Novel Human Expression System Glycoengineered for Optimal Glycosylation of Biotherapeutics

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With the second and third generations of therapeutic proteins and the new generation of antibodies, large efforts are concentrating on improving their activity. Companies are working to increase therapeutic effects, reduce applied doses, lessen the costs of therapy, and further prevent undesired side effects. A very promising strategy is to optimize the glycosylation of such biotherapeutics.

The rationale behind that strategy is that the naturally occurring counterparts of most therapeutic proteins are glycosylated, but available production systems do not take this sufficiently into account. Unlike protein structure, which does not change when a protein is expressed in heterologous systems, glycan structures attached to proteins differ considerably among cell types used for production (**1, 2**). Bacterial cells do not glycosylate as mammalian cells do (**1, 2**). Yeast and insect cells have very restricted abilities to glycosylate (**1, 2**). Mammalian cells, such as Chinese hamster ovary (CHO) cells, have complex mammalian glycosylation machinery, with more than 200 enzymes and transporters available. Yet they still produce glycoproteins that differ significantly from human glycoproteins because of species-specific differences in glycosylation machinery (**1, 2**).

Why should glycosylation matter? The activities of some proteins (such as insulin) do not seem to be affected by glycosylation. However, other proteins need to be produced in mammalian cells or their activity drops dramatically; these include recombinant antibodies, certain growth factors, cytokines, and blood coagulation factors. In addition to that direct effect on protein activity, most glycosylated therapeutic proteins are characterized in vivo by a much better stability, serum half-life, antigenicity, and/or immunogenicity — also significantly improving their bioactivity (**3**). This means, for instance, that all glycans attached to the "natural" human counterpart are present on a therapeutic protein, species-specific differences in glycosylation are absent, and attached glycans are complete (e.g., either no or undersialylation or -galactosylation). Consequently, the correct and human glycosylation of therapeutic proteins is becoming increasingly important to pharmaceutical companies and regulatory authorities alike.

The quest for processing genuine human proteins is best addressed with human cell lines, but limited access to such cell lines makes only a few available for biopharmaceutical production. Human embryonic kidney (HEK) 294 cells, believed to be of neuronal origin, and HKB11 cells, generated by fusion of HEK293 with a human B-cell lymphoma cell line, are primarily used for transient expression of proteins (**4, 5**). The retinoblastoma-derived cell line PER.C6 (Crucell, www.crucell.com) is currently the most favored human cell line for production of viral vaccines and therapeutic antibodies (**6, 7**). In the future, new human cell lines generated by transformation of primary human amniocytes may prove useful for biotherapeutic production (**8**).

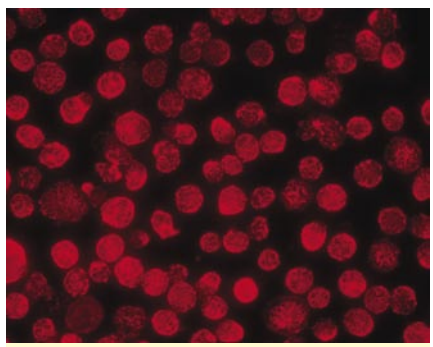


Photo 1: A human cell line glycoengineered for expression of a sialic acid free glycotope (after 20 month in culture)

Leukaemia-derived NM cell lines (GlycoExpress, developed by Glycotope GmbH, www.glycotope.com) enable production of biotherapeutics with a very high degree of sialylation and/or an optimized degree of sialyation. Negatively charged sialic acids are the most prevalent terminal monosaccharides on the surface of eukaryotic cells. It is generally observed that the more a glycoprotein is sialylated, the longer its serum half-life will be in circulation. Because therapeutic proteins from most production systems are sialylated at very low levels, various strategies have been tested to increase those levels, including in vitro enzymatic sialylation of purified products (**9**) and genetic modification of CHO cell lines to improve cellular expression of sialyltransferases (**10, 11**). GlycoExpress technology makes available a human cell line that needs no additional steps to produce highly sialylated proteins.

Beyond their effect on bioavailability, negatively charged sialic acids can influence

biotherapeutic interaction with in vivo targets and therefore affect bioactivity. This is shown in case of the follicle-stimulating hormone (FSH), which circulates in various forms and different degrees of sialylation. As expected, highly sialylated FSH has the longest half-life, but the most active FSH has a low degree of sialylation (**12**). The lack of an appropriate test system makes the situation less clear in other cases.

NM-F9 cells enable expression of glycoproteins with various degrees of sialylation — between almost 0% to 100% — just by adding a sugar metabolite to the cell culture medium (**13**). Because NM-F9 cells are characterized by excellent biotechnological features (Table 1), optimal culture conditions, once identified through a screening process, could be transferred to a production process relatively easily.

Table 1: Biotechnological characteristics of GlycoExpress*

Culture characteristics	GlycoExpress
Type of cell line	Myeloid cell line from CML patient without viral transformation
Genetic translocations	Ph+, BCR-ABL+
Genetic stability	High genetic stability
Outstanding glycoprofile	Very high degree of sialylation (NM-wt) Reconstitutable defect in sialylation (NM-F9)
Standard culture medium	10% FCS, 1% glutamine in RPMI 1640
Serum-free culture medium	AIM V, X-VIVO-20 or Panserin 401 Rapid and nonproblematic adaptation
Cell viability	>95%
Culture conditions	Robust and homogenous suspension culture
Maximal cell density	1–3 × 10^6 cells/mL
Year of first establishment	2001 (glycoengineered NM-F9; wild type before 1980)
Doubling time	20–24 h (15–18 h for wild type cells)
Production rate in lab scale	≥ CHO cells in lab scale
Transfection efficiency	40%–60% by electroporation
Single cell cloning efficiency	Up to 85% by limited dilution
Sensitivity to antibiotics (lowest concentration resulting in rapid cell death)	Hygromycin B (100 µg/mL) Neomycin (400 µg/mL), Puromycin (500 ng/mL), Zeocin (100 µg/mL)
Viral status analysed by:	
PCR	EBV-, HBV-, HCV-, HHV-8-, HIV-, HTLV-I/II-
Electron microscopy	Negative
Cell culture assay	No infectious particles detected with virus-sensitive human (MRC-5, Vero und HeLa) and bovine (BT) cell lines
Up-scaling system successfully tested	Spinner flasks High shear force resistance

*Published by (**13**) with updated information

Results and Discusion

Expressing Glycoproteins in Various Degrees of Sialylation: GlycoExpress technology is based on NM-F9 cells, which have been generated by glycoengineering (a technique described later in more detail) of NM-wt (wild-type) cells. Unlike NM-wt cells, NM-F9 cells have a certain genetic defect in the sialic acid biosynthesis pathway, resulting in their inability to sialylate (**14**). However, special medium supplementation can then restore that ability, enabling control over the degree of sialylation from almost 0% to 100%. This differential sialylation was demonstrated in a series of experiments using NM-F9 cells in serum-free or serum-containing medium.

Figure 1 shows how the degree of sialylation can be controlled by medium supplementation. Six batches of NM-F9 cells were cultured in the presence of 0, 10, 30, 50, 70, and 90 mM N-acetylmannosamine (ManNAc), an intermediate of the cellular sialic acid biosynthesis. After four days, cells were analyzed for binding of the lectin PNA (peanut agglutinin) to monitor the degree of cellular sialylation. (PNA binding is strongly inhibited by sialic acids, so the lower the signal, the higher the sialylation.) NM-F9 cells bind very efficiently to PNA, whereas the low amount of PNA bound by wild-type cells (NM-wt) reflects a high degree of sialylation (Figure 1). When the ManNAc concentration in the medium is increased from 10 mM to 90 mM, the capacity of NM-F9 cells to bind PNA decreases, and the sialylation degree of NM-F9 cells rises gradually to the level of wild-type cells.

This gradual control of sialylation was confirmed with antibodies that recognize sialic-acid–free carbohydrate epitopes (A78-G/A7, Nemod-TF1 and -TF2, and 63-C/A9), with lectins that bind α2-3 (MAA) or α2-6 (SNA) bound sialic acids, and by the thiobarbiturate acid method (see "Material and Methods").

In the next step we wondered whether it is possible to use NM-F9 cells and ManNAc to control the sialylation degree of recombinantly expressed proteins. Recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) was produced by NM-F9 cells in absence or presence of ManNAc and analyzed for its sialylation degree (Figure 2). Using a PNA Sandwich

Two Powerful Techniques

Glycoprofiling
Selects suitable cell lines for glycoengineering by identifying

- Irrelevant glycosylation processes during cell cloning
- Aberrant glycosylation processes during cell cultivation
- Missing glycosylation processes during cell expansion

Screens novel host cells for production of glycoproteins.

Glycoengineering
Generates host cell lines with stable phenotypes.

Table 2: Technical approaches to glycoengineer novel host cells

Technical Approach	Reference
Overexpression of a single gene	**10**
Random mutagenesis and selection of cells by phenotype	**13**
Genetic knock-out of a single gene encoding a key enzyme	**17**
Recombinant expression of antisense constructs to downregulate expression of a single gene	**18**

Figure 1: Differential sialylation of the cell surface by metabolic complementation with ManNAc

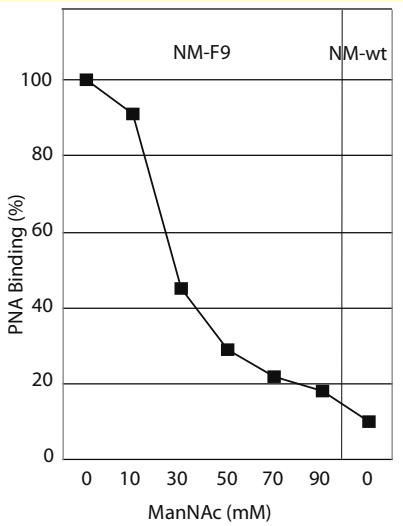
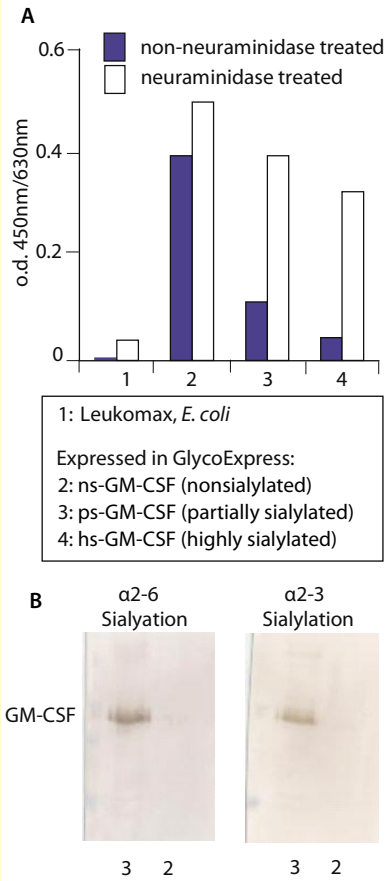


Figure 2: Differential sialylation of secreted GM-CSF expressed by NM-F9 cells cultivated with and without ManNAc; analyzed by a lectin ELISA (A) and a Western blot (B).



ELISA (Figure 2A), it is demonstrated that three distinct batches of GM-CSF could be produced: nonsialylated (ns)-, partially sialylated (ps)-, and highly sialylated (hs)-GM-CSF, which bind PNA at high, medium or a very low level, respectively. When ps- and hs-GM-CSF were desialylated by a neuraminidase treatment, PNA binding increased to a similar level, showing that binding of PNA is a tool to analyze the sialylation degree. In contrast, a very low increase in PNA binding was observed in case of ns-GM-CSF and the nonglycosylated Leukomax (Schering-Plough, www.schering-plough.com) when treated with neuraminidase. This is probably due to the neuraminidase treatment because the nonglycosylated Leukomax should have no PNA binding capability either before or after neuraminidase treatment.

Figure 2B demonstrates by Western blot analysis of ps-GM-CSF that the sialic acids are α2-6 and α2-3 linked to the glycans, whereas no sialylation could be detected in case of ns-GM-CSF. Mammalian cells, such as CHO cells, often lack α2-6 linked sialic acids and the corresponding sialyltransferase (**2**). Because of the importance of a complete sialylation of recombinant proteins, CHO cells have been genetically modified to conjugate sialic acids in the α2-6 position (**10, 11**). NM-F9 cells and NM-wt cells (results not shown) have a very high capacity to sialylate (in case of NM-F9 cells in the presence of ManNAc) including the α2-6 sialylation which renders unnecessary any genetic modification.

Therefore, the capacity to reconstitute sialylation through addition of suitable intermediates of the sialic acid precursor pathway enables a controlled and defined differential sialylation of membrane-bound glycoproteins and, importantly, of secretory glycoproteins.

Increasing the Bioavailability of a Glycoprotein: The influence of the degree of sialylation on the bioavailability of human GM-CSF was investigated in mice. The three sialylation forms of GM-CSF (as described above) and Leukomax were injected subcutaneously. The human GM-CSF concentration in blood was measured by ELISA between 0.5 and 25 hours postinjection (Figure 3).

The results demonstrate that bioavailability of hs- and ps-GM-CSF is about five times longer than that of the nonsialylated ns-GM-CSF and Leukomax. Both were detected only five hours postinjection at a similar level as hs- and ps-GM-CSF at 24 hours postinjection.

The highest and lowest bioavailability seems to correspond to the highest and lowest degree of sialylation (hs- and ns-GM-CSF, respectively), whereby ns-GM-CSF seems to have an even shorter circulation time than that of Leukomax. Indeed, for many glycoproteins, it is known that the higher the degree of sialylation, the longer is a protein's serum half-life.

One way of eliminating nonsialyated glycoproteins is by binding to the asialoglycoprotein receptor on liver cells followed by clearance of the glycoproteins (**15**). This could be the reason for the somewhat more rapid clearance of ns-GM-CSF from the bloodstream compared with that of Leukomax, which is not glycosylated and therefore does not expose desialylated galactose residues, the ligands of the asialoglycoprotein receptor. Considering the production of glycosylated biotherapeutics, it is possible to elongate significantly the bioavailability of a glycosylated therapeutic by increasing the sialylation degree that can be achieved by expression in NM-F9 cells or for higher sialylation in NM-wt cells.

Alternatively, polyethylene glycol (PEG) conjugation has been used to prolong the circulating half lives of therapeutic proteins. Attachment of PEG increases protein size and thus reduces its clearance rate. On the other hand, coupling of PEG can often decrease protein functionality and may increase immunogenicity.

In the case of GM-CSF, PEGylation is problematic in moderate loss of protein bioactivity and increased serum half lives (**16**), with potentially higher immunogenicity.

Glycoprofiling and Glycoengineering: The sialylation-defective NM-F9 cell line has been developed by glycoengineering NM-wt cells. Glycoengineering represents a technology to modify cells or cell lines to achieve novel stable cellular phenotypes with respect to specific glycostructures or glycosylation patterns. Table 2 outlines different approaches.

NM-F9 cells were glycoengineered by inducing random mutations in NM-wt cells using ethyl methanesulfonate (EMS) at a concentration of 0.5 mg/mL cell culture medium and by selecting sialylation-defective cells using Nemod-TF2 antibody immobilized on magnetic beads (Miltenyi Biotec, www.miltenyibiotec.com). Nemod-TF2 antibodies specifically recognize the O-glycosidic-bound disaccharide Thomsen-Friedenreich (TF), which is usually covered by sialylation. The sialylated TF is not bound by Nemod-TF2 (**19**).

After three rounds of single cell cloning and selection with Nemod-TF2, a cell clone was engineered with a very homogenous phenotype expressing the sialylation-free TF antigen (Photo 1). This approach turned out to be fast, and the novel phenotype is stable without the need to apply antibiotic pressure (by now for more than 20 months in culture). The novel cell line is not considered a genetically modified organism (GMO), which is important for regulatory issues.

The first step in glycoengineering is glycoprofiling to better understand the cellular glycosylation machinery and glycostructures on a cellular surface. Glycoprofiling comprises a set of techniques (Figure 4) to detect glycosylation-relevant mRNA transcripts by real time PCR or array analysis. Messenger RNA (mRNA) expression is validated by analyzing the presence of corresponding proteins by FACS (fluorescence-activated cell sorting), immunocytochemistry, or Western blot; by analyzing cellular extracts for glycosyltransferase activities; and/or by proving the presence of certain carbohydrates linked to the cell or specific product. All techniques are standard molecular biological and/or biochemical methods and are performed as described elsewhere (**14, 19**).

In glycoengineering NM-F9 cells, the glycoprofile of a set of human cell lines was analyzed to identify a cell line with the appropriate prerequisites to engineer the NM-F9 phenotype. NM-wt cells were chosen because of their very high sialylation capacity, the presence of high amounts of sialylated TF antigen, and the lack of core-2 transferases that could elongate the TF antigen in absence of sialylation (**20**). The high sialylation capacity was determined on the cell surface by flow cytometry using a range of lectins and antibodies (see above) and at the level of sialyltransferase enzyme activity and mRNA expression. The latter revealed a broad spectrum of sialyltransferases, enabling the cell to sialylate many different glycostructures efficiently.

In addition to selecting a suitable cell line for glycoengineering, glycoprofiling would be useful for identifying irrelevant, aberrant, or missing glycosylation processes during cell cloning, cultivation, or expansion (see the "Two Powerful Techniques" box).

Figure 3: Bioavailability of sialylated GM-CSF is extended.

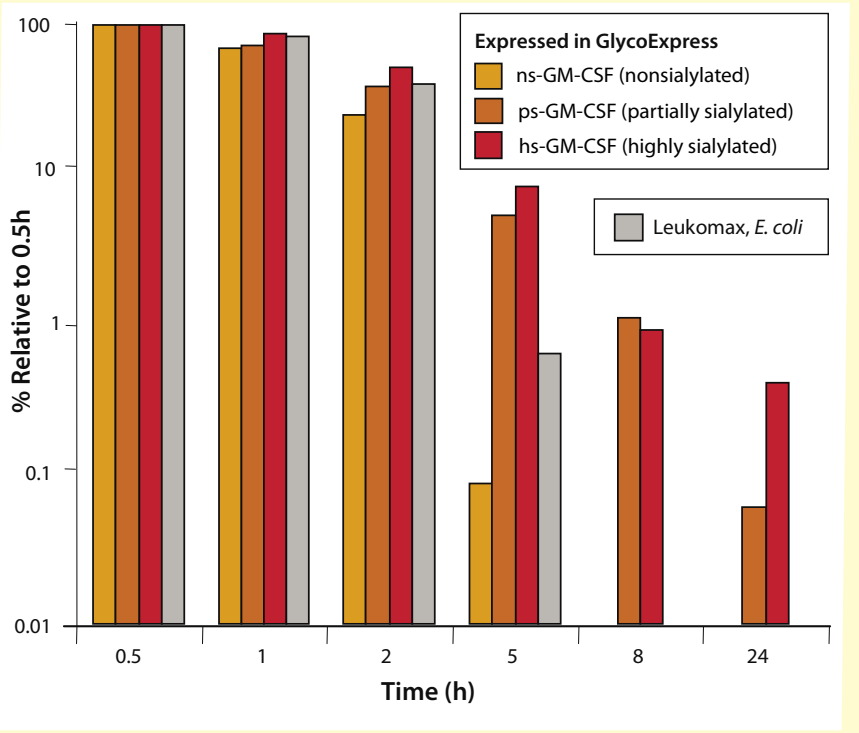
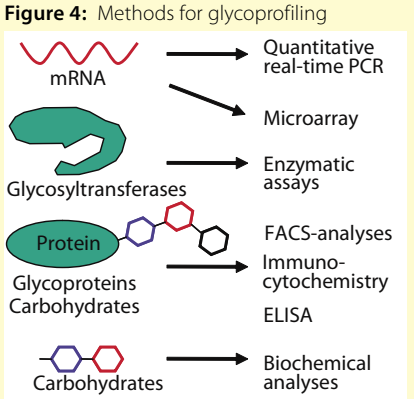


Figure 4: Methods for glycoprofiling



Materials and Methods

Flow Cytometry with Lectins and Antibodies: 1–3 × 10^5 cells were stained with 20 µg/mL FITC-labeled lectins (such as SNA or MAA from Vector Laboratories, www.vectorlabs.com) or Vector's biotinylated lectin PNA, which is detected by FITC-conjugated streptavidin; or with antibodies (A78-G/A7, Nemod-TF1, Nemod-TF2, A63-C/A9, all available from Glycotope). Those were used as hybridoma supernatants in a dilution of 1:2. Their binding was detected by a secondary Cy3-conjugated, antimouse-IgM antibody (Dianova, www.dianova.de) at 1:200 dilution. All measurements were performed with the Coulter-Epics flow cytometer and analyzed by using the Expo 32

ADC-software (both Beckman-Coulter, www.beckmancoulter.com).

Sandwich ELISA to Analyze the Sialylation Degree of GM-CSF: NM-F9 cells that do not express detectable levels of human GM-CSF were stably transfected with a human GM-CSF expression vector (Invivogen, www.invivogen.com). Recombinant human GM-CSF, which was secreted into the cell culture medium of NM-F9 cells, was quantified by a sandwich ELISA available at Becton-Dickenson (BD, www.bdeurope.com). To analyze the sialylation degree of GM-CSF, the protein was detected with the biotinylated lectin PNA (2 µg/mL) and POD (horseradish peroxidase)-conjugated streptavidin. Alternatively, the sialylation degree was analyzed by the thiobarbituric acid method (**21**).

Preparation of ns-, ps- and hs-GM-CSF: GM-CSF expressed in NM-F9 cells was produced in three degrees of sialylation: non-, partially, and highly sialylated GM-CSF (ns-, ps-, and hs-GM-CSF, respectively). All three forms were purified by affinity chromatography using rat antihuman GM-CSF antibody. The sialylation degree was analyzed by the sandwich ELISA described above. For comparison, Leukomax (Schering-Plough, www.schering-plough.com) was used, which is produced in *E. coli* with no glycosylation or sialylation. Desialylation of GM-CSF was achieved by incubation of GM-CSF with 0.1 units/mL of the neuraminidase isolated from Vibro Cholerae (Behring Dade, www.dadebehring.com) for one hour under standard conditions described in the package insert.

Western blot Analysis: GM-CSF was separated in a 15% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The presence of GM-CSF on that blot was shown by staining with the rat anti-human GM-CSF antibody and a secondary anti rat peroxidase-coupled antibody. The α2-3 and α2-6 linked sialic acids were detected by binding of the biotinylated lectins *Sambucus nigra* agglutinin (SNA) and *Maachia amurensis* lectin II (MAL II, both from Vector, www.vectorlabs.com) and peroxidase-coupled streptavidin (Vector).

Analysis of the Bioavailability in mice: Groups of three nu/nu mice received 2 µg/mouse of ns-, ps-, and hs-GM-CSF, respectively, as well as Leukomax by subcutaneous injection. At hours 0.5, 1, 2, 5, 8, and 24 postinjection, blood samples were taken, and the concentration of the human GM-CSF was measured by ELISA as described above.

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