Genetic knock-out *via* site-specific recombination;
3. Stable transfection of glycosylation enzymes.

Figure 1 gives an example of a glyco-engineered cell line that express stably a novel glycan that was absent from the original cell line. In that case the relatively fast approach of induction of mutagenesis was successful to generate a small number of cells with the desired glycoprofile (see Figure 1, panel A) which could be selected, enriched and single-cell-cloned to establish the novel glyco-engineered cell line with the new phenotype (Figure 1, panel B). This glyco-engineering approach by induction of mutagenesis and phenotypic selection convinces by speed, good success rates and the lack of genetically modified organisms that are generated when the cells are genetically engineered as in approach 2 and 3. However, the phenotypic selection makes specific tools necessary that are not available for all glycostructures of interest. Therefore, the other two techniques are necessary add-ons in the repertoire of glyco-engineering techniques.

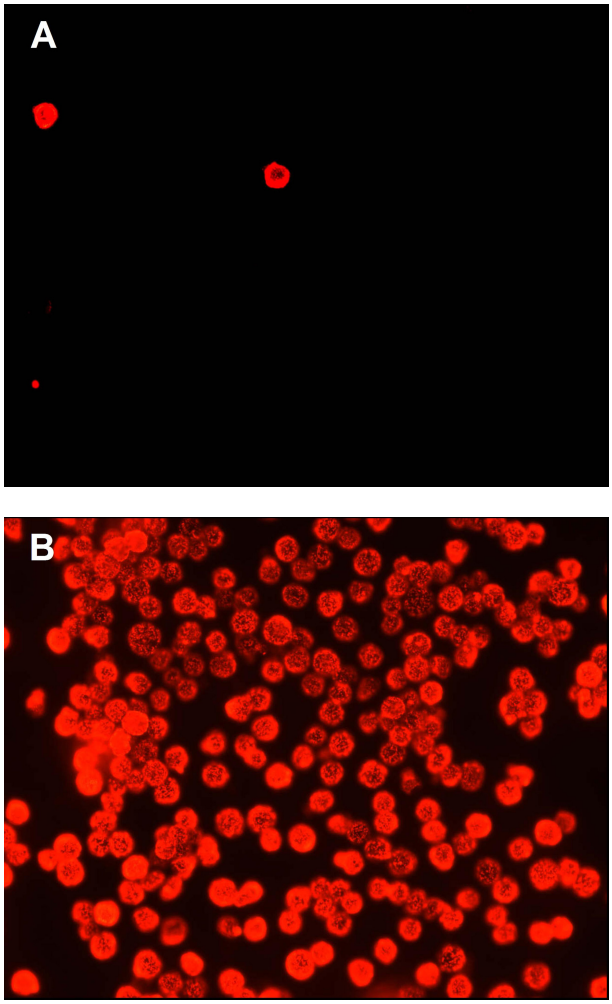


Figure 1. Cells stained for a desialylated key glycan analysed before (A) and after (B) glyco-engineering. In both panels the same cell number is shown.

A Toolbox of Glyco-engineered Human Cell Lines

The current set of cell lines that have been generated by glyco-engineering address the fucosylation, the α-2,3- and α-2,6-sialylation, the galactosylation, the antennarity and the presence of bisecting GlcNAc on glycoproteins. A given glycoprotein expressed in these cell lines can be produced *e. g.* in absence or presence of fucose, in absence or presence of 2,3- and 2,6-sialylation, at a very high degree of galactosylation (important for CDC activity of antibodies and to achieve high degrees of sialylation), in presence of bisecting GlcNAc and with antennarities from lower to higher complexity. To control the levels of sialylation, one

cell line has been engineered, that is characterized by the capability to sialylate glycoproteins at a very high degree, an important feature to elongate the circulating half-life of a therapeutic glycoprotein. In addition, two glyco-engineered cell lines allow controlling the degree of sialylation or fucosylation to levels in between 0% and the naturally possible maximum by means of medium supplementation (metabolic engineering) and in-process control.

Hence, a glycoprotein expressed in these glyco-engineered cell lines can be made available in various glycoforms and screening in suitable human bioassays allows the identification of the particular glycosylation pattern that confers optimal product characteristics to the product. The whole procedure of glycooptimization is depicted in Figure 2 which also outlines another highlight of GlycoExpress to directly use one of the chosen GlycoExpress cell lines for development of a high yield production cell line for the GMP production process of the glycooptimized protein. The reason for the integration of screening and production are the excellent biotechnological features of the GlycoExpress cell lines which will be described in more detail below. In the following, two of twelve biotherapeutic products that were successfully glycooptimized are described in more detail.

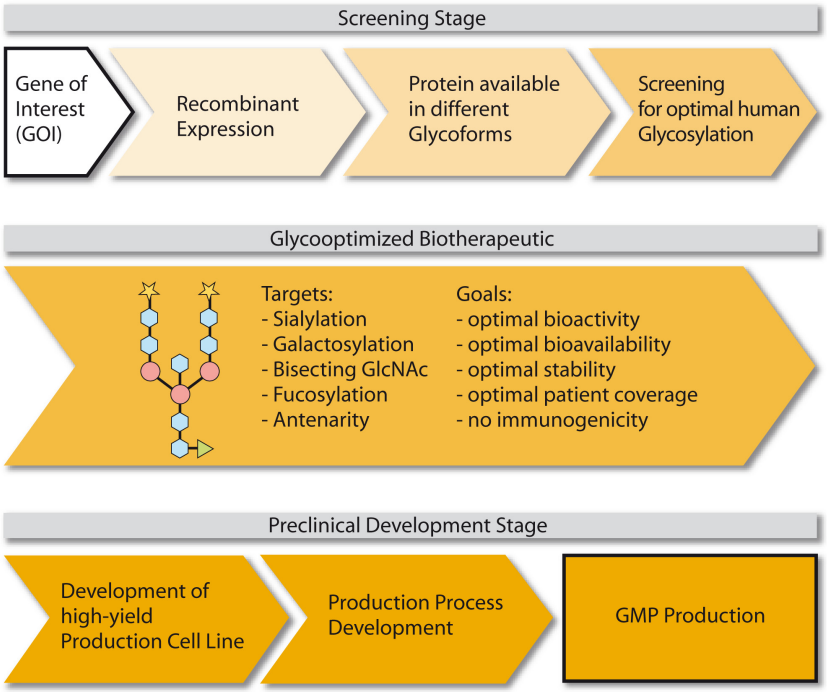
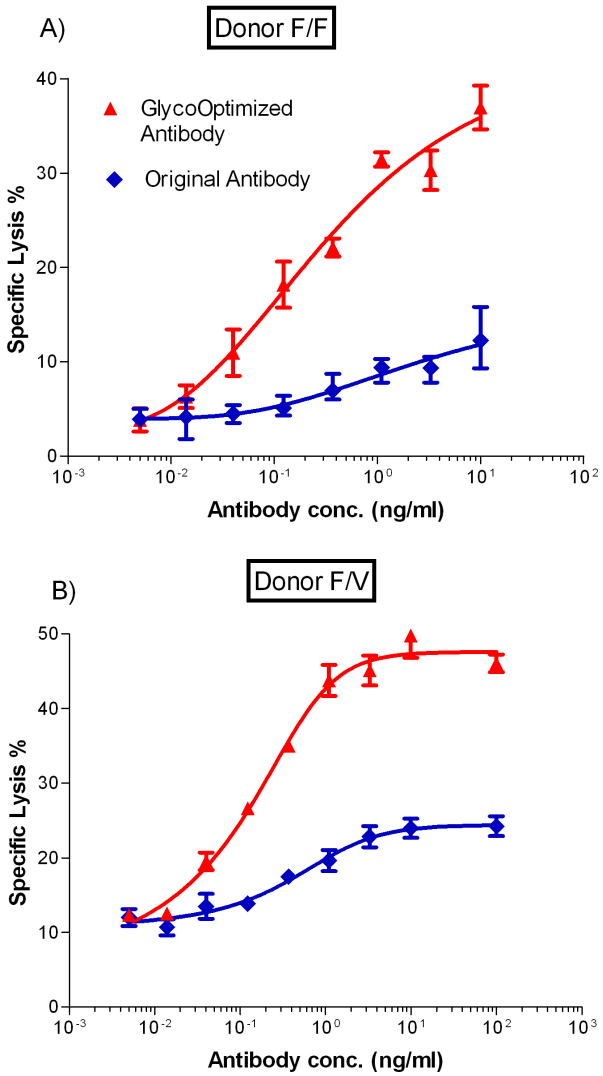


Figure 2. Schematic depiction of the integrated process of screening for glycooptimization and production of the glycooptimized product.

Glycooptimization of Antibodies

Antibodies are an important target for glycooptimization because of the remarkable economic success therapeutic antibodies have enjoyed in the last years and the observations that glycosylation can greatly affect the activity of an antibody *in vitro* and *in vivo*. Antibodies are glycoproteins with two N-linked glycans attached to the Fc part of IgG molecules plus additional glycans in the Fab protein in some of the antibodies.

Figure 3 A-C gives an example of an antibody that mediates tumour cell killing by activating natural killer (NK) cells and their antibody dependent cell cytotoxicity (ADCC) activity.



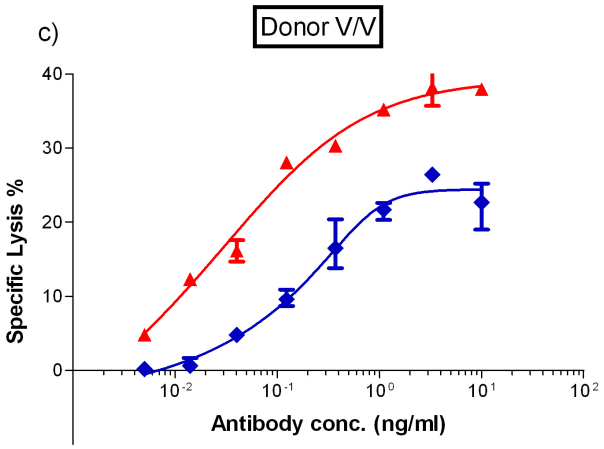


Figure 3. Glycooptimization of an antibody. The antibody-dependent cellular cytotoxicity (ADCC) is analysed *in vitro* for different concentrations of the original antibody (in blue) and the glycooptimized antibody (in red) using the cells of three donors of different FcγIIIa receptor phenotype F/F (A), F/V (B) and V/V (C).

By producing this antibody in the MabExpress toolbox of GlycoExpress cell lines, a fully human glycosylated and glycooptimized IgG1 antibody was selected with an up to 10fold to 250fold higher anti-tumour cytotoxicity (ADCC) compared to the original antibody produced in a rodent cell lines and depending on the donor type. Hence up to 250 fold less material is needed for achieving the same activity in human *in vitro* ADCC assays using human peripheral blood mononuclear cells (PBMC) as source for NK cells and human tumour cells as target. This is combined with an up to 4 fold increase in maximal tumour cell lysis activity, resulting in a potent ADCC anti tumour activity for all donor types with the three different FcγRIIIa ADCC receptor allotypes F/F, V/F and V/V (Fig. 3A-C). In consequence, the patient coverage is expected to be broadened by making the key anti-tumour activity (ADCC) available for all patients compared to about 20% when antibodies are produced in rodent systems (Table 1 and more details see below). In addition to the increase in potency, the half-life of the glycooptimized antibody was strongly elongated as shown in cynomolgous monkeys (data not shown). And the immunogenic carbohydrate moieties, such as the non-human Gallili-Epitope and non-human sialic acids present on the antibody from rodent production cells, which were reported to be the major reason for clinically severe hypersensitivity in up to 33% of the patients [6], was removed. These effects were obtained by combination of fucose removal and bisecting GlcNAc addition as well as a high sialylation and galactosylation degree and fully human glycosylation of the antibody product.

The ADCC increase by glycooptimization of the antibody is mainly caused by a much better binding and activation of the FcγRIIIa receptor by defucosylation of the antibody [7]. The FcγRIIIa receptor is located on NK cells and responsible for mediating the ADCC activity

against tumour cells. A polymorphism of this receptor in the population causes the high variability of ADCC activity deviation of up to 10 – 250fold observed with the glycooptimized antibody. Figure 3 shows representative ADCC data for a glycooptimized antibody with PBMC from the various FcγIIIa allotypes and in comparison to the same antibody produced in rodent cells (SP2/0). The F/F and F/V phenotypes respond to the glycooptimized antibody 200fold/100fold better than to the original antibody (Fig. 3A and B), while donor cells of the V/V phenotype are less responsive to improvements by glycooptimization (10fold) still resulting in a clear increase in anti-tumour activity (Fig. 3C). This large increase of bioactivity especially with cells of F/F and V/F donors is largely explained by the fact that the antibody expressed in rodent cells is hardly able to activate the receptor of these cells because the binding affinity to the F allotype of the FcγIIIa receptor is much lower for the non-glycooptimized antibody. The responsible glycooptimization for the ADCC improvement is mainly due to a removal of 1,6-fucosylation and/or the addition of bisecting GlcNAc, both achievable with the GlycoExpress system. The original antibody produced in rodents is most active with PBMC of the V/V phenotype which correlates with a much better clinical outcome for V/V tumour patients in comparison to F/F and V/F patients for several non-glycooptimized antibodies such as Trastuzumab (Herceptin®), Rituximab (Rituxan®) and Cetuximab (Erbitux™) [8 – 10]. Glycooptimization increases the anti-tumour efficacy and makes it available not only for V/V patients who represent only 4 – 25 percent of the patient population but to all patients including the F/F and F/V phenotypes (Table 1).

Table 1. The distribution of the three different FcγIIIa receptor allotypes V/V, V/F and FF in the European and Asian population and its response to a glycooptimized antibody.

Receptor Type	V/V	V/F	F/F
Europe (in %)	8 – 27	48 – 60	33 – 49
Asia (in %)	4 – 11	35 – 47	43 – 54
ADCC increase (fold)*	10 x	100 x	250 x

* Compare with Figure 3A-C

This effect of strongly increased ADCC activity was observed with a number of antibodies when expressed in the MabExpress cell line. One of the antibody was largely ADCC improved not by removal of fucose but by addition of bisecting GlcNAc in combination with high degree of human α-2,3- and α-2,6-sialylation and galactosylation, showing that in contrast to the literature removal of 1,6-fucosylation is not the only reason for improvement of ADCC.

It will be very interesting to see the potency of these glycooptimized antibodies in the clinic. Currently among four glycooptimized antibodies in late stage development the first glycooptimized antibody, GT-Mab2.5-GEX, a novel, highly potent and tumour specific antibody recognizing 90% – 100% of all patients in all major carcinoma indications and multiple myeloma being effective already at 0.5 mg/kg in mouse tumour models, is in clinical trails.

Two other antibodies, GT-Mab5.2-GEX and GT-Mab7.3-GEX are glycooptimized and highly improved antibodies of which the originals are already marketed block busters (biobetters or largely improved 2nd generation antibodies). The clinical test for GT-Mab5.2-GEX has been started in the middle of 2010 and for GT-Mab7.3-GEX is expected to start early in 2011.

Glycooptimization of Non-antibody Glycoproteins

Not only antibodies can be successfully glycooptimized, also therapeutic proteins such as growth factors, glycoprotein hormones, cytokines, certain enzymes, blood factors and thrombolytica are all by nature glycosylated and good candidates for glycooptimization.

For example, a marketed growth factor currently produced in *E. coli* and yeast with either no glycosylation at all or irrelevant glycosylation lacking human sialylation was glycooptimized with optimized and fully human carbohydrates by GlycoExpress. Using the SialoFlex cell line various glycoforms with different degrees of human α-2,3- and α-2,6-sialylation where generated using metabolic engineering. SialoFlex has a defect at the key enzyme of the enzymatic pathway responsible for synthesis of human sialic acids. Therefore in a serum free production environment SialoFlex cells are lacking CMP-sialic acid, the substrate for sialyltransferases, which disenables the cells from sialylation. This defect of an epimerase in the precursor synthesis pathway can be by-passed by addition of ManNAc to the cell culture medium in a concentration dependent manner which allows the adjustment of the sialylation degree by in process controls resulting in different sialylation glycoforms of the product. Five variants with increasing sialylation degree were analysed in a mouse model for determining the bioavailability and in *in vitro* assays for the bioactivity. As shown previously [9], the degree of sialylation clearly had a strong impact on the activity in the chosen experimental *in vitro* setting, with the highest activity at a high, but interestingly not the highest sialylation degree. The comparison of these activities with that of the commercial products revealed a manifold higher activity especially for the high but not highest sialylated isoform. When comparing the *in vivo* half-life, the optimized form with the high degree of sialylation was detectable by far for the longest time after injection in mice (Figure 4), which is in accordance to the theory that large amounts of sialic acids result in an elongated serum half life. Interestingly, the unsialylated (but nevertheless galactosylated) glycoform exhibited a half-life even shorter than that of the not-glycosylated *E. coli* product, which is assumed to be due to the large amount of free terminal galactoses.

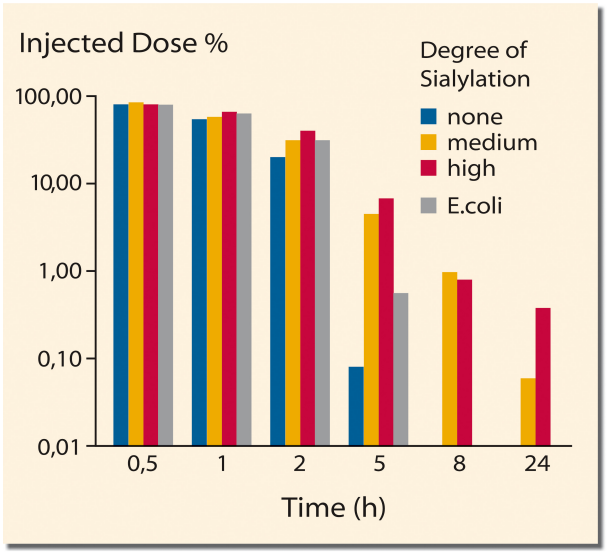


Figure 4. Improvement of the bioavailability of a glycooptimized growth factor in mice. Three glycosylation forms of one growth factor expressed in GlycoExpress (not-sialylated in blue, medium-sialylated in yellow and highly-sialylated in red) and the commercially available protein produced in *E. coli* (in grey) were injected at comparable concentrations into mice and the amount of circulating growth factor (shown in percentage of injected dose) was analysed at the indicated time after injection.

Glyco-engineered Cell Lines Chosen for Production of Therapeutic Proteins Need to Have Excellent Biotechnical Characteristics

Besides a fully human and glycooptimized glycosylation a high and competitive productivity in large scale fermentation processes are needed for pharmaceutical GMP production.

The glyco-engineered human cell lines are of leukemic origin and, by nature, suspension cells and resistant to high sheer forces. Prerequisite for biopharmaceutical production is a fully serum free system lacking any viral particles, which was proven for the various GlycoExpress cell lines by extensive RT-PCR tests, cellular reporter assays and by electron microscopy. Further advantages are the easy and rapid single cell cloning and the high transfection efficiencies as well as a very stable expression for more than 55 – 80 cell generations without selection pressure at a very rapid cell division (14 – 24 h) in high density cell cultures at outstanding cell viability rates. A proprietary gene amplification vector system and secretion signal peptides were developed for high-yield expression and an automated cell screening and cloning system was established. In consequence, GlycoExpress based production cell lines are very fast developed in 4 – 8 month with productivities of up

to 45 pg of antibody/cell/day under serum-free conditions. To meet the regulatory requirements as a production cell line for biopharmaceuticals it was of advantage that the cells are of non-fetal origin, that the transformation/immortalization was non-virally (in contrast to *e.g.* Per.C 6 cells) and that the transformation events are known. It was essential that no virus or virus particle, either human or bovine, could be detected (in contrast to CHO cells) and that the history of the cell lines was documented.

A high-yield, high cell density perfusion process was established allowing over 1 – 5 g/l volumetric productivity over 14 – 20 days thereby comparable to the best CHO fed batch systems, with the ability to prolong and expand the production to several weeks. The big advantage over CHO and other fed batch systems is the uniform, highly reproducible and scalable glycosylation pattern. This was shown up to now in many non-GMP and about 10 productions under GMP conditions with up to 6000 l production volumes.

In summary, the human GlycoExpress cell lines are highly competitive in speed and productivity to the other production systems such as the most broadly used and evolved mammalian production system based on CHO cells or human Per.C 6 cells and are even superior in a whole set of characteristics. Several regulatory authorities approved the use of biopharmaceutical products produced from the GlycoExpress system for use in clinical trails in humans.

Conclusion

Glycosylation has increasingly shifted into the focus of drug development due to its potency of largely improving biopharmaceuticals in respect to bioactivity, bioavailability, immunogenicity, stability, solubility and patient coverage as shown for varies molecule types by the GlycoExpress system. Molecule classes that can be largely improved are not only antibodies but also non-antibody molecules such as protein hormones, growth factors, blood factors, cytokines, interferons, co-stimulatory factors and other glycosylated molecules covering the whole spectrum of biopharmaceuticals for humans. Today, no other technology is known to be able to improve this broad spectrum of molecules and to such an extend as shown herein in a few examples. The technology matured now to a state where production of the glycooptimized products is highly competitive and the product quality is superior, with first products in the clinic. The glycooptimized biotherapeutics are and can be further protected by new IP. Therefore, it is expected that further glycooptimized products will enter the clinic and will be of benefit for the patients.

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