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Exploiting the Di-Sialyl Galactose Activity of $\alpha$2,6 Sialyltransferase from Photobacterium Damselae to Generate a Highly Sialylated Recombinant Alpha-1-Antitrypsin

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Supporting Information Placeholder

ABSTRACT: Sialic acids are cell surface sugars present in many animal glycoproteins and are of particular interest in biopharmaceuticals, where lack of sialylation can reduce bioactivity. Here we describe how $\alpha$-2,6-sialyltransferase from Photobacterium damsela can be used to markedly increase sialylation of CHO produced alpha-1-antitrypsin. Detailed analysis of the sialylation products showed that in addition to the expected $\alpha$-2,6-sialylation of galactose, a second di-sialyl galactose motif was produced, which had never been recorded on a mammalian glycoprotein. We exploited this di-sialyl galactose activity of the Photobacterium damsela in a multi enzyme reaction to produce a highly sialylated alpha-1-antitrypsin. The influence of this unique di-sialylation on the in vitro activity of alpha-1-antitrypsin was studied and a toolkit of mass spectrometry methods to identify this new di-sialyl galactose motif in complex mixtures was developed.

The importance of sialylation on certain biotherapeutics has led to the development of several methods and patents for increasing biotherapeutic sialylation. For example, introduction of additional glycosylation sites, and gene engineering have been explored as methods to increase biotherapeutic sialylation.5,6 Another approach to increasing sialylation without altering the protein is using sialyltransferases (SiaTs) in vitro to covalently link N-acetylneuraminic acid (Neu5Ac) to the glycans of AAT or other glycoconjugates. In vitro sialylation has been shown to be a versatile method to increase sialylation of biotherapeutics. 7,8

Table 1: Sialylation of rAAT following glycosyltransferase incubations

| Enzymes Used During Incubation | Sialylation (%) | Average $^*$ | SA Count |
|-------------------------------|----------------|-------------|----------|
| None                          | 48±3           | 2.6±0.3     |          |
| $\alpha$2,6PTB                | 56±1           | 3.3±0.2     |          |
| $\alpha$2,6PTB + GaIT         | 73±3           | 3.8±0.2     |          |
| $\alpha$2,3PM + GaIT + $\alpha$2,6PTB | 93±1 | 6.6±0.1 |          |

$^*$ SA Count = $\frac{\text{Σ Glycans with 1 sialic acid x Σ Glycans with 2 sialic acid} \times \text{etc. N}}{100}$

(Where: %1SA = % Glycans with 1 sialic acid, %2SA = % Glycans with 2 sialic acid, etc. N = number of glycosylation sites on a protein. (for rAAT N = 3))

α2,3-2,6-sialyltransferase from Photobacterium damselae can be used to markedly increase sialylation of CHO produced alpha-1-antitrypsin. Detailed analysis of the sialylation products showed that in addition to the expected α-2,6-sialylation of galactose, a second di-sialyl galactose motif was produced, which had never been recorded on a mammalian glycoprotein. We exploited this di-sialyl galactose activity of the Photobacterium damselae in a multi enzyme reaction to produce a highly sialylated alpha-1-antitrypsin. The influence of this unique di-sialylation on the in vitro activity of alpha-1-antitrypsin was studied and a toolkit of mass spectrometry methods to identify this new di-sialyl galactose motif in complex mixtures was developed.

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Alpha-1-antitrypsin (AAT) is a protease inhibitor that has numerous roles in the body most notably the inhibition of neutrophil elastase in the lungs. The genetic disease AAT deficiency (AATD) is caused by a lack of AAT in the body leading to complications ranging from chronic obstructive pulmonary disease to liver cirrhosis.1 Augmentation therapy of severe AATD suffers involves human serum derived plasma AAT. Unfortunately, the augmentation treatment is prohibitively expensive owing to AATD patients requiring weekly intravenous drug.

During an in vitro reaction, two di-sialyl galactose motifs were produced, which had never been recorded on a mammalian glycoprotein. We exploited this di-sialyl galactose activity of the Photobacterium damselae in a multi enzyme reaction to produce a highly sialylated alpha-1-antitrypsin. The influence of this unique di-sialylation on the in vitro activity of alpha-1-antitrypsin was studied and a toolkit of mass spectrometry methods to identify this new di-sialyl galactose motif in complex mixtures was developed.

The importance of sialylation on certain biotherapeutics has led to the development of several methods and patents for increasing biotherapeutic sialylation. For example, introduction of additional glycosylation sites, and gene engineering have been explored as methods to increase biotherapeutic sialylation.5,6 Another approach to increasing sialylation without altering the protein is using sialyltransferases (SiaTs) in vitro to covalently link N-acetylneuraminic acid (Neu5Ac) to the glycans of AAT or other glycoconjugates. In vitro sialylation has been shown to be a versatile method to increase sialylation of biotherapeutics. 7,8 A challenge is the high cost of glycosylation remodelling which involves expensive nucleotide sugar donors and mammalian glycosyltransferases, which can lack the desired substrate specificity and can be difficult to access.

Bacterial SiaTs can be easily produced in E.coli and several have been shown to be versatile catalysts towards both acceptor and donor substrates. However, few of these versatile SiaTs have yet to be studied for their usefulness for in vitro glycan remodelling or biopharmaceuticals. 17,18
We therefore decided to explore how the unique attributes of bacterial sialyltransferase could be used to produce highly sialylated biotherapeutics, such as AAT, where level of sialylation has been shown to be crucial for drug half-life.

Initially, α,2,6 sialyltransferase from *Photobacterium damselae* (α2,6PTB) was used for the sialylation of AAT produced recombinantly in Chinese Hamster Ovary (CHO) cells (rAAT). The N-glycans of the resulting products were analysed by glycopeptide analysis using LC-MS/MS techniques following tryptic digestion. Initially, increases in relative sialylation of rAAT was small with only an 8% increase in the number of glycans sialylated following incubation with α2,6PTB (Table 1, increase from 48% to 56%). The SA count takes into account how highly sialylated a glycoprotein is by analysing the level of sialylation at each glycosylation site i.e if the glycans are, mono, di, tri etc sialylated. An unexpected rise in average SA count to 3.3 was observed despite the small increase in the number of glycans that were newly sialylated (Table 1). An increase of 7% in glycans that were tri and tetra sialylated (Table S1-S2) suggested that the α2,6PTB has sialylation activity towards already sialylated glycans leading to a surprisingly large increase in average SA count considering the small increase in newly sialylated glycans.

Forty-four percent (44%) of glycan species expressed on the CHO-rAAT in our study had terminal N-acetyl-hexosamine (HexNAc) residues (Table S1-S2), which are not substrates for CHO-rAAT in our study had terminal HexNAc residues (Table S1-S2), which are not substrates for CHO-rAAT in our study had terminal HexNAc residues (Table S1-S2). Analysis using LC-MS/MS techniques following tryptic digestion confirms the observed hypersialylation is the result of the α2,6PTB activity (Figure 1).

The nature of the hypersialylated N-glycan species is of some interest. In mammalian N-glycans there are typically two main types of hypersialylation (Figure 2). Firstly, in N-glycans of Fetuin (*Bos taurus*) hypersialylation can arise due to a bisecting sialic acid (BiS) on N-acetyl-glucosamine (GlcNAc) (Figure 2a). Sialylation could also result from polysialic acid disialylation (PSD) (Neu5Ac-a2,8-Neu5Ac-a2,3-Gal) sometimes seen on N-glycans (Figure 2b). However, α2,6PTB has no recorded sialylation activity for GlcNAc substrate or polysialylation activity. The LC-MS/MS also does not support either of the BiS or PSD sialylation. There is a lack of HexNAc Neu5Ac C/Zo/B3 Y3 product ions (m/z 495) normally observed in the MS/MS for any alternative HexNAc Neu5Ac C/Zo/B3 Y3 product ions (m/z 495).

To better understand α2,6PTB activity towards already sialylated glycans the LC-MS/MS data were further investigated. A number of glycans were hypersialylated (contained two Neu5Ac on a single glycan antennae). Inspection of the MS/MS for those hypersialylated glycopeptides showed HexNAcHexose Neu5Ac Neu5Ac: B1 (m/z 948) product ion fragments in the MS/MS (Figure S1). No significant m/z 948 product ions were observed in the extracted ion chromatogram of any rAAT glycopeptides prior to α2,6PTB incubation, confirming the observed hypersialylation is the result of the α2,6PTB activity (Figure 1).

![Figure 1](image1.png)

*Figure 1. Extracted ion chromatogram in the LC-MS/MS at m/z 948 for; untreated (red) and α2,6PTB treated (blue) rAAT during glycopeptide elution of the three rAAT glycopeptide sites Asn-46, Asn-83 and Asn-247 (50-100 mins).*

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![Figure 2](image2.png)

*Figure 2. Proposed hypersialylation structures and potential diagnostic fragments of N-Glycans (a) Bisecting Neu5Ac (a2-6) sialylation of GlcNAc residue (b) Neu5Ac-a2,8-Neu5Ac-a2,3-Gal polysialylation (c) Neu5Ac-a2,3-(Neu5Ac-a2,6)-Gal double sialylation of a terminal galactose.*
Ethyl-esterification will lactonize α2,3/α2,8 sialic acids with subsequent loss of water (m/z -18), while the same reaction upon α2,6 sialic acids would yield an ethyl group (m/z +28). Therefore, if DSG is present we expected to see a m/z +10 shift (0±28-18+28) for some disialylactose standards (S7, Figure S3-S6). Hypersialylated tryptic MS/MS and a unique product ion at m/z 948 product ion fragment from the ethylation reaction, followed by LC-MS/MS analysis. The ethylated hypersialylated glycopeptides were identified in the LC-MS/MS and a unique product ion at m/z 958 was observed (Figure S7). This product ion is consistent with a HexNAcHexoseNeu5AcC2 product ion fragment with a m/z +10 shift compared with the B3 m/z 948 product ion fragment prior to the ethylation reaction. These data suggested that the hypersialylated structures observed on the rAAT following α2,6PTB treatment could be the result of DSG sialylation with PSD eliminated as a potential isomer. However, no HexoseNeu5AcC2 product ions were observed that would help eliminate the BiS isomer as BiS could also produce 948 product ions upon ethylation. Having failed to see the HexoseNeu5AcC2 product ion during the ethyl-esterification analysis permethylation of the released glycans was investigated to identify whether the BiS or DSG is present on the rAAT glycans.

Permethylation of glycans can be used to stabilize glycans allowing for comprehensive MS and MS/MS analysis even on labile glycan residues such as sialic acids. We released N-glycans from a trypsin digest of α2,6PTB treated rAAT, permethylated and analysed the glycans on a MALDI-TOF-MS/MS. A number of molecular species consistent with hypersialylated N-glycans were observed (Table S5, Figure S8-S10). The HexNAcHexoseNeu5AcC3 permethylated glycan ([M+Na]+ = m/z 3327.61) was analysed by MALDI-TOF-MS/MS to confirm DSG sialylation. The MS/MS of the HexNAcHexoseNeu5AcC3 produced unique fragments when compared to other sialylated species in the sample (Figure 3). A unique C3 HexoseNeu5AcC3 product ion at m/z 981 was observed. This is consistent with a single hexose containing two sialic acids, a product ion which cannot be produced by BiS sialylation, confirming DSG is indeed present on the rAAT. Other product ions consistent with DSG but not diagnostic include an intense B3 HexNAcHexoseNeu5AcC2 product ion at m/z 1208. Increases in intensity of the product ions at m/z 588 & 833 due to the increased scarring of hexose, as a result of the double sialylation on the terminal hexose residue were also observed, and are consistent with DSG being present. The diagnostic C3 fragment in combination with other product fragments confirms DSG as opposed to BiS being present on the rAAT.

As a further confirmation of structure, ion-mobility spectrometry (IMS) was used to distinguish DSG and BiS. IMS is an emerging tool in glycomic analysis, able to separate a variety of glycan isomers in the gas phase during MS analysis. We performed LC-MS/MS-IMS experiments on the DSG B1 m/z 948 product ion fragment from the α2,6PTB treated rAAT and compared it to the BiS B1 m/z 948 product ion fragment from a Fetuin standard. We observed a CCS 282.7±0.6 Å for the BiS species while the DSG product ion had a CCS of 295.1±0.2 Å (Figure 4). This highlighted that ion-mobility can differentiate these two isomeric structures.
suggests that MS glycomics can now comprehensively identify the DSG isomer if present in a complex sample for the first time.

Having confirmed the DSG on rAAT we attempted to exploit the DSG activity of α2,6PTB and generate a highly sialylated rAAT. First the amount of α2,3 sialylation already present on the rAAT needed to be increased. To achieve this we incubated the rAAT with the α2,3 sialyltransferase from Pasteurella multocida (α2,3PM) and GaIT for 16hr. α2,6PTB was added into the reaction mixture and incubated for a further 4hr with rAAT. A sequential enzyme incubation ensures a large amount of α2,3 sialic acids are present on the galactose of rAAT prior to introduction of the α2,6 PTB to the reaction. This reduces the α2,6 PTB activity towards any un-sialylated galactose. The activity of α2,6PTB is therefore pushed towards production of DSG formation as α2,3 sialylated galactose is in much larger abundance on the rAAT compared with the free galactose. The resulting LC-MS/MS analysis of the tryptic glycopeptides showed large increases in the multiply sialylated glycans and average SA count rose to 6.6 for the rAAT (Table 1 & S4).

The significant increase of SA count to 6.6 demonstrates how exploiting the DSG activity of α2,6PTB can be a powerful tool to significantly increase sialylation of biotherapeutics such as AAT. A SA count of 6.6 is higher than the reported 5.85 SA count of native AAT.29 It is well known in the literature that the greater the level of sialylation of a biotherapeutic the longer the half-life of the drug in the body, resulting in improved patient benefits.3 Our highly sialylated rAAT may therefore offer significant benefits for AATD patient treatment by extending the life time of the drug in the body. This highlights how α2,6PTB DSG activity can be used to help generate the new generation of heavily sialylated biotherapeutics.

The influence of DSG on the activity of rAAT was next investigated. After purification, using affinity chromatography, an ELISA elastase activity assay showed that the activity before (89.3%) and after glycan remodelling of the rAAT (95.4%) was comparable (Table S6). The enzyme treated rAAT having activity of 95.4% is also comparable to native human plasma rAAT (100%). These data suggest that the DSG sialylation and the incubation process has no significant effect on the in vitro activity of the remodelled rAAT.

Finally, reactivity of DSG towards sialidase from Arthrobacter ureafaciens treatment was investigated. The tryptic glycopeptides of the α2,6PTB treated rAAT were treated with sialidase from and subsequently analysed by LC-MS/MS. The results of the sialidase testing showed that the DSG offered no apparent sialidase resistance with overall sialylation dropping from 93% to 2% similar to that seen in a control (87% drop to 4%) (Table S7). Although DSG does not appear to be resistant to (ABS) it would be of significant interest to test a wider range of sialidases especially human and viral sialidases however, that was beyond the scope of this study.

Further biological studies into DSG beyond those presented here are required to assess possible immunogenic or unwanted biological response to DSG. In vivo neonatal rat studies by Xi Chen et. al. of the DSG structure on a small oligosaccharide reported no adverse effects, which gives some evidence any extreme biological response would be unlikely29, although the response to DSG when conjugated to a protein such as AAT remains to be evaluated. Little is known about the biological role and/or function of DSG and further wide ranging biological studies would be of significant interest, especially to the glycobiology community. Our methods now provides an easy way for researchers to prepare DSG onto protein probes and identify DSG in any subsequent MS analysis. This will hopefully allow for wider study of DSG and its potential applications and biological roles.

In this study we present a method for generation of a highly sialylated therapeutic glycoprotein by installing the unique DSG motif to rAAT. We are able to demonstrate a robust method to add two sialic acids to a single galactose on a glycoprotein by exploiting the DSG activity of α2,6PTB. This new method for increasing sialylation of biotherapeutic could be used for increasing the pharmacokinetic properties of recombinant biotherapeutics, such as AAT. In particular the half-life maybe significantly increased due to the increase of sialylation. More pharmacokinetic, immunogenic and biological studies of DSG are required however, previous in vivo studies of DSG reported no adverse affects and we showed sialylation due to DSG does not affect in vitro activity of rAAT. We also present a detailed and in-depth analysis of DSG through hyphenated MS analysis techniques including ion mobility spectrometry. The identification of diagnostic peaks and features should enable other groups to easily identify DSG in future MS glycomic studies or even to reassess previous work.

ASSOCIATED CONTENT
Supporting Information
Supporting Information is available free of charge on the ACS Publications website.

Supporting Information Contains: All Experiments, Supplementary Figures and Supplementary Tables (PDF).

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Notes
The authors declare no competing financial interests.

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