N-Acetylmannosamine and Mammalian Cell Culture Production of Recombinant Therapeutic Human Glycoproteins[1]

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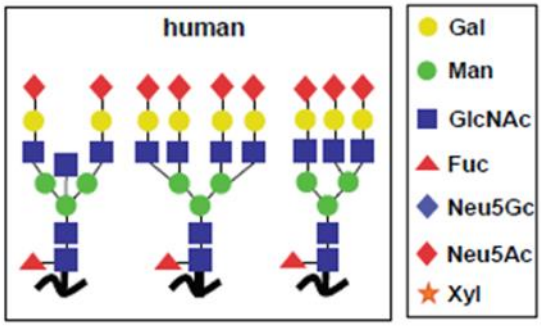
Summary

This mini-review covers developments in the application of N-acetyl-**D**-mannosamine, a compound that improves sialylation during the production of recombinant human glycoproteins.

Introduction

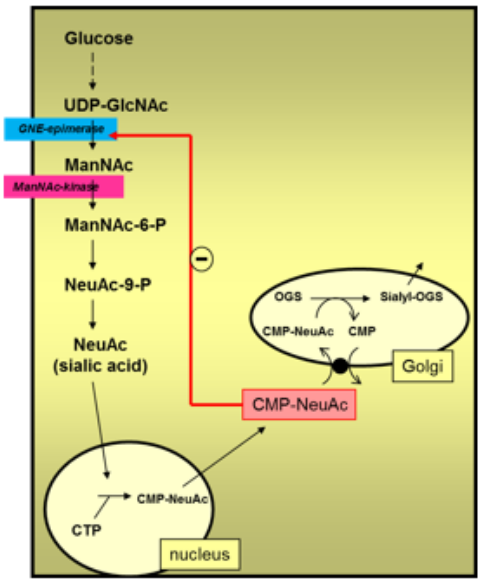
Sialic acid (N-acetylneuraminic acid, NeuAc, Neu5Ac) is an essential terminal sugar on the glycan part of functional and structural human glycoproteins (Figure 1). The first committed intermediate in the biochemical process to form Neu5Ac is the monosaccharide, N-acetyl-**D**-mannosamine (ManNAc), which is formed by the bifunctional enzyme UDP-N-acetylglucosamine / N-acetylmannosamine epimerase kinase (GNE enzyme, formerly GNE/MNK). The final product of the GNE enzymatic transformation, ManNAc-6-phosphate, condenses with phosphoenolpyruvate via an aldolase to form the 9-phosphate of Neu5Ac. At this point the Neu5Ac is activated in the cell nucleus to form the nucleotide CMP-Neu5Ac, which can be further modified to other sialic acids[2] prior to bonding to galactose (the intermediate terminal sugar of the glycan in glycoprotein formation, by way of a sialyltransferase[3], Figure 2). This is normally the last step in the biosynthesis of a wide range of glycoproteins which are found in all human tissues.

Figure 1. Typical Human Glycan Structures



There is a feedback inhibition mechanism for the GNE enzyme. When an excess of Neu5Ac is detected, the GNE enzyme essentially shuts down, thereby limiting the supply of endogenous ManNAc leading to a reduction in the formation of Neu5Ac. When there is a biosynthetic demand for additional Neu5Ac, ManNAc supplementation is very effective as it makes the feedback inhibition mechanism irrelevant.

Figure 2. Sialic Acid Biosynthesis and conjugation



When GNE fails to fulfil its biological role in humans, the disease GNE myopathy[4] is manifested as progressive muscle weakness. GNE myopathy is a rare genetic disorder with no available therapy[5]

[1] The original paper was accepted for publication in November 2012; Chemistry in New Zealand, **2013**, 77(4) 18. Updated November, 2014.

[2] Muchmore, E.A., et al, *J. Biol Chem.,* **1989**, 264, 20216-20223

[3] Bork, K.; Horstkorte, R.; Weidermann, W.; *J. Pharm. Sci*., **2009**, 98(10), 3499.

[4] GNE myopathy was formerly known as "Hereditary Inclusion Body Myopathy" (HIBM).

[5] Huizing, M.; Krasnewich, D.M.; *Biochim. Biophys. Acta*, **2009**, 1972(9), 881. And An Open Label

caused by hyposialylated muscle proteins and glycosphingolipids[6] because there is insufficient metabolic ManNAc to form the Neu5Ac terminal sugar.

There is a growing body of evidence that the lack of function of the GNE enzyme in the sialylation pathway in kidney tissue could contribute to several glomerular kidney diseases[7] due to the lack of the Neu5Ac terminal sugar on several kidney glycoproteins.

Keppler et al [8] demonstrated that the GNE enzyme was rate limiting in human hematopoietic cell lines and affected efficiency in cell surface sialylation.

The activity of the GNE enzyme is now recognised as one of the defining features in the efficient production of recombinant sialylated glycoprotein therapeutic drugs[9]. Improved sialylation not only increases manufacturing yield, but also improves therapeutic efficacy by increasing solubility, increasing half-life and reducing immunogenicity by reducing the formation of antibodies[10] to the therapeutic glycoprotein.

Sialylation of glycoproteins

There is normally some level of glycan sialylation within a glycoprotein, but with the observation that incomplete sialylation can lead to reduced therapeutic activity, it becomes relevant to assess the cell-lines and culture media to "humanise" the glycoprotein to improve performance and yield and reduce manufacturing costs. The structural heterogeneity of glycans is sensitive to fed-batch or interval feeding, culture type and environment, nutrient balance, waste accumulation, oxygen levels, pH and temperature, therefore process control is critical to improving the production of the glycoproteins. ManNAc is a non-nutrient culture medium ingredient that can increase sialylation to therapeutically effective levels. This report assesses the benefits of using ManNAc in CHO cell cultures to produce sialylated glycoproteins.

A secondary benefit from the addition of ManNAc is the increase in the ratio of Neu5Ac to of N-glycolylneuraminic acid (Neu5Gc) on the glycoprotein. The increase in the proportion of Neu5Ac over Neu5Gc helps to "humanise" the glycoprotein because Neu5Gc is not produced in the human body[11]. The ability to increase the Neu5Ac versus Neu5Gc ratio was also exemplified by Varki[12] by the addition of Neu5Ac to the culture medium but the many papers cited in this review indicate that ManNAc is the preferred choice for overall process improvements.

Recombinant proteins as therapeutic drugs

The application of therapeutic recombinant human glycoproteins is escalating as new discoveries are harnessed through mammalian cell culture technologies. The compound annual growth rate of recombinant human glycoproteins is 16% and growing; double the average growth rate of the pharmaceutical sector[13].

The defined molecular structure of a glycoprotein results from multiple intra-cellular processes that include co- and post-translational biosynthetic modifications. However, it is important to recognise that the structure of a "natural" glycoprotein is defined in human cells and body fluids environment. In contrast, and with severe regulatory implications, human recombinant glycoprotein therapeutics are produced in cultured "non-human" tissues such as Chinese hamster ovary cells (CHO cells, the mammalian "work-horse" cell for the production of recombinant glycoproteins) that may yield products without the necessary humanised co- and post-translational modifications. Enforcing high cell productivity may

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Phase 2 Study of DEX-M74 in Subjects With GNE Myopathy. ClinicalTrials.gov Identifier: NCT02346461.

[6] Patzel, KA.; et al, *J Inherit Metab Dis.* **2014**; 37(2):297-308. doi: 10.1007/s10545-013-9655-6.

[7] Galeano, B, et al.; *J. Clin. Invest.* **2007**,117(6), 1585.

[8] Keppler OT., et al. *Science* 284, 1372 **1999**; DOI: 10.1126/science.284.5418.1372

[9] Gu, X.; Wang, D.I.C. *Biotech and Bioeng*., **1998**, 58(6), 642.

[10] Weiss, P.; Ashwell, G.; *Prog. Clin. Biol. Res*.; **1989,** 300, 169.

[11] Varki, N.M. and Varki, A., *Lab. Invest*., **2007**, 87(Sept), 851

[12] Ghaderi, D., et al., *Biotechnology and Genetic Engineering Reviews*, **2012**, 28(1),147, DOI:10.5661/bger-28-147

[13] Werner, R.G.; Kopp, K.; Schlueter, M.; *Acta Paediatrica*, **2007,** 96(s455), 17.

compromise the cellular machinery with consequent poor product quality and an increase in isoforms.

Other cell types

Human embryonic kidney (HEK) cells are an exception because they are of human origin regarding co- and post-translational modifications, although larger scale production difficulties inhibits the application of HEK cells in commercial manufacturing processes.

Glycoprotein synthesis using insect cells has been patented[14]. The conventional substrates in insect cells are not efficiently converted to CMP-Neu5Ac, but it was observed that improvement in the production of CMP-Neu5Ac and subsequent sialylation of the recombinant glycoprotein is improved by the addition of ManNAc to the insect cell culture.

In all cell culture technologies[15] used to produce therapeutic glycoproteins, be it CHO, NS0, HEK, BHK or PERC.6 cell lines, there are issues with expressing the complete human glycoprotein, not only in the protein but also with constraints in formation of the glycans.

Issues related to ManNAc and glycoprotein sialylation

The timing of the addition of ManNAc to the culture as well as the time the culture is left to produce the glycoprotein can affect the degree of sialylation. Extended production times especially in fed-batch systems should increase the production of the protein but could adversely affect the glycan because once formed, the sialylated glycan could be degraded by extracellular enzymes excreted by the cells. Nutrient depletion in batch technology can limit glycan formation and thereby produce more isoforms.

The benefits of using ManNAc as an ingredient in a CHO cell line to produce recombinant human interferon-γ was investigated in depth.[2] ManNAc was chosen over Neu5Ac as the preferred additive to the culture medium because it is the specific precursor for intracellular synthesis of Neu5Ac and it has greater cell membrane permeability than Neu5Ac and CMP-Neu5Ac, at physiological pH.

The authors were able to measure a nearly 30-fold increase in intracellular concentration of CMP-Neu5Ac upon ManNAc feeding at 20 mM concentration and increased incorporation of the precursor into the sialylated product. While sialylation was significant, with a 15% gain, it was always incomplete on the pre-sialylated biantennary glycan structures at specific asparagine glycosylation sites. Incomplete sialylation with CMP-Neu5Ac might occur because a sialidase removed the Neu5Ac once it was bound to the glycan, or perhaps there was limited access of the CMP-Neu5Ac to the Golgi apparatus or perhaps limited steric accessibility for the sialylation to occur. The authors concluded that more dramatic sialylation could occur for proteins with low sialylation profiles and more easily accessible sialylation sites.

Werner[9] noted that while the CMP-Neu5Ac pool increased, they did not identify increased sialylation of the human tissue inhibitor of metalloproteinase (TIMP-1) glycoprotein either in CHO cells or in the NS0 cell lines. The Werner group concluded there could be three basic variables that could affect sialylation (i) availability of sialyltransferases, (ii) abundance of competing acceptor sites; and (iii) availability of nucleotide-sugar substrate in the Golgi. These three issues continue to be the key problems today. However, as research continues and as this review identifies, ManNAc with the inclusion of additional ingredients can improve sialylation.

Sialylation and monoclonal antibodies (mAbs)

While earlier papers indicated that the addition of uridine, manganese chloride and galactose (UMG) could increase galactosylation in mAbs, it was Gramer et al[16] who proved that the addition of the three compounds to their mAb medium synergistically increased the amount of galactose on the mAb Fc glycan.

[14] Betenbaugh, MJ; et al.; **2010**, US Patent 7,776,565. Also, see references cited within.

[15] Hossler, P.; Khattak, S.F.; Zheng, J.L.; *Glycobiol.*; **2009**, 19(9), 936.

[16] Gramer, MJ., et al. *Biotech. Bioeng*. **2011**, 108, 1591-1602 DOI 10.1002/bit.23075.

For immunoglobulin G (IgG) antibodies to effectively remove pathogens and toxic antigens, including cancer cells, sialylation is an unwelcome step in recombinant mAb production, so there are cases where the addition of ManNAc is clearly undesirable.

Intravenous Immunoglobulin (IVIG) are IgGs pooled and purified from blood donor serum with the sialylated fraction contributing about 10% of the total IgGs. To ensure ongoing supplies of IVIG are available for therapeutic applications, it is a reasonable strategy to produce recombinant sialylated igGs[17,18]. Further to the potential of using UMG along with ManNAc to increase sialylation of IgG, Kaneko[19] noted that increased sialylation of IgG reduced the need for high dose IVIG to be used to suppress inflammation. Thus, increasing the Neu5Ac content in the Fc region of IgG increases the anti-inflammatory properties.

For mAbs where the presence of a terminal Neu5Ac on the Fc glycan is desirable for the anti-inflammatory effect, the addition of ManNAc along with UMG to the culture medium could increase sialylation, when the addition of the galactose is optimised.

The glycans in the Fab region of mAbs can be more easily manipulated as there is less steric hindrance compared to the Fc region; that is, the Fab regions are more accessible to enzymes, leading to greater glycoform variability and the modification of this region can impact on both drug safety and drug properties[20].

Other glycoproteins

Examples include erythropoietin (EPO), factors, lectins, Siglecs and viral particles. EPO is a heavily sialylated glycoprotein hormone and recombinant human EPO is used therapeutically to enhance the production of red blood cells. There are up to 14 sialic acids per EPO molecule with 4 sialic acid molecules on each tetra-antennary *N*-glycan and 2 sialic acid molecules on the *O*-glycans. By use of a novel CHO-EPO cell line, it was demonstrated[21] that the addition of ManNAc increased the sialylation of the EPO. However, genetic modification of cell lines is a competitive R&D area and a commercially very sensitive and secretive operation, and scale up to commercial production introduces technical and regulatory complications.

As noted earlier, Keppler[10] demonstrated that the GNE enzyme was rate limiting, and determined that supplementation with ManNAc improved the efficiency of the production of cell surface adhesion glycoproteins.

Byrne [22] suggests that additional sialylation residues on a glycoprotein are an effective alternative to PEGylation (attachment of polyethylene glycol (PEG) polymer to the glycoprotein), with sialylation being a less technically challenging modification. PEG has a higher immunogenic potential compared to terminal Neu5Ac and often PEGylation reduces the biological activity whereas sialylation enhances activity. A key feature of many protein interactions is that binding to Neu5Ac represents the initial event instigating biological activity. For readers unfamiliar with the range of biologically important *sialo*glycoproteins, the Byrne paper includes a table of fifteen different examples.

Hills [23] and Baker [24] noted that ManNAc supplementation increased the availability of CMP-Neu5Ac in NS0 (murine myeloma) cells, but despite the increased CMP-Neu5Ac pool, sialylation of their glycoproteins did not increase. However, this author notes that these two papers do not include supplementary ingredients that could enhance the sialylation of the glycans once the CMP-Neu5Ac is formed, that have been

[17] Sibéril, S., et al., *Annals of the New York Academy of Sciences*. Volume 1110, **2007**. Autoimmunity, Part B Novel Applications of Basic Research, 497–506. DOI: 10.1196/annals.1423.052

[18] Raymond, C., et al., *Intech* **2012**, 397-418. http://dx.doi.org/10.5772/51301

[19] Kaneko, Y., et al. *Science* **2006**, 313 670-673.

[20] Jedrzejewski, P., et al. *Pharm. Bioprocess*, **2013,** 1(1), 51-69.

[21] Bork, K.; Reutter, W.; Weidemanna, W.; Horstkorte, R.; FEBS *Lett*., **2007**, 581(22), 4195.

[22] Byrne, B., Donohoe, G., O'Kennedy, R. *Drug Discovery Today*, **2007**, 12, (7-8), 319. DOI: 10.1016/j.drudis.2007.02.010

[23] Hills, A. E. et al, *Biotech. Bioeng*., **2001**, 75, 239.

[24] Baker, K.N.; Rendall, M.H.; Hills, A.E.; Hoare, M.; Freedman, R.B.; James, D.C.; *Biotech. Bioeng.*, **2001**, 73(3), 188.

described in this and other more recently publications. Alternatively, it could be a problem associated with NS0 cells, a cell type that has not been widely adopted for commercial production. Baker demonstrated that the addition of ManNAc increased the Neu5Ac : Neu5Gc ratio in the glycan structure.

Bork[19] reported that ManNAc reduced cell proliferation and differentiation, which are indicative signs of toxicity but proliferation and differentiation inhibition can also be a desirable trigger to enable the cell to commit more energy to producing the recombinant glycoprotein. In other reports high concentrations of ManNAc reduced proliferation but did not reduce glycoprotein production. As a general comment on the safety profile for ManNAc, high dose cell culture and oral toxicity studies have been undertaken and there was no significant toxicity detected [25].

In a departure from research on CHO cell lines, Hayashi[26] studied the supplementation of ManNAc to enhance sialylation using Baby Hamster Kidney fibroblasts (BHK cells) to improve recombinant EPO production. They noted that ManNAc at 20 mM did not affect cell proliferation, whereas at 200mM ManNAc inhibited cell proliferation, although cell viability was unaffected at all concentrations. Despite the inhibitory effect, the production of EPO was significantly higher with ManNAc supplementation and perhaps the anti-proliferative effect contributed to EPO production. EPO concentrations and sialylation were measured by ELISA and two-dimensional immunoblots.

In a study on the impact of feeding nucleoside sugar precursors to CHO cells [27] it was demonstrated that ManNAc and ManNAc plus cytidine at 20 mM did not significantly affect cell growth. However, the addition of ManNAc alone increased the CMP-Neu5Ac pool 12-30-fold in line with published results from Wang[2] but with the addition of cytidine the CMP-Neu5Ac pool increased 120-fold! The CMP-Neu5Ac pool increased the overall sialylation of interferon-γ by 32%, a significant and commercially valuable increase. The addition of cytidine alone contributed a small additional increase (2-8% depending on conditions) in sialylation of interferon-γ.

In another advance on sialylation technology in CHO cells, Follstad[28] demonstrated a 36% increase in sialylation from the addition of 4 mM each of ManNAc, fructose, galactose and mannose to induce sialylation of Tumour Necrosis Factor Receptor:Fc (TNFR:Fc) fusion protein (Etanercept, commercially known as Enbrel).

Butler[29] cites three recombinant drugs in which additional glycans created additional Neu5Ac sites, which increased the *in vivo* half-life. The three drugs are the EPO derivative, darbepoetin, the follicle stimulating hormone and the thyroid stimulating hormone. It is possible that additional ManNAc would assist with the increased sialylation to overcome rate limiting issues with the epimerase when there is greater pressure put on that enzyme to perform efficiently.

The German company, Glycotope [30], offers a human cell line which is evidently more effective at sialylated glycoprotein production with added ManNAc. Recombinant human granulocyte macrophage colony stimulating factor (rh GM-CSF) was produced in the absence or presence of ManNAc in the medium and analysed for the degree of sialylation. There was a substantial increase in sialylation after the inclusion of ManNAc at 90 mM. The Glycotope team also noted that sialylated GM-CSF fared much better than the PEGylated variant because the increased sialylation does not reduce biological activity.

[25] A completed toxicity study showed ManNAc is well tolerated in animals with no pathological changes (NZP; private communication).
[26] Hayashi, M., Doi, K. and Terada, S; In *Animal Cell Technology: Basic and applied Aspects* **2009**, 15, 81; DOI 10.1007/978-1-4020-9646-4_13
[27] Wong, N.S.C.; et al., *Biotech. Bioeng*; **2010**, 107**(**2), 321.
[28] Follstad BD., *Cell Culture Engineering IX* Poster **2004**. US Patent No. 7,645,609.
[29] Butler, M., *Cytotechnology*, **2006**, 50: 57-76.
[30] Baumeister, H., et al., *BioProcess International Supplement* 200636-41. US 8,609,370

The excessive accumulation of ammonia (or ammonium salts; pH effect) decreases sialylation[31]. There are two possible mechanisms for ammonia to reduce sialylation, with the first being UDP-GlcNAc competing with the transport of CMP-Neu5Ac into the Golgi and secondly, ammonia increases the alkalinity from the optimal pH for the sialyltransferases to operate.

A reduction in incubation temperature might also assist sialylation by enabling an increased glycoprotein residence time in the Golgi thereby increasing the exposure of the partially formed glycoproteins, as noted above, to sialyltransferases. Therefore, two additional attributes which are already used in glycoprotein production, pH control and lower culture temperatures should enhance sialylation after ManNAc supplementation.

In order to overcome the problems associated with increased levels of the CMP-Neu5Ac but with poor utilisation for increasing glycoprotein sialylation, there have been attempts to insert improved transporters and sialyltransferases in the CHO cell.[32] At the same time, the investigators improved the function of the GNE enzyme, but this author considers it to be somewhat pointless exercise considering ManNAc feeding has already been proved to increase the CMP-Neu5Ac pool.

Several papers[21,32] suggested that ManNAc is too expensive for commercial applications, so NZP undertook calculations to assess the cost of its cell culture grade ManNAc[33] versus the value of the glycoprotein in question. ManNAc becomes a minimal contribution at just a few dollars per gram compared to some therapeutic glycoproteins valued well over US$1,000 per gram. The cost issue raised by Bork, Son and others, is inconsequential and ManNAc addition to the culture medium is the first choice for increasing intracellular Neu5Ac concentrations in large-scale production processes[25].

The role of metal ions and their impact on glycoprotein sialylation

The role of metal ions appears to be quite significant in ManNAc promoted sialylation and this subject could benefit from further investigation. In a thesis[34], which is a development of the Wang paper[7], a "high throughput method" for the quantification of sialylation of glycoproteins was developed and the authors' assessment confirmed earlier observations that there are different mechanisms operated by different mammalian cells to produce glycoproteins. This is unsurprising and within their own work they detected significant interclonal variability in the sialylation of the interferon-γ glycoprotein model. A key result of this thesis demonstrates that the addition of ManNAc at concentrations as low as 2mM (plus a specific metal ion cofactor, Cu^{2+}) almost doubled the sialylation and production of interferon-γ.

Lithium chloride has a history with its application to inhibit the glycogen synthase kinase-3β activity, a treatment for manic depression. Inhibition of this ubiquitous enzyme inhibits cell life-cycle regulation and proliferation, which is a route to increasing, for example, Fc-fusion protein productivity. The addition of lithium chloride to the medium in an experiment to enhance Fc-fusion protein production reduced sialylation, but when the culture medium was supplemented with ManNAc (10 mM), sialylation increased[35].

Crowell [36] found the addition of manganese chloride, a cofactor for beta-1,4-galactosyltransferase enhanced galactosylation and increased the abundance of the fraction of sialylated glycans. Crowell's results were further

[31] Anderson DC and Goochee CF. *Biotech. Bioeng.*, **1995**, (47), 1, 96–105. DOI: 10.1002/bit.260470112.

[32] Son, Y.D.; Jeong, Y.T.; Park, S.Y.; Kim, J.H.; - *Glycobiol*, **2011**, 21(8), 1019.

[33] www.nzp.co.nz/index.php/products/microbiological-media-and-fermentation-ingredients/n-acetyl-d-mannosamine-mannac.html.

[34] Markley, L.R.A.; Wang D.I.C.; *DSpace@MIT.*, **2011**, http://dspace.mit.edu/handle/1721.1/65762.

[35] Ha, T. K., et al., Biotechnology *Products and Process Engineering*, In *Appl. Microbiol. Biotechnol.* **2014**, DOI 10.1007/s00253-014-6012-0

[36] Crowell CK, et al. *Biotech. Bioeng*. **2007** Feb 15;96(3):538-49. DOI: 10.1002/bit.21141

improved by Gramer[37] whose UMG experiments (see above) improved galactosylation of their mAbs. While Gramer was seeking to prevent the undesirable drop in mAb Fc galactosylation, it requires only a little extrapolation to propose that if the galactose platform is fully availably in a timely manner, ManNAc supplementation should lead to enhanced sialylation.

Are there other formulations that could utilise ManNAc to enhance recombinant therapeutic glycoprotein function?

The most recent publication contributing to this literature review, is the Coherus patent application which uses ManNAc (20 mM) in combination with galactose and dexamethasone to specifically reduce the amount of "incorrectly folded" fusion protein, the tumour necrosis factor inhibitor, Etanercept (also, see above). Improved folding occurs because the glycoprotein contains more complete sialylation producing a more desirable glycosylation profile[38].

Supplementary galactose feeding alone can assist with sialylation but not in all models[39]. In an Interleukin model, decreased galactose feeding decreased the sialylation, whereas additional galactose improved the sialylation rate. Nevertheless, as noted in other models, the addition of ManNAc could counterbalance galactose supplementation.

Issues to be considered when experimenting with ManNAc

As CHO cultures move to higher density production systems, the post-translational glycan synthesis needs to be boosted with the addition of supplementary compounds to enhance the "natural" glycan profiles. ManNAc supplementation is not a universal solution and more work needs to be undertaken to gain a complete understanding of the drivers of the ManNAc technology. For example, issues can occur if there is steric hindrance (eg in the Fc region in mAbs), limits to the sialyltransferase machinery and enzymatic degradation of the sialylated glycoprotein once it has been formed. However, given the appropriate cell line, solutions based on ManNAc supplementation do increase sialylation.

ManNAc supplementation is likely to enhance a range of culture types without breach of specific patents and furthermore supplementation may have limited impact on the regulatory environment because it is not changing the way the cell synthesises the glycoprotein. This is particularly important when a company files an Investigational New Drug (IND) application with the FDA in the USA and should be carefully reviewed on a case by case basis.

Several ideas stem from this review;

1. The ManNAc concentration range of 2-40 mM seems to be a good starting point for the working range for CHO cells without causing inhibition of cell growth. If cell growth inhibition is observed as being caused by ManNAc, then glycoprotein production could still be enhanced rather than be reduced.
2. There are examples of improved sialylation when other additives are used along with the added ManNAc, including galactose, uridine, cytidine, manganese and lithium, although not necessarily all together. More research here would be quite enlightening.
3. Neu5Ac is conjugated exclusively to galactose, so does galactosylation efficiency impact on sialylation efficiency?
4. Increasing ammonia concentration is a problem in CHO cultures and it inhibits sialylation, so this by-product needs to be monitored when supplementing with ManNAc.
5. A lower temperature could improve sialylation after ManNAc supplementation.
6. While there are several ways to enhance sialylation without supplementing the medium with ManNAc, could these methods be more efficient in reducing isoforms after ManNAc supplementation?
7. For systems in which overexpressing the CMP-Neu5Ac transporter, the galactosyltransferase and the α(2,6)-

[37] Gramer MJ., et al., *Biotech. Bioeng*., **2011**, 108(7), 1591. DOI.10.1002/bit.23075.
[38] Puchacz E., and Grove, JR., U.S. Patent Application, 2014/0296489.
[39] Clark KJ., et al. *Biotech Bioeng*. **2005,** 90, 568-577.

sialyltransferase genes is operating, sialylation efficiency could still enhanced by the addition of ManNAc to the medium.

8. If an increase in the Neu5Ac:Neu5Gc ratio is considered to be important, the Neu5Ac fraction can be increased over Neu5Gc by the addition of ManNAc, even if overall sialylation does not increase.
9. If cells are designed to overexpress CMP-Neu5Ac synthase and CMP-Neu5Ac transferase there is a noted feedback inhibition on the GNE enzyme which reduces production of ManNAc[32]. ManNAc supplementation could readily assist production of the required glycoprotein rather than genetic engineers trying to overexpress the GNE epimerase kinase.

Conclusion

The addition of ManNAc to the culture medium can make a special and essential contribution to maximising the sialylation of certain recombinant glycoproteins. Some mammalian cell cultures are more efficient with the sialylation mechanism, although there are many factors that can influence the sialylation yield. Several early papers noted that ManNAc was not particularly useful for increasing sialylation of glycoproteins despite an increase in the CMP-Neu5Ac pool. It is the more recent papers that are convincing in their observations that while ManNAc alone can assist with sialylation, ManNAc dosing along with specific additional ingredients definitely improves sialylation and protein folding.

It is likely that the best utility of ManNAc will be in the production of proteins that do not have sterically hindered sites that prevent the sialyltransferase from operating efficiently in the final step of the glycosylation process.

The addition of ManNAc to the medium offers additional value where the Neu5Gc content in a glycoprotein should be minimised.

Increased sialylation increases solubility, reduces the risk of immunogenicity and extends the half-life of therapeutic glycoproteins, while maintaining or even increasing a high level of biological activity.

Owing to the recent availability of ManNAc in large quantities at modest cost, it has become an essential culture medium ingredient for research and development with growing importance in large scale recombinant human therapeutic glycoprotein production.