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Sialic acids: carbohydrate moieties that influence the biological and physical properties of biopharmaceutical proteins and living cells

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Sialic acids are structurally diverse molecules that have important roles in the physiological reactions and characteristics of prokaryotes and eukaryotes. These include the ability to mask epitopes on underlying glycan chains and to repulse negatively charged moieties. Here, we describe the metabolism and immunological relevance of sialic acids and outline how their properties have been exploited by the pharmaceutical industry to enhance the therapeutic properties of proteins such as asparaginase and darbepoetin α.

Developing proteins as potential therapeutics

The development of proteins as potential therapeutic agents is the focus of intensive medical and industrial research, mainly because of the economic and clinical importance of these products, whose biological activities range from anti-cancer agents to treatments for rheumatoid arthritis. When introduced into a patient, a glycosylated protein (glycoprotein) can be subject to several host-generated responses, including acid denaturation in the gastrointestinal tract (when administered orally) and cleavage by renal and hepatic enzymes (parenterally administered proteins). Glycoproteins can also be recognized as antigenic, resulting in the stimulation of an immune response. The antibodies generated as a result might be directed towards either proteinaceous or carbohydrate elements of the molecule, ultimately causing its premature removal from the host. The resulting reduction in efficacy might necessitate dose escalation, which is undesirable for both the patient and the health-care provider. Hence, the development of biopharmaceutical preparations with reduced antigenicity and improved stability is of great clinical importance, and, in this review, we discuss approaches that enables this to occur.

Improving pharmokinetic profiles of therapeutic proteins

Increase in half-life through pegylation

Several protocols have been developed and implemented to improve the pharmokinetic properties of many biopharmaceutical proteins (Table 1), with the main aim of increasing the elimination half-life and stability of the protein in question. One such modification is to increase the overall size of the circulating protein, because molecules that are not associated with plasma-based proteins and with molecular weights <5 kDa tend to be excreted rapidly via the kidneys [1]. This enlargement results in a reduced rate of glomerular filtration, giving the protein more time to interact with the target tissue or antigen. Such increases in size can be achieved by pegylation, involving the covalent attachment of either linear or branched chains of polyethylene glycol (PEG) via a chemically reactive side-chain, such as a hydroxysuccinimidylester or an aldehyde group, for linking to either the α or the epsilon amino groups on the protein. The use of activated double bonds or disulphide bonds for conjugation to thiol groups on the protein of interest is also a possibility. PEG is highly water-soluble and reduces proteolytic cleavage by masking available cleavage sites on the protein backbone [2]. The value of pegylation was demonstrated for several proteins, including asparaginase [3], an enzyme used in the treatment of leukaemia, and adenosine deaminase [4], which participates in purine metabolism. Pegylation was also used to enhance the biological activity of immunologically important entities such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) [5], tumour necrosis factor (TNF), interferon α-2a (IFN α-2a) and IFN α-2b [2].

Although pegylation is a modification procedure that might enhance biopharmaceutical pharmokinetic properties, it is not without drawbacks. After conjugation, many different monomeric isoforms of varying biological activity can be generated, which

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TABLE 1

Modifications that increase the half-life of a biopharmaceutical protein

Modification	Target molecule	Effect	Refs
Truncation and/or alteration of the amino acid sequence	Octreotide	Enhanced enzymatic stability	[60]
N- and C-terminal acetylation (peptide capping)	MART-1(27–35) immunopeptide	Increased plasma stability by reducing proteolytic digestion	[61]
Disulphide bond cyclization	Indolicidin	Enhanced resistance to proteases	[62]
Albumin fusion proteins (human serum albumin)	Hirudin Albutropin	Decreased clearance in rabbits Decreased clearance in rats and monkeys	[63] [64]
Pegylation	TNF IFN α-2a, IFN α-2b Asparaginase Adenosine deaminase	Reduced proteolytic cleavage Increased molecular size	[2] [2] [3] [4]
Hypersialylation	Asparaginase Leptin Luteinizing hormone Cholinesterase Erythropoietin	Masking of galactose residues Reduced immunogenicity Increased serum half-life	[22] [23] [24] [25] [26,27,30,31]

might compete for binding to receptors in the host. This observation occurs as a result of the polydisperse nature of the polymer. Concerns have also been raised about introducing into the body a synthetic polymer that does not appear to be completely biodegradable. This is despite the observation that mono- and di-carboxylated metabolites of PEG are detected in bile; these metabolites are generated by cytochrome P450 oxidation via a mechanism that is analogous to that acting on more chemically inert polyethylene to generate ketone and aldehyde groups [6]. Furthermore, the extended half-life of pegylated proteins might be accompanied by reduced biological activity related to the structural change in the molecules as a result of conjugation [7].

Effects of sialylation

An effective alternative to pegylation involves the introduction of additional sialic acid residues to a protein. This modification, termed sialylation, is not as challenging technically as pegylation, and can be as effective in increasing the half-life of therapeutic

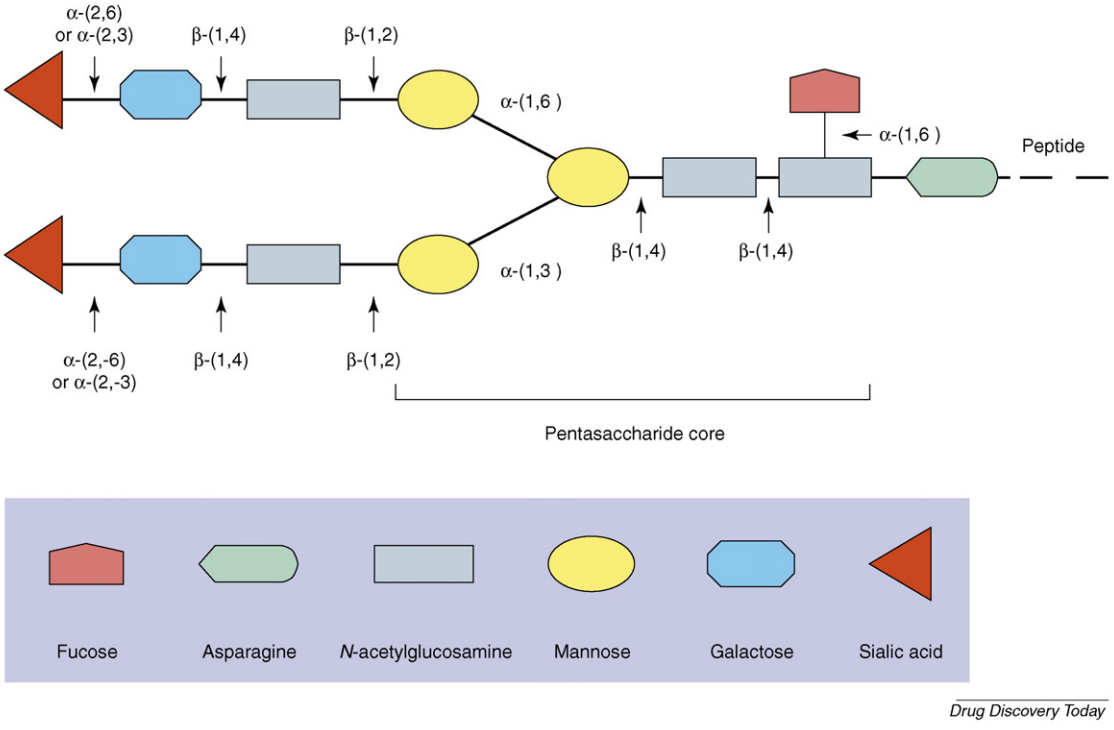

FIGURE 1

Spatial arrangement of sialic acid residues on an *N*-linked glycoprotein. A typical oligosaccharide, which includes a conserved pentasaccharide core, is shown. Greater degrees of mannosyl-branching, concomitant with increased sialylation, are also possible.

proteins such as asparaginase [22] and erythropoietin [26] (*vide infra*).

A contributory factor to the efficacy of sialylation in biopharmaceutical processes arises from the biological traits conferred by sialic acids on prokaryotic and eukaryotic cells. The term 'sialic acid' (derived from 'sialos', the Greek word for saliva) describes a large family of structurally diverse 9-C carbohydrate residues whose nomenclature was established through the collaborative efforts of Guntar Blix, Alfred Gottshalk and Ernst Klenk [8]. Sialic acids have been reported in a range of eukaryotic hosts, including fungi and protozoans [9]. In mammalian cells, sialic acids occupy the terminal positions on oligosaccharide chains (Figure 1) and are constituents of several biologically important glycoproteins (Table 2). Binding to sialic acids might, therefore, represent an initial event in the binding of macromolecules to instigate biological activity. Examples include lectins [10], siglecs [11] and viral particles such as the H5N1 influenza virus [12], which has received significant media interest.

The structure and function of sialic acids

There are in excess of 50 natural analogues of sialic acid [9] that result from modifications to the sialic acid backbone, such as the introduction of lactoyl, sulphate, methyl and phosphate groups at the hydroxyl groups at C-4, C-7, C-8 and C-9 [13]. A solitary substitution at C-5 presents the most common sialic acid derivatives (Figure 2): namely, *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) and neuraminic acid (Neu). Residues of Neu5Ac interact with each other in a conjugation process that results in the formation of polysialic acid (PSA) [14]. These homopolymers, which are referred to as colominic acids, are added post-translationally to the mammalian neuronal cell adhesion molecule (NCAM), a glycoprotein that is present on the surface of glial cells and skeletal muscle. This association enables sialic acids to participate in several neurological processes, such as the maintenance of plasticity during neuron development, fasciculation and axonal branching by regulating homophilic interactions. It is also conducive to reduced cell adhesion and altered patterns of migration [15]. The negative charge presented at C-1 also enables the attraction and repulsion of charged molecules and other entities.

Neuroinvasive prokaryotic strains such as *Neisseria meningitidis* [16] and *Escherichia coli* K1 [17] evade host-immune responses by decorating their exterior with PSA. The structural similarity of this polysaccharide to PSA-NCAM suppresses complement and phagocytosis-based activity of the host [18] as a result of the ability of sialic acid residues to conceal underlying epitopes on a glycan chain, including the penultimate galactose residue that is detected by liver-based receptors [19].

Using the masking properties of polysialylation in biopharmaceuticals

The introduction of protocols for the polysialylation of biopharmaceuticals results primarily from the ability of sialic acids to mask and reduce biological degradation and neutralization because no known receptors are specific for sialic acids that are independent of

TABLE 2

Examples of biologically important sialoglycoproteins

Sialoglycoprotein	Biological function(s) and application(s)
Human α-Fetoprotein (HAFP)	Used to monitor foetal development during gestation; biomarker for testicular and hepatocellular carcinoma
Ascites sialoglycoprotein-1 (ASGP-1)	Constituent of MUC4, a membraneous mucin that is present in rats and also on luminal surfaces of blood vessels and milk in humans; biomarker for breast cancer
CD44	Cell-surface glycoprotein involved in haematopoiesis, immune recognition and tumour metastasis
Follicle stimulating hormone	Involved in the development of Graafian follicles in females and the stimulation of semen production in males
GP20	Occurs on human sperm and is involved in capacitation (the process by which sperm acquire the ability to penetrate an ovum)
GP44	Present on tumour cells and participates in platelet aggregation
GP120	Envelope sialoglycoprotein present on the surface of human immunodeficiency virus (HIV) particles; enables targeting and infection of host cells
Glycophorin A	Glycoprotein on erythrocyte membranes that acts as a receptor for binding proteins introduced by the malaria parasite *Plasmodium falciparum*
Glycophorin C	Interacts with human erythrocyte proteins 4.1 and p55 to enhance the stability of red blood corpuscles; also acts as a receptor for *P. falciparum* proteins
Human chorionic gonadotropin	Hormone produced during the early stages of gestation. Used to detect pregnancy, and to monitor the onset of carcinoma in developing foetuses and in germ-cell tumours in adults
Luteinizing hormone	Stimulates ovulation in females and the production of testosterone in males by the interstitial cells of Leydig
MG-160	Membraneous protein on the medial cisternae of the Golgi apparatus that acts as a receptor for E-selectin, a cell surface-adhesion molecule on endothelial cells, and a fibroblast growth factor
Osteopontin	Glycoprotein used as a biomarker for several carcinomas, including colorectal, ovarian, breast and lung cancers
Thyroid stimulating hormone	Regulation of endocrine activity of the thyroid gland
Antibody (e.g. IgG)	Key component of immune system Structural basis for many biopharmaceuticals Used in immunoassay development Presence of pathogen-specific antibodies is used to detect infection Major therapeutic where sialylation is key for biological activity and half-life

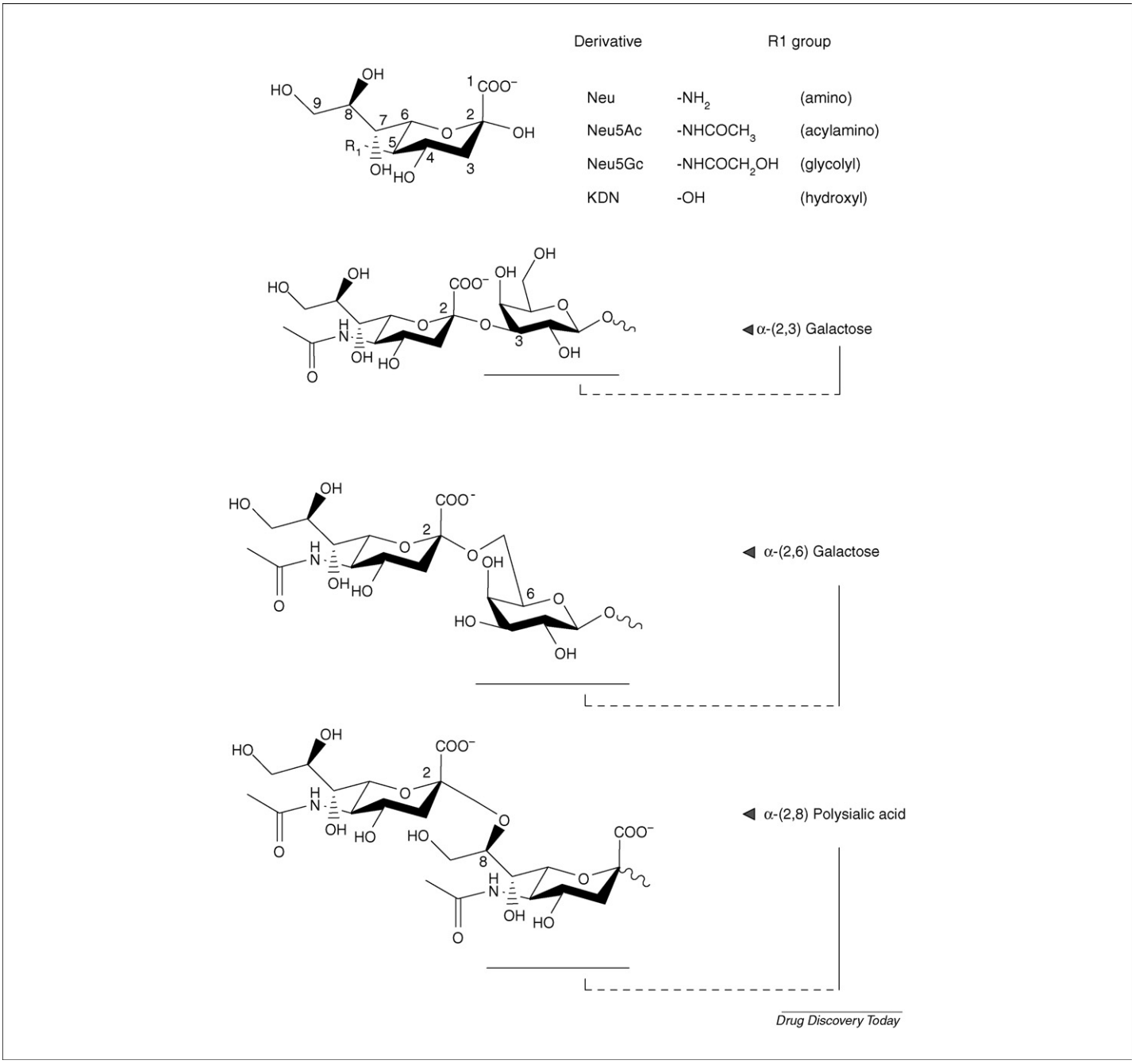

FIGURE 2

The structure and linkages of some common, natural derivatives of sialic acid. Sialic acids can be linked with galactose by either an α-(2,3) or α-(2,6) linkage, whereas the sialic acid residues in PSA molecules interact with each other through an α-(2,8) linkage.

glycan chains. Typically, circulating antibodies that target PSA and most other carbohydrate residues are polyvalent IgM isotypes that tend to have low affinity and might lack the ability to seroconvert to higher affinity IgG [20]. This indicates that sialic acids contribute as part of the innate immune system, and highly sialylated proteins, when introduced to a host, are prone to generate only weak, host-based immune responses. This is in contrast to PEG, which has a much greater immunogenic potential [21]. For biopharmaceutical processes, the α-(2,8)-linked group B capsular polysaccharide of *E. coli* K1 or colominic acids have been selected most frequently for use, with the terminal residues modified to facilitate conjugation with the target protein. There is a direct correlation between the chain length of the conjugated PSA and the imparted increase in half-life of the target protein [21].

There are several examples of how hypersialylation improves the overall therapeutic efficacies of important biopharmaceutical proteins. These include asparaginase [22], leptin [23], luteinizing hormone [24] and cholinesterase [25]. Arguably one of the more interesting examples is erythropoietin (EPO), a glycoprotein that is synthesized by the peritubular cells in the kidney and whose overall function as a stimulator of erythropoiesis is in the regulation of the production of red blood corpuscles [26]. This protein, which is produced in a recombinant form (recHuEPO) in Chinese hamster ovary (CHO) cells, is used to treat anaemia [27]. The

glycoproteins that are secreted by CHO cell lines, in particular, have a different carbohydrate composition compared with their human counterparts, being comprised of heterogeneous mixtures of different glycoforms. Most are *N*-linked glycans, attached to asparagine residues on the protein of interest. As with *O*-glycosylation, where carbohydrates are attached to the hydroxyl groups of either serine or threonine residues, the presence of these glycans is instrumental in promoting biological activity in a range of different glycoproteins [28]. *N*-linked glycans also contribute to protein folding in the endoplasmic reticulum [29]. RecHuEPO contains three *N*-glycans at asparagine residues 24, 38 and 83. Each of these can accommodate up to four sialic acid residues, and the ability of the additional *O*-linked glycan (at serine 126) to accommodate a further two sialic acid residues means that there is a potential for 14 sialic acid residues as part of a complex, *N*-linked glycan.

The introduction of additional glycans to EPO

The introduction of two additional consensus-attachment sites for glycans at asparagine residues 30 and 88 of the EPO molecule (providing a total of five *N*-linked oligosaccharides) [26], using recombinant DNA technology, significantly increased the serum half-life when intravenously administered to mice, dogs, rats [27] and humans [26,30]. A contributory factor to this, in addition to the increased size of the molecule, is the increase in sialic acid content. This molecule, which is referred to as either darbepoetin α or novel erythropoiesis stimulating protein (NESP), accommodates 22 sialic acid residues. This increased sialic acid content results in a threefold increase in serum half-life compared with native EPO [26,27]. When darbepoetin α is administered subcutaneously to human subjects, the half-life of the molecule is ~50 h [31], which is approximately twofold greater than after intravenous administration (25.3 h) [30]. The reduction in receptor binding affinity that has been observed by Egrie and co-workers [27] also results in lower clearance.

The masking ability of sialylation on darbepoetin α is illustrated further by the inability of two commercially available sandwich enzyme-linked immunosorbent assays (ELISAs) to detect sialylated darbepoetin α in 13 patients and 37 control subjects, whereas neuraminidase-treated samples gave detectable signals [32]. Immunological responses to darbepoetin α have been examined by Glaspy and co-workers [33] who show that, over a 16-week period, darbepoetin α given to patients did not stimulate an immune response of clinical significance. MacDougall [31] observed a similar pattern in >1500 subjects.

The production of sialylated proteins by eukaryotic cells

Eukaryotic expression systems are used routinely to secrete correctly folded, sialylated therapeutic proteins, including EPO, with these processes occurring in the rough and smooth endoplasmic reticulum, and the Golgi apparatus of the cell. In addition to CHO cell lines (mentioned earlier), other mammalian cell lines, such as murine myeloma NS0 and baby hamster kidney (BHK) cells, are also commonly selected for use. When secreted by a eukaryotic cell, the initial stages of processing involve the trimming of mannose residues by mannosidase II enzymes and the subsequent introduction of GlcNAc, fucose, galactose and, finally, sialic acid residues, to generate an operational oligosaccharide (Figure 1).

Engineering sialic acid biosynthetic pathways in CHO and NS0 cell lines

Since the introduction of sialic acid is of particular interest for the synthesis of therapeutically active products, knowledge of the biosynthetic pathway used by eukaryotic cells is conducive to the engineering of hypersialylated glycoproteins. In particular, interest has focused on engineering the sialyltransferase enzyme, which is ultimately responsible for introducing a Neu5Ac residue to the penultimate galactose residue. In CHO cells, this enzyme is specific for α-(2,3)-linked sialic acid moieties [34]. The overexpression of this enzyme, and an α-(2,6)-specific sialyltransferase that introduces linkages similar to those found on human cells, is responsible for the introduction of elevated amounts of sialic acid to recombinant proteins. Minch *et al.* [35] have targeted a recombinant tissue plasminogen activator by transfecting α-(2,6) sialyltransferase from rat liver β-galactosidase into a CHO cell line. The presence of elevated α-(2,6)-sialylation on the hybrid product is illustrated by the increase in fluorescence when a lectin derived from *Sambucus nigra* is used to detect Neu5Ac linked specifically to galactose via an α-(2,6) linkage. Control cells did not show a fluorescence-based response.

A similar protocol was used by Bragonzi and co-workers [36] to modify human IFN-γ and the tissue-inhibitor of metalloproteinases-1. Analysis of the former product showed >40% content of α-(2,6)-linked sialic acid. Fukuta *et al.* [37] have overexpressed both α-(2,3) and α-(2,6)-specific enzymes (derived from mouse and rat cells, respectively) to hypersialylate IFN-γ in engineered CHO cells and, in doing so, observed a significant increase in sialic content in mutant cells. A rat α-(2,6)-specific sialyltransferase was introduced to a CHO cell line by Jassal and co-workers [38] to improve the therapeutic activity of a recombinant IgG3. Recently, Nakagawa *et al.* [39] successfully overexpressed α-(2,6)-specific sialyltransferase in Neuro2A and CHO cell lines to hypersialylate endogenous amyloid precursor protein, and Wong *et al.* [40] have overexpressed a cytidine monophosphate (CMP)–sialic acid transporter to enable the hypersialylation of IFN-γ.

Mammalian cells are sensitive to environmental changes during fermentation processes [41]. Alterations to several parameters, including pH, process time and temperature, might influence the carbohydrate content of the secreted product directly by introducing greater *N*-glycan heterogeneity [42]. However, this might be advantageous if the outcome of the fermentation is focused on generating a range of analogues with varying degrees of sialylation. Elevated sialic acid content is also introduced by feeding with precursors of *N*-acetyl-D-mannosamine (ManNAc), although this approach has varying degrees of success. Gu and Wang [43] found that feeding these residues to CHO cell lines synthesizing IFN-γ significantly increases sialylation, which results directly from elevated amounts of intracellular CMP−Neu5Ac. Hills and co-workers [44] attempted to feed identical amounts of ManNAc precursors (20 mM) to NS0 cells producing a recombinant IgG_1. Although the content of CMP–Neu5Ac increased intracellularly, no elevated sialylation was observed on the antibody. Baker *et al.* [45] also recorded similar observations in CHO and NS0 cells secreting a tissue inhibitor of metalloproteinase 1, but concluded that precursor feeding might alter the sialic acid content of the product.

Minimising cleavage of sialic acid

Another protocol to ensure that the sialic acid content of therapeutic proteins is elevated focuses on the suppression of sialidase activity. Sialidases are cytosol-derived glycosylhydrolase enzymes that cleave sialic acid residues and, in a CHO cell-culture process, are released into the culture medium after cell lysis. The enzymatic cleavage of Neu5Ac residues is problematic for two main reasons; the exposure of galactose and the decrease in serum half-life that is introduced by decreased sialylation. It is possible to monitor desialylation by monitoring sialidase activity by using a coumarin-bound fluorescent substrate [2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid], which fluoresces when cleaved [46]. Gramer and co-workers [47] found that addition of the transition state analogue 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (also called DANA or 2,3-D) to CHO culture supernatant suppresses sialidase activity. Such activity is also inhibited by transfecting CHO cells with small interfering RNA [48].

Modification of the linkage that joins a Neu5Ac residue to a galactose residue might also prevent sialidase-based cleavage of sialic acid. This involves introducing a thioglycoside linkage in which a sulphur atom replaces an oxygen atom. This might be introduced enzymatically by incorporating mutant glycosidases [49]. Mülleger *et al.* [50] modified an endo-xylanase from a *Bacillus circulans* strain using glycosynthase and thioglycoligase methodologies to generate a novel, stable, thioglycosylated protein that is resistant to cleavage. This modification protocol might provide a solution to the premature removal of sialidase-cleaved glycoproteins.

Neu5Gc: a non-human sialic acid

This discussion has focused primarily on the Neu5Ac derivative of sialic acid. However, the products that are secreted by CHO cells might also contain the Neu5Gc isoform. This derivative occurs frequently in animal cells, but is predominantly absent in humans because of an internal frame-shift mutation in the gene that encodes CMP–Neu5Ac hydroxylase [51]. The consumption of animal produce, such as red meat and milk, introduces traces of this residue into human hosts [52]. This sialic acid derivative is essentially 'foreign' and, hence, monitoring the Neu5Gc content of biopharmaceutical samples is important. The primary epitope on this moiety, which is referred to as the Hanganutziu-Deicher antigen, might stimulate an immune response from the host. This might be problematic in xenotransplantation procedures in which porcine organs that contain Neu5Gc residues are introduced to human hosts who are immunocompromised at the time of organ transplantation. This has led to a significant research concerning the antigenicity of this carbohydrate. In contrast to Neu5Ac (mentioned earlier), a more varied immune response is generated towards these moieties. Typical responses consist of IgM, IgG and IgA antibodies [52], with activated T cells and human T leukaemic cells incorporating this carbohydrate [53], which indicates that these residues are potentially immunogenic. Recently, Martin and co-workers [54] have demonstrated that when human embryonic stem cells are cultured in a specialized medium (serum replacement) that is derived from animal sources, the native Neu5Gc residues might be introduced into the host. It has also been demonstrated that free sialic acid (from the growth medium) is incorporated by mutated human fibroblast and neuroblastoma cells, chimpanzee lymphoblasts and CHO-K1 cell lines. This uptake involves a non-clathrin-mediated mechanism (pinocytosis) that involves a human sialic acid transporter and a lysosomal sialidase [9,55].

The production of humanized glycoproteins by fungal cells

In addition to mammalian cell lines, fungal strains, such as *Pichia pastoris*, have been engineered to synthesize sialylated glycoproteins. The versatility of this eukaryote is illustrated by several experiments showing that human glycosylation patterns can be replicated and stringently controlled in this host through recombinant DNA technology [56]. This has led to the production of humanized, *N*-linked glycoproteins [57,58], in contrast to the heavily mannosylated glycans that are associated with wild-type strains [42]. These glycosylation mutagenesis experiments do not alter the viability of the recombinant strain [57].

Additional benefits of selecting fungal expression systems include cost-effectiveness and the ease with which strains such as *P. pastoris* are propagated and, ultimately, scaled-up. In a recent development, Hamilton *et al.* [59] successfully engineered the glycosylation and sialylation patterns in this strain to produce recombinant cells that synthesizes heavily sialylated glycoproteins, including recombinant EPO. This development should be of great importance for the pharmaceutical industry, and could lead to the accelerated production of sialylated glycoproteins of therapeutic importance in this fungal strain.

Conclusion

Here, we have focused on the addition of sialic acids to enhance the therapeutic properties of a variety of biopharmaceutical proteins. The versatility of these carbohydrate moieties, coupled with developments in sialyltransferase engineering, should enable more glycoproteins to be hypersialylated and, subsequently, to be evaluated for clinical studies. The optimization of protocols for the large-scale production of hypersialylated proteins in mammalian cell lines should be considered as a mechanism for generating large amounts of highly effective pharmaceutical preparations. However, the regulation of the Neu5Gc content needs to be addressed with reference to the potential immunogenicity associated with the introduction of multiple glycan chains that contain this sialic acid residue. Further research on fungal cell lines should also yield more heavily sialylated glycoproteins of therapeutic importance.

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