MINI-REVIEW

Increasing the Sialylation of Therapeutic Glycoproteins: The Potential of the Sialic Acid Biosynthetic Pathway

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Received 29 August 2008; accepted 10 December 2008

Published online 6 February 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.21684

ABSTRACT: The number of therapeutic proteins has increased dramatically over the past years and most of the therapeutic proteins in the market today are glycoproteins. Usually, recombinant glycoproteins are produced in mammalian cell lines, such as Chinese-hamster-ovary-cells to obtain mammalian-type of glycosylation. The terminal monosaccharide of N-linked complex glycans is typically occupied by sialic acid. Presence of this sialic acid affects absorption, serum half-life, and clearance from the serum, as well as the physical, chemical and immunogenic properties of the respective glycoprotein. From a manufacturing perspective, the degree of sialylation is crucial since sialylation varies the function of the product. In addition, insufficient or inconsistent sialylation is also a major problem for the process consistency. Sialylation of over-expressed glycoproteins in all mammalian cell lines commonly used in biotechnology for the production of therapeutic glycoproteins is incomplete and there is a need for strategies leading to homogenous, naturally sialylated glycoproteins. This review will shortly summarize the biosynthesis of sialic acids and describe some recent strategies to increase or modify sialylation of specific therapeutic glycoproteins. J Pharm Sci 98:3499–3508, 2009

Keywords: sialic acid; recombinant glycoproteins; therapeutic proteins; protein stability

RECOMBINANT THERAPEUTIC PROTEINS AND GLYCOSYLATION

Major demographic shifts in the industrial nations will prompt numerous changes in social and health systems. The need for novel drugs fighting serious diseases such as cancer or disorders of the central nervous system will grow rapidly. Two very different strategies are used for the development for those novel drugs: (I) High-throughput screens of small molecules libraries to identify new drug candidates[1,2] and (II) Development of specific therapeutic proteins using recombinant DNA technologies.[3,4] Both strategies have advantages and disadvantages. Here we will focus on therapeutic glycoproteins. Due to their outstanding specificity, therapeutic glycoproteins will revolutionize our possibilities to treat serious diseases. However, there are several restrictions and problems during the production process, purification and application. In nature, nearly all proteins outside the cell are glycoproteins and glycosylation represents the most common posttranslational modification of

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Journal of Pharmaceutical Sciences, Vol. 98, 3499–3508 (2009)

proteins.[5–7] Glycosylation has dramatic impact on the function, stability, solubility and immunogenicity of recombinant glycoproteins.[8,9] This explains the general interest for novel strategies to engineer glycosylation and to increase the value of therapeutic proteins. However, glycosylation is not directly encoded by the genome and by far not understood.[10] Glycans of glycoproteins are synthesized in the Golgi apparatus by specific glycosyltransferases, which attach nucleotide-activated monosaccharides to specific sugar residues of glycoproteins.[11] Although these glycans have a common core structure (Fig. 1A) the combination of different monosaccharides and different linkage-type allows the cell to create an enormous number of different structures. The terminal and most exposed monosaccharide of most glycoproteins is sialic acid,[12] which will be in the focus of this review (Fig. 1B).

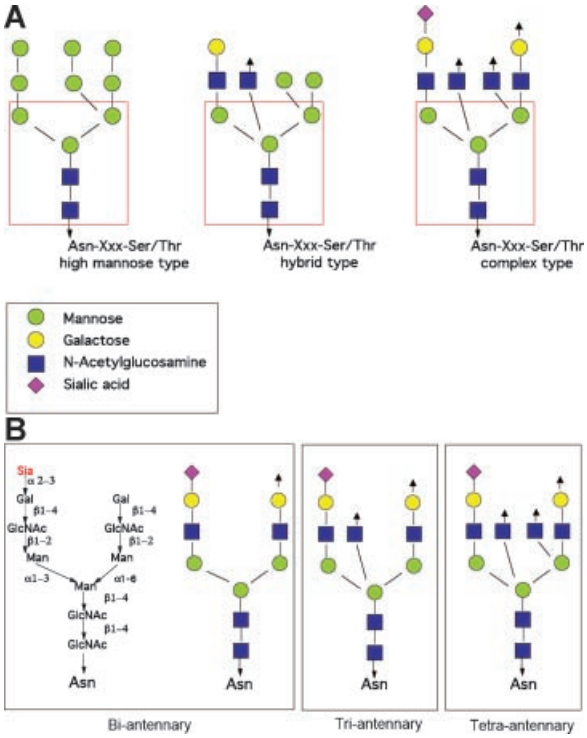
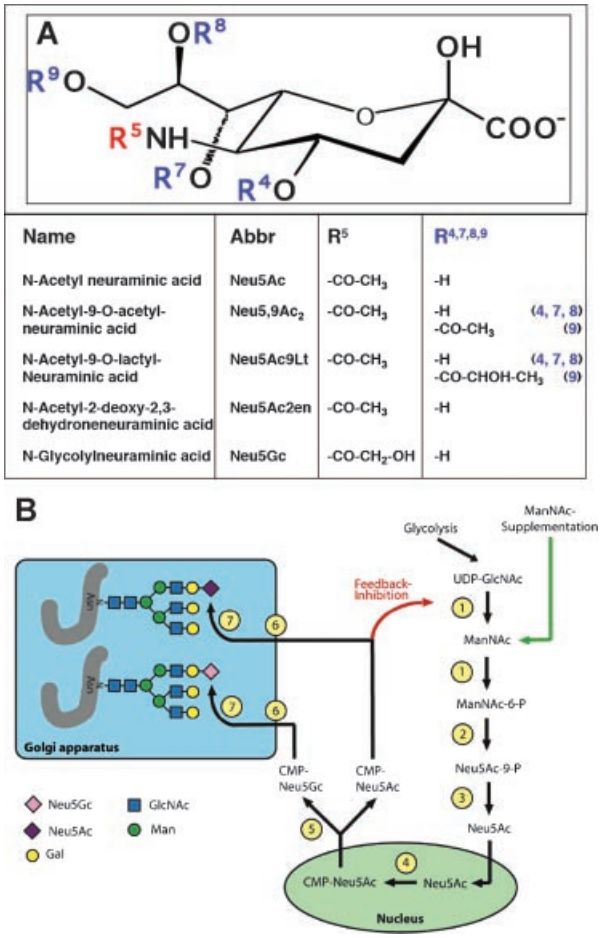


Figure 1. Diversity of glycosylation. (A) Schematic structure of three major types of *N*-glycans. The arrows indicate further diverse glycosylation. A red box indicates the common core structure. (B) Branching of typical *N*-glycans. The arrows indicate further diverse glycosylation. The potential linkages are shown in the left bi-antennary structure.

SIALYLATION OF (THERAPEUTIC) GLYCOPROTEINS

Sialic acids represent a family of aminosugars with 9-carbons with over 50 members derived from *N*-acetylneuraminic acid[12,13] (Fig. 2A). Most mammals express *N*-glycolylneuraminic acid, the hydroxylated form of N-acetylneuraminic acid at position C5. However, humans express predominantly *N*-acetylneuraminic acid, due to a



Name	Abbr	R^5	$R^{4,7,8,9}$
N-Acetyl neuraminic acid	Neu5Ac	$-CO-CH_3$	-H
N-Acetyl-9-O-acetyl-neuraminic acid	$Neu5,9Ac_2$	$-CO-CH_3$	-H (4, 7, 8) $-CO-CH_3$ (9)
N-Acetyl-9-O-lactyl-Neuraminic acid	Neu5Ac9Lt	$-CO-CH_3$	-H (4, 7, 8) $-CO-CHOH-CH_3$ (9)
N-Acetyl-2-deoxy-2,3-dehydroneuraminic acid	Neu5Ac2en	$-CO-CH_3$	-H
N-Glycolylneuraminic acid	Neu5Gc	$-CO-CH_2-OH$	-H

Figure 2. (A) Structure of sialic acids. (B) Biosynthesis pathway of sialic acid and feedback inhibition of the GNE. Enzymes: (1) GNE = UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase, (2) *N*-acetyl neuraminic acid-9-phosphate synthase, (3) *N*-acetyl neuraminic acid-9-phosphatase, (4) CMP-*N*-acetyl neuraminic acid synthase, (5) CMP-*N*-acetyl neuraminic acid hydroxylase, (6) CMP-*N*-acetyl neuraminic acid Golgi transporter, (7) Several specific sialic acid transferases. Figure modified and printed with permission from Bork et al.[49]

homozygous mutation in the CMP-neuraminic acid hydroxylase gene in the human genome. *N*-glycolylneuraminic acid is antigenic to humans,[14,15] is enriched in tumor cells and is originated most probably from the diet.[16] This is an important issue since this is one of the reasons why the nonhuman *N*-glycolylneuraminic acid has to be avoided in any production process of recombinant therapeutic glycoproteins. This problem has been overcome recently by using antisense strategies to reduce the activity of the CMP-neuraminic acid hydroxylase in CHO-cells.[17] The respective sialic acids possess different highly specific recognition and binding properties for a variety of cellular receptors.[18] This structural and functional diversity of sialic acid is exploited by viruses, bacteria and toxins, and by the sialoglycoproteins and sialoglycolipids involved in cell-cell, cell-matrix or molecular recognition.[19]

Sialic acid is only one component out of several monosaccharides building glycans of glycoproteins, but has an outstanding impact on the quality and stability of any therapeutic glycoproteins for several reasons: (I) terminal galactose residues are one of the major factors determining the serum half-life of glycoproteins. The serum half-life is regulated by the expression of liver asialo-glycoprotein receptors. These receptors bind nonsialylated glycoproteins (on free galactose residues, see Fig. 1) and bound asialoglycoproteins are removed from the serum by endocytosis.[20] As a consequence, expression of terminal sialic acid on galactose residues prevents serum glycoproteins from degradation.[21] (II) Sialic acids are important for masking antigenic determinants or epitopes.[12] It is known that the receptors of the immune system (T- and B-cell receptors) often prefer nonsialylated structures. Therefore, the possibility of the generation of antibodies (neutralizing antibodies) against the therapeutic glycoproteins[22] correlates with the degree of its sialylation. (III) Negatively charged sialic acids influence protein-specific parameters such as the thermal stability,[23] the resistance to proteolytic degradation[24] or its solubility.[25]

For these reasons, sialic acids are crucial for the production process and especially for the approval of therapeutic glycoproteins. Manufactures have to ensure the homogeneity of each batch of therapeutic glycoproteins. This includes the degree of sialylation. Therefore, great efforts have been done to standardize sialylation during the fermentation process since in many cases only one third of the primary glycoprotein after the fermentation fulfils the standard of homogenous gylcosylation/sialylation.

In the following, we will introduce the biosynthesis of sialic acid and consequently present some aspects and possibilities to improve and/or engineer higher levels of sialylation.

BIOSYNTHESIS AND ACTIVATION OF SIALIC ACID

The initial reaction in the pathway to form free sialic acid is a conversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) to *N*-acetyl D-mannosamine (ManNAc) since the physiological precursor of all sialic acids is ManNAc (Fig. 2B). ManNAc is formed from UDP-*N*-acetylglucosamine (UDP-GlcNAc) by epimerization of the hydroxyl-group in position 2 and cleavage of UDP by the UDP-*N*-acetylglucosamine 2-epimerase.[26] Cardini and Leloir originally discovered this enzyme in rat liver.[27] All ManNAc produced by the UDP-*N*-acetylglucosamine 2-epimerase is metabolized to sialic acid. The biosynthesis of sialic acid is regulated by the feedback inhibition of the key enzyme of sialic acid biosynthesis, the UDP-*N*-acetylglucosamine 2-epimerase/ManNAc kinase (GNE). GNE is a bifunctional enzyme, which catalyzes the conversion of UDP-GlcNAc to ManNAc and the phosphorylation of ManNAc to ManNAc-6-phosphate[26,28] (see Fig. 2B). The next step is a condensation of ManNAc-6-P and pyruvat resulting in sialic acid-9-phosphate by the *N*-acetyl-D-neuraminyl-9-phosphate synthase.[29,30] The formation of *N*-acetyl neuraminic acid is completed by specific phosphatase acting on sialic acid-9-phosphate.[31] Then, the resulting primary sialic acid (*N*-acetyl neuraminic acid) is either modified to the other members of the sialic acid family or is activated in the nucleus to CMP-sialic acid.[32,33] It is very important to mention, that CMP-sialic acid inhibits the epimerase activity of GNE in a feedback dependent manner.[34] CMP-sialic acids are than transported to the Golgi apparatus by a specific CMP-sialic acid transporter[35] and attached to free galactose residues by specific sialyltransferases.[36] Since not only mammalian, but also plant cells, which are not able to generate mammalian type of glycosylation are used for the production of glycoproteins, these cells have to be engineered with the proper glycosylation machinery.[37] However, plant cell

glycosylation and sialylation is not the focus of this review.

INCREASING SIALYLATION OF RECOMBINANT GLYCOPROTEINS

Several approaches have been made to enhance sialylation of glycoproteins and to maximize the yield of high quality glycoproteins for therapeutic use in mammalian cell lines.

Application of ManNAc

As described above, *N*-acetylmannosamine (ManNAc) is the specific precursor for increasing intracellular sialic acid pools.[38] To bypass the GNE-feedback mechanism (the rate-limiting step in the biosynthesis of sialic acids), cells can be supplemented with ManNAc, which intercept the pathway beyond the feedback mechanism of GNE (see Fig. 2B), since only the epimerase activity of the GNE is feedback-controlled. Any cellular ManNAc is phosphorylated and subsequently converted to sialic acid. Supplementation with ManNAc therefore leads to an increased intracellular concentration of sialic acids. However, aminosugars such as ManNAc does not easily pass the cell membrane[39] and there is no detailed cellular uptake mechanism known (most probably, ManNAc is taken up during endo- or pinocytosis). This is one reason, why very high concentrations (mM range) have to be used to increase intracellular ManNAc levels and sialylation.[40] Recent studies revealed that application of peracetylated ManNAc, which can cross the plasma membrane, helps to reduce the concentration needed to increase sialylation.[41]

It was reported that ManNAc feeding to Chinese-hamster-ovary-cells (CHO-cells), producing recombinant human interferon-gamma (CHO IFN-γ) increased intracellular sialic acid concentration.[42] Interferons (IFNs) are proteins produced by a wide variety of cells of the immune system of many vertebrates in response to challenges by foreign pathogens such as viruses, parasites or tumor cells.[43] IFNs belong to the large class of glycoproteins known as cytokines. They assist the immune system to response by (I) inhibiting viral replication within the host cells, (II) Activating natural killer cells and macrophages, (III) Increasing antigen presentation to lymphocytes, and (IV) Inducing the resistance of host cells to viral infection. Interferon-γ exists in three sialylated glycoforms: double-glycosylated (at Asn25 and Asn97), single glycosylated (at Asn25) or nonglycosylated.[44] Without further efforts, 25% of the total IFN-γ produced in CHO-cells is nonsialylated after 140 h,[45] which is a nontolerated reproducibly for a production process. Application of ManNAc led to a 15% increasing in the sialylation of IFN-γ and saturation was reached with the addition of 40 mM ManNAc.[42]

This approach was also successfully adopted to increase the sialylation of erythropoietin (EPO). EPO is a glycoprotein hormone that is a cytokine for erythrocyte precursors cells.[46] Of all clinical approved recombinant growth factors, EPO has the broadest indication spectrum and economic potential. EPO is available as a therapeutic agent produced by recombinant DNA technology in mammalian CHO-cells and is used in treating anemia resulting from chronic kidney disease, from the treatment of cancer (chemotherapy and radiation), and from other critical illnesses (heart failure or chronic infections). EPO contains three complex type N-glycans located on Asn residues at position 24, 38, and 83, and an O-glycosylation site at position 126 and has a maximum of 14 sialic acids per molecule.[47] These terminal sialic acids determine the biological activity of EPO.[47] Less sialylated or nonsialylated EPO has dramatically decreased *in vivo* activity compared to sialylated EPO. Interestingly, mutated and nonsialylated EPO has strong *in vitro* activity, indicating the importance to the asialo glycoprotein receptor system.[48] Application of 10 mM ManNAc to EPO-producing CHO-cells led to increased and more homogenous sialylation as demonstrated by two-dimensional gel electrophoresis.[49]

However, although ManNAc supplementation does increase the intracellular sialic acid pool dramatically,[49] ManNAc-application does not automatically lead to an increase of sialylation of the product. This was shown for NS0 cells producing a recombinant humanized IgG1[50] and to CHO- or NS0-cells producing TIMP-I.[51] In addition, there exist first reports in the literature that ManNAc application influences cell proliferation and differentiation.[52] Finally, from a manufacturer point of view, ManNAc is an expensive supplement, which has to be used in very high concentration during the fermentation process. Furthermore, the distribution of ManNAc and the process lead-through is difficult to control. Therefore, ManNAc application is not first choice to

increase intracellular sialic acid concentrations in a large-scale production process.

Overexpression of Enzymes Involved in the Biosynthesis of Glycans

CHO-cells are widely employed to produce glycosylated recombinant glycoproteins. However, CHO-cells do not express alpha-2,6-sialyltransferase[53] and therefore cannot produce glycoproteins similar to human glycoproteins that are characterized by both α2,6- and α2,3-linked terminal sialic acid residues.[54] Several approaches have been performed to enhance the sialylation machinery of CHO-cells. Coexpression of 2,3-sialyltransferase together with CMP-sialic acid synthase did not further increase sialylation, although a further increase in the intracellular pool of CMP-sialic acid was measured. As explanation, it was postulated that the transport capacity of CMP-sialic acid into the Golgi lumen was limited, thereby causing the reduced availability of CMP-sialic acid substrate for sialylation[55] and be responsible for the lack of (further) increase in sialylation of the recombinant protein. As CMP-sialic acid transport affects the availability of CMP-sialic acid substrate in the Golgi, in addition the CMP-sialic acid transporter was over-expressed. The result was increased sialylation of EPO.[55] Similar results were obtained in CHO-cells producing recombinant human interferon gamma (CHO IFN-γ).[56] Using this approach it could be demonstrated that over-expression of the CMP-sialic acid transporter represents a novel and powerful approach to improve sialylation during recombinant glycoprotein production.[55,56] As described before, terminal sialylation of human proteins is characteristically in alpha-2,3 and alpha-2,6 linkage to galactose. Therefore, CHO-cells were stably transfected with human alpha-2,6-sialyltransferase cDNA to increase the sialylation of recombinant glycoproteins.[57,58] Using this approach it could be demonstrated that sialylation and clearance of IFN-γ was improved compared to untransfected CHO-cells.[57] However, in one study analyzing the EPO-bioactivity after engineering CHO-cells to express alpha-2,6-sialyltransferase, no significant differences could be detected.[58]

Over-Expression of GNE

The intention of this approach is trying to enhance the intracellular precursor pools for the biosynthesis of the sialic aids, ManNAc, and thereby increasing sialylation of glycoproteins in the respective eukaryotic cell by the endogenous cellular metabolism. In first line experiments, GNE was over-expressed and it was possible to increase GNE activity in *in vitro* assays.[59] But unfortunately the pool of intracellular sialic aids was not increased.[60] This could be explained by the feedback inhibition of the GNE by activated CMP-sialic acid. To avoid or bypass the feedback inhibition of the epimerase activity of the GNE, a human sialic acid biosynthesis disease could be one way out. Sialuria is a rare human inborn error of the sialic acid metabolism.[34] A defect of the feedback inhibition of the GNE is caused by point mutations within the epimerase domain of GNE (263L, 266Q, 266W) leads to excessive synthesis of sialic acid[34] (see Fig. 1). In several experiments it could be shown that transfection of CHO-cells with sialuria-mutated GNE increases the intracellular pool a sialic acids and consequently increases the sialylation of some model glycoproteins, such as EPO.[49,60]

Resialylation

Coagulation factor IX produced by CHO-cells exhibited complex-type glycosylation with carbohydrate chains capped with sialic acid in alpha 2–3 linkage.[61] Human plasma-derived coagulation factor IX contains terminal sialic acid in alpha 2–6-linkage. Using a strategy of desialylation followed by resialylation with specific sialyltransferases it was possible to convert of CHO-cell-derived sialylation pattern into human-like sialylation pattern in vitro.[62] To increase the serum half-life of Etanercept®,[63] a recombinant fusion protein, which is used to treat patients suffering from ankylosing spondylitis, juvenile rheumatoid arthritis, psoriasis, psoriatic arthritis or rheumatoid arthritis, a resialylation strategy after expression was developed. Etanercept® is a disulfide-linked dimer of a polypeptide composed of the extracellular portion of the human type 2 (p75) tumor necrosis factor receptor (TNFR) fused to the hinge and Fc regions of the human IgG1 heavy chain.[64] This bivalent antibody-like molecule contains two N-glycosylation sites per polypeptide in the receptor domain.[60] The heterogeneous N-linked oligosaccharides of TNFR-IgG fusion protein contain sialic acid, galactose, and *N*-acetylglucosamine as terminal sugar residues. To increase the level of terminal sialylation,

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