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Gly-Q

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Glycan Remodeling of Therapeutic Proteins using Galactosyltransferase and Sialyltransferases

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ABSTRACT

Glycosylation of therapeutic proteins can play critical roles in product quality and efficacy; controlling or optimizing glycosylation has many potential benefits. Terminal galactosylation has been shown to directly impact complement-dependent cytotoxicity (CDC); terminal sialylation can affect serum half-life; core fucosylation modulates antibody-dependent cellular cytotoxicity (ADCC); terminal and bisecting N-acetylglucosamines and high mannose glycans can impact ADCC as well as rate of clearance. An alternative strategy for modifying glycosylation is through the use of selected glycosyltransferase enzymes in a post-expression setting. In this way, glycomodified products can be quickly generated for various applications, such as structure-activity relationship (SAR) studies and quality-by-design (QbD) experiments. There is even potential for the use of glycosyltransferases as a means for generating glyco-opitimized therapeutics. Presented here are the results from glycomodifications performed on Rituxan® and Enbrel® using β(1–4)-Galactosyltransferase (GalT) and two sialyltransferases, α(2–6)-Sialyltransferase (ST6) and α(2–3)-Sialyltransferase (ST3). Analysis of 2-AB labeled N-glycans by UPLC®-FLR demonstrates the ability to generate biotherapeutics with varying degrees of galactosylation and sialylation.

INTRODUCTION

During the drug development process, companies often perform experiments to better understand the relationship between structure and activity (SAR studies). Because of the nature of glycosylation, the generation of both a broad range of glyco-variants and an adequate quantity of each variant can be extremely difficult. A more expeditious alternative to modulating glycosylation by adjusting/modifying cell culture conditions is secondary remodeling using glycosyltransferases. By using glycosyltransferases in a post-expression setting, specific glycosylation attributes can be adjusted, which allows for more direct control in the generation of glyco-variants. In the same way, glycosyltransferases offer an interesting alternative to traditional Quality by Design (QbD) experiments. Implementing glycosyltransferases could greatly increase the process space with regard to glycosylation; in effect, compensating and correcting for conditions that do not yield a product with the correct glycosylation.

Additionally, the expiration (or impending expiration) of the patents for many innovator biotherapeutics has resulted in widespread activities toward the development of biosimilars. One of the key product characteristics that companies must pay close attention to is glycosylation; target glycosylation profiles have been set by innovator drugs, and therefore biosimilar developers must develop products with comparable profiles. The most common means of controlling or adjusting a product's glycosylation is by closely controlling cell culture conditions; unfortunately, this may not be adequate. A potential alternative approach to obtaining the desired glycosylation profile is secondary remodeling using glycosyltransferases. Further, glycosyltransferases may offer the potential for a more direct means of improving glycosylation to generate glyco-optimized "biobetters".

MATERIALS AND METHODS

Samples

Rituxan (rituximab) is a recombinant chimeric monoclonal antibody. It contains predominantly neutral, core-fucosylated biantennary N-glycans; lower levels of high-mannose and trace amounts of sialylated species are also present. Enbrel (etanercept) is a fusion protein composed of the Fc domain of human IgG and the p75 tumor necrosis factor receptor (TNFR). It contains a mixture of neutral and sialylated biantennary N-glycans. The majority of the Fc-domain N-glycans are neutral core-fucosylated biantennary species (predominantly G0F and G1F), while the majority of the TNFR N-glycans are sialylated biantennary species (predominantly A1, A1F, A2, A2F).

Transferases

- GalT (EC 2.4.1.38) (product code GKT-GA14) is used for the synthesis of Galβ1-4GlcNAc by transfer of galactose from UDP-Gal to GlcNAcβ1–2Man.
- ST6 (EC 2.4.99.1) (product code GKT-S26) is used for the synthesis of NeuAc2-6Gal by transfer of NeuAc from CMP-NANA to Galβ1-4GlcNAc.
- ST3 (Roche developmental product) is used for the synthesis of NeuAc2–3Gal by transfer of NeuAc from CMP-NANA to Galβ1–4GlcNAc.

Transferase Reactions

All transferase reactions were performed at 37°C for 6 hours. The reactions consisted of 50 µg of protein and varying amounts of the transferase enzymes (0.5–2.5 µg GalT with 150 µg UDP-Gal; 0.5–5 µg ST6 or 0.1–5 µg ST3 with 150 µg CMP NANA; or a combination) in a 100 mM MES, 10 mM MnCl2, pH 6.5 buffer. For reactions utilizing only ST6, a 50 mM Tris-acetate buffer at pH 7.5 was used. Samples were purified using AssayMAP PA50 Cartridges (product code G5524-60010).

Preparation and analysis of N-glycans

N-glycan samples were prepared using GlykoPrep® Rapid N-Glycan Sample Preparation with 2-AB (product code GP96NG-AB). UPLC analysis was performed with a Waters ACQUITY UPLC® (H-class) with a fluorescence detector (ex: 360 nm; em: 428 nm). A 1 µl aqueous injection of 2-AB glycans was separated over Waters ACQUITY UPLC BEH glycan column (1.7 µm, 2.1 x 100 mm) at 60°C using a 10 minute method.

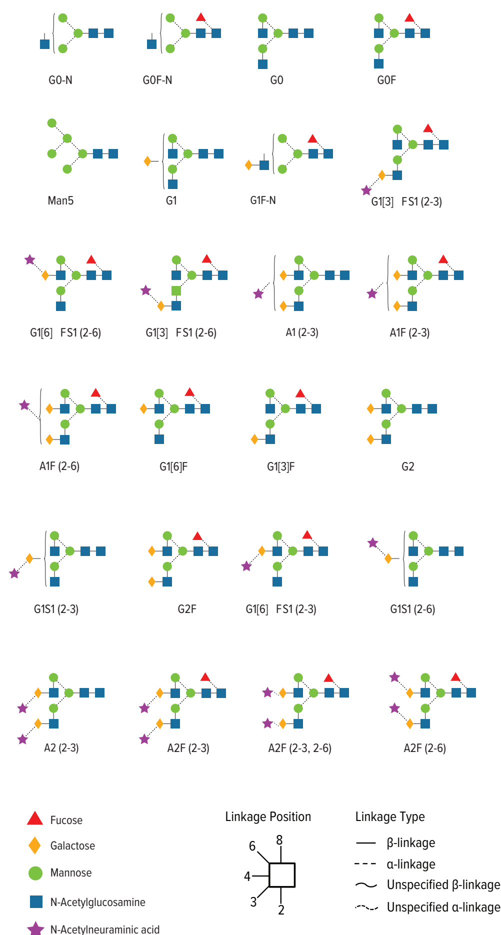


Figure 1: Structures of the N-Glycan profiles referred to in Figures 2–6, Tables 1–3, and the text. Numbers in brackets, e.g. [3 or 6], refer to arm structure; numbers in parentheses, e.g. (6), refer to linkage position.

RESULTS AND DISCUSSION

Galactosyltransferase Reaction

Rituxan was treated with varying amounts of GalT (Figure 2 and Table 1). At lower amounts (0.5 & 1 µg GalT), the non-galactosylated species (G0F) was converted to a mixture of mono-galactosylated (G1F) and fully-galactosylated (G2F) species. At higher levels of GalT (2.5 µg), all of the target glycans were converted G2F (comprising >90% of the total N-glycan population). The remaining 8% of glycans consists of those that are either galactosylated as much as possible (e.g., G1F-N, G2, A1F, A2F) or are not substrates for GalT (e.g., Man5).

Sialyltransferase Reaction

Rituxan was separately treated with ST3, ST6 as well a combination of ST3 and ST6 (Figure 3 and Table 1). Treatment with ST6 resulted in a nearly complete conversion of G1[3]F(8.6%) and G2F (10.1%) to the 2–6 linked sialylated species of G1FS1, A1F, and A2F. There was also conversion of G1[6] F; a decrease from 30.8% to 18.2% was observed. ST6 is known to have a preference for the α(1–3) Mannose arm over the α(1–6) Mannose arm, which could explain the lack of full conversion.1,2 Treatment with ST3 did not result in complete conversion of either G1F isomer, but bias was not observed; G2F was completely converted. Treatment with a combination of both ST3 and ST6 resulted in a distribution of sialylated glycans; the addition of the mixed linkage 2–6, 2–3 A2F is a notable difference. Interestingly, 17.5% of G1[6]F remained.

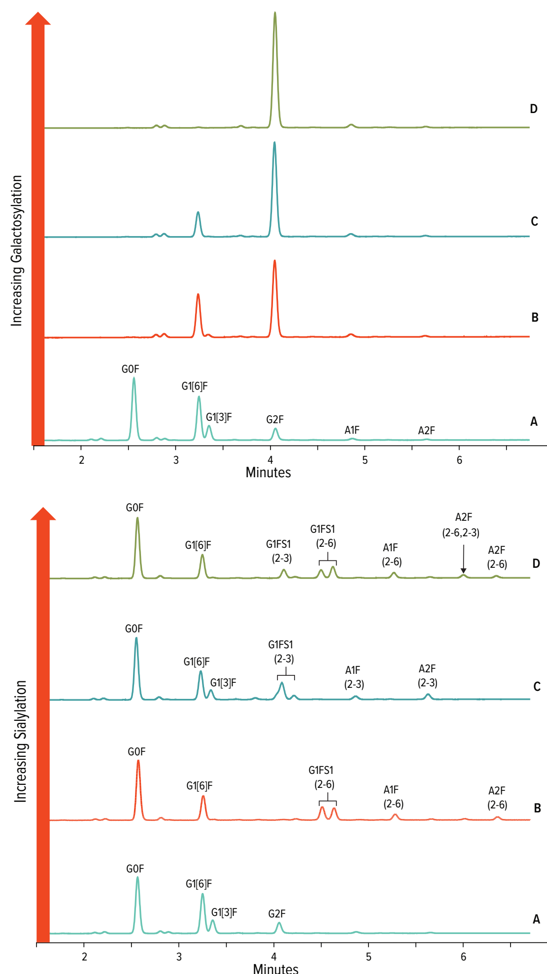


Figure 2: Modification of Rituxan with Galactosyl-transferase (GalT). UPLC profile of (A) 2AB-labeled Rituxan N-glycans after incubation with (B) 0.5 µg, (C) 1 µg, and (D) 2.5 µg GalT. Decreases in G0F and G1F correlate with increases in G2F.

Figure 3:Modification of Rituxan with Sialyltrans-ferases (ST3 and ST6). UPLC profile of (A) 2AB-la-beled Rituxan N-glycans after incubation with (B) 5 µg ST6, (C) 5 µg ST3, and (D) both 5 µg ST6 & 5 µg ST3. A moderate amount of sialylation is occurring on G1F and G2F to produce mono- and di-sialylated glycans.

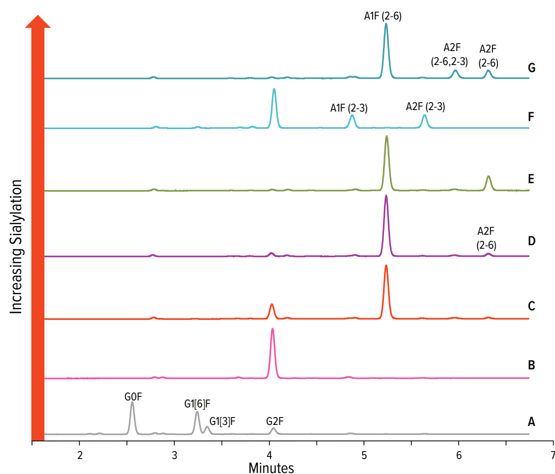


Figure 4: Modification of Rituxan with GalT, ST6, and ST3 to Obtain Higher Degree of Sialylation. UPLC profile of (A) 2AB-labeled Rituxan N-glycans after modification with (B) 2.5 µg GalT, (C) 2.5 µg GalT & 0.5 µg ST6, (D) 2.5 µg GalT & 1 µg ST6, (E) 2.5 µg GalT & 5 µg ST6, (F) 2.5 µg GalT & 5 µg ST3, and (G) 2.5 µg GalT, 5 µg ST6, & 5 µg ST3. Increasing amounts of ST6 result in a greater conversion of G2F to mono- and di-sialylated glycans; ST3 generates a smaller amount of these glycans. In a one pot reaction, ST6 shows greater activity than ST3.

One-pot Galactosyltransferase and Sialyltransferase Reaction

The combination of both the galactosyltransferase and sialyltransferase(s) in the same reaction resulted in a much higher degree of sialylation than either sialyltransferase alone; see Figure 4 and Table 1 for details. Individual treatment with either ST6 or ST3 did not yield significant increases in sialylation of Rituxan; see Figure 3. Following treatment with GalT and ST6, galactosylation was nearly complete and sialylation was markedly elevated; the major glycans were A1F (2–6) at 70.1% and A2F (2–6, 2–6) at 19.1% and A2F (2–3, 2–6) at 2.5%. Treatment with the combination of GalT and ST3 was not as productive; A1F showed an increase of less than 20% (from 1.5% to 20%), A2F increased from 0.7% to19.4% and a large percentage (53.7%) of G2F remained unmodified by ST3. In reactions with all three transferases, most of the sialyltransferase activity was performed by ST6, which is evident in the increase of A1F and A2F peaks bearing only 2–6 linked sialylation; ST3 activity was evident from the appearance of G1FS1 (2–3) and A2F (2–3, 2–6). The total population of sialylated glycans was increased to greater than 96%.

Name	Rituxan	+0.5 µg GalT	+1 µg GalT	+2.5 µg GalT	+5 µg ST6	+5 µg ST3	+5 µg ST3 +5 µg ST6	+2.5 µg GalT +0.5 µg ST6	+2.5 µg GalT +1 µg ST6	+2.5 µg GalT +5 µg ST6	+2.5 µg GalT +5 µg ST3	+2.5 µg GalT +5 µg ST3 +5 µg ST6
G0-N	0.1											
G0F-N	0.9				0.9	0.8	0.9					
G0	1.1				1.0	1.0	1.1					
G0F	42.2				43.8	41.7	43.3					
Man5	1.9	1.9	2.0	2.0	1.9	1.8	1.8	1.7	1.8	1.8	2.2	1.7
G1 & G1F-N	1.2	2.5	2.3	1.8	0.1							
G1[6]F	30.8	31.7	18.6		18.2	20.4	17.5					
G1[3]F	10.1	2.0			0.6	6.9	0.6				1.8	
G2	0.2	0.7	1.1	1.6	0.3			0.7			1.0	
G1S1 (2-3)	0.3			0.3	0.5	1.4	0.6	0.5	0.6	0.6	2.0	0.7
G2F	8.6	57.7	72.5	90.7	0.3			18.5	4.2	1.4	53.7	1.5
G1[6]FS1 (2-3)					0.4	16.1	8.2					
G1S1 (2-6)					1.2			1.3	1.3	1.5		
G1[3]FS1(2-3)	0.2					3.0	1.5					1.2
G1[6]FS1 (2-6)					11.4		6.0					
G1[3]FS1 (2-6)					9.7		8.5					
A1F (2-3)	1.5	2.7	2.7	2.9	0.5	2.7	0.7	1.6	2.5	2.3	20.0	2.0
ND												1.7
A1F (2-6)					4.7		4.1	71.0	83.3	70.1		69.1
A2F (2-3)	0.7	0.9	0.8	0.7	0.8	4.3	1.6	0.8	0.7	0.8	19.4	0.8
A2F (2-3, 2-6)					1.0		2.2	1.8	2.0	2.5		11.0
A2F (2-6)					2.7		1.5	1.5	3.6	19.1		10.3

Table 1: % Peak Area of Rituxan N-glycans. Modification of Rituxan with GalT, ST6, & ST3.

Transferase Reactions on Enbrel

Galactosyltransferase reaction (Figure 5 and Table 2). Enbrel was treated with varying amounts of GalT. At lower amounts (0.5 & 1 µg GalT), G0F and G1FS1 were converted to galactosylated species; a moderate amount of G1F remained. At higher levels of GalT (2.5 µg), nearly complete galactosylation was observed with just over a total of 1% galactosylatable species remaining. Both G0F and G1F were reduced to 0.3% from 18.3% and 11.9%, respectively, while G2F increased from 4.9% to 36%. The sialylated glycans were not greatly affected; those with an available site for galactose became further galactosylated.

Sialyltransferase Reaction

Treatment of Enbrel with ST3 (Figure 6 and Table 2) resulted in an overall increase in sialylation. Glycans with available sialylation sites (G1F, G2, G2F, A1 and A1F) showed marked decreases in peak area, and a concomitant increase in the peak areas for sialylated species: G1FS1 increased from 3.2% to 11.1%, A2 increased from 4.2 to 17.8% and A2F increased from 10.7 to 29.6%.

One-pot Galactosyltransferase and Sialyltransferase Reaction

The combination of both the GalT and ST3 in a single reaction resulted in nearly complete galactosylation and a much higher degree of sialylation than treatment with ST3 alone (Figure 7 and Table 3). An increase of ~10% for A1F (2–3) and ~14% for A2F (2–3) were observed.

Controlling sialylation of Enbrel to obtain desired profile. The production of therapeutic glycoproteins can result in a wide variety of glycosylation profiles. Even with tightly-controlled and monitored cell culture conditions, the desired profile may not be achieved. In these cases, secondary remodeling may be a useful alternative. To demonstrate the ability to modulate sialylation to a desired target, Enbrel was treated with Sialidase A to remove all of the sialic acids and then re-sialylated to varying degrees using ST3. Varying amounts (0.1 to 5 µg) of ST3 were applied. A profile comparable to that of the original unmodified Enbrel material was achieved by treatment with 0.5 µg ST3.

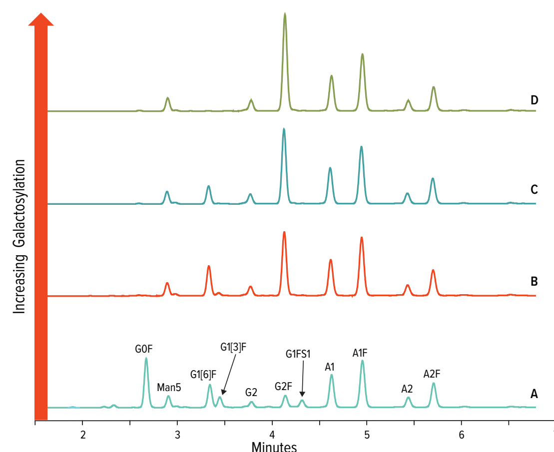


Figure 5: Modification of Enbrel with GalT. UPLC profile of (A) 2AB-labeled Enbrel N-glycans after incubation with (B) 0.5 µg, (C) 1 µg, and (D) 2.5 µg GalT. Decreases in G0F and G1F correlate with increases in G2F.

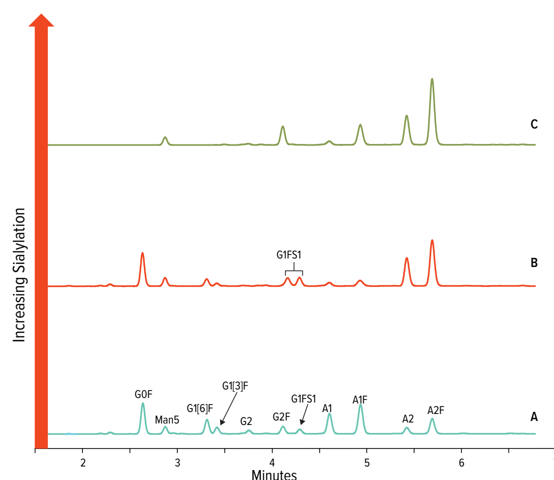


Figure 6: Modification of Enbrel with GalT and ST3 to Obtain Higher Degree of Sialylation. UPLC profile of (A) 2AB-labeled Enbrel N-glycans after modification with (B) 5 µg ST3, which results in partial conversion of G1F into G1FS1 and of A1/A1F into A2/A2F. (C) Modification of Enbrel with 2.5 µg GalT & 5 µg ST3 results in complete galactosylation of G0F and G1F and further increases the pool of completely sialylated glycans.

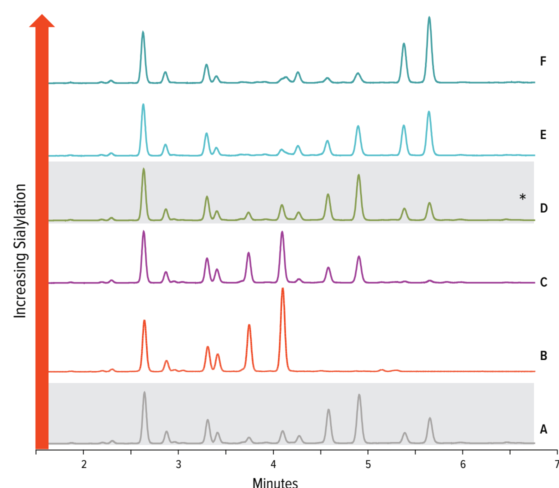


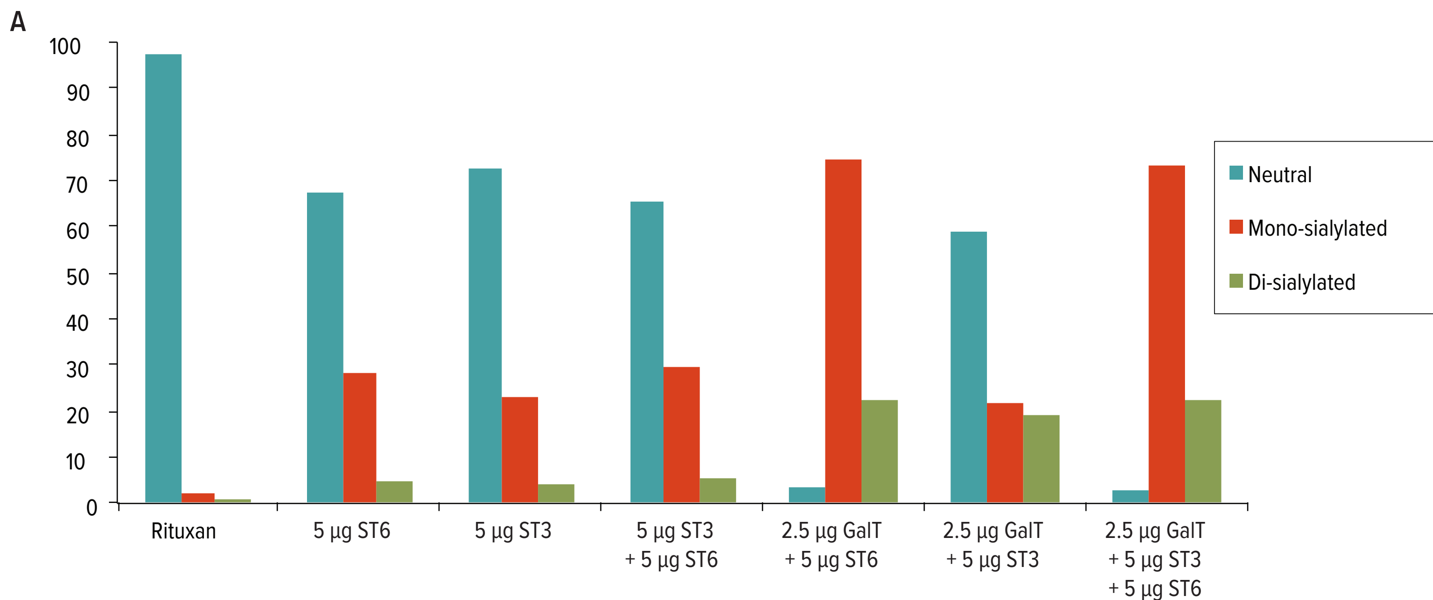
Figure 7: Controlling Sialylation of Enbrel to Obtain Desired Profile. UPLC profile of (A) 2AB-labeled Enbrel N-glycans followed by (B) digestion with Sialidase A. After removal of sialic acids, glycomodification with increasing amounts of ST3 results in a wide range of sialylation; (C) 0.1 µg ST3, (D) 0.5 µg ST3, (E) 1 µg ST3, and (F) 5 µg ST3. *Profile most similar to Enbrel.

Name	Enbrel	+0.5 µg GalT	+1 µg GalT	+2.5 µg GalT	+5 µg ST3	+2.5 µg GalT +5 µg ST3
G0-N	0.2				0.2	
G0F-N	0.3				0.3	
G0	0.9				1.0	
G0F	18.3	0.2	0.2	0.3	18.3	
Man5	4.3	4.6	4.5	4.7	4.6	4.4
G1	0.6	0.7	0.6	0.3	0.2	
G1[6]F	9.0	10.8	6.6	0.3	4.2	
G1[3]F	4.2	1.1	0.3		1.7	0.4
G2	2.4	3.9	4.0	4.2	0.6	1.1
G1S1 (2-3)	0.5				0.6	0.3
G2F	4.9	24.0	28.3	36.1		11.3
G1[6]FS1 (2-3)	0.2	0.3	0.3	0.2	5.8	0.4
G1[3]FS1 (2-3)	3.0	0.2	0.2	0.2	5.3	
A1 (2-3)	13.8	14.1	14.5	13.9	2.7	3.0
A1F (2-3)	20.4	23.3	23.6	23.2	4.3	14.2
A2 (2-3)	4.2	4.2	4.3	4.1	17.8	18.9
A2F (2-3)	10.7	10.4	10.6	10.3	29.6	43.7

Table 2: % Peak Area of Enbrel N-glycans. Modification of Enbrel with GalT & ST3.

Name	Enbrel	+Sial A	+Sial A +0.1 µg ST3	+Sial A +0.5 µg ST3	+Sial A +1 µg ST3	+Sial A +5 µg ST3
G0-N	0.2	0.2	0.2	0.2	0.2	0.2
G0F-N	0.3	0.3	0.4	0.4	0.4	0.4
G0	0.9	0.9	1.0	0.9	0.9	0.9
G0F	18.3	19.4	19.7	19.5	19.1	18.9
Man5	4.3	4.3	4.3	4.2	4.1	4.2
G1	0.6	0.7	0.6	0.4	0.3	0.2
G1[6]F	9.0	9.9	9.9	9.5	8.7	7.2
G1[3]F	4.2	7.0	5.7	3.9	3.2	2.7
G2	2.4	19.7	13.0	3.2	0.4	0.4
G1S1 (2-3)	0.5	0.2	0.2	0.6	0.6	0.5
G2F	4.9	34.4	21.7	6.6	3.6	
G1[6]FS1 (2-3)	0.2			0.3		3.9
G1[3]FS1 (2-3)	3.0		1.3	3.3	4.1	4.5
A1 (2-3)	13.8		6.4	11.6	6.9	2.4
A1F (2-3)	20.4		11.9	20.1	13.1	4.9
A2 (2-3)	4.2		0.6	5.1	12.9	16.9
A2F (2-3)	10.7		1.2	7.9	19.1	28.9

Table 3: % Peak Area of Enbrel N-glycans. Modification of Enbrel with Sial A & ST3 to obtain desired profile.



The sialylation for Rituxan normally comprises ~3% of its glycan species. By treatment with either ST3 or ST6, alone, that value was increased to 27–35%. When treated with a combination of GalT and ST3, sialylation was increased to 41%, while the combination of GalT and ST6 resulted in an increase of sialylation to greater than 96%. The combination of GalT and both ST3 and ST6 also resulted in an increase of sialylation to greater than 96%. The distribution of neutral and sialylated glycans are displayed in Figure 8A. Enbrel is normally more sialylated (>50%) than Rituxan. After treatment with ST3, sialylation increases to greater than 65%. When treated with a combination of GalT and ST3, sialylation increases to greater than 80%. The distribution of neutral and sialylated glycans are displayed in Figure 8B.

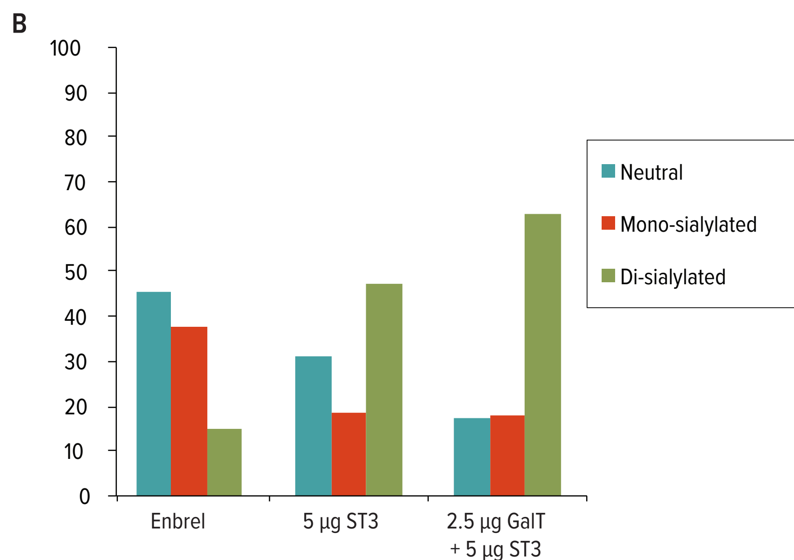


Figure 8: Overview of Sialylation. N-glycans from (A) Rituxan and (B) Enbrel and their glyco-modified forms are divided into neutral, mono-sialylated, or di-sialylated glycans.

CONCLUSIONS

The effectiveness of glycosyltransferases to generate glycomodified therapeutics has been demonstrated:

1. Reactions can be accomplished in 6 hours
2. GalT is effective in altering the glycosylation profile of therapeutic proteins such as Rituxan and Enbrel to achieve a range from partial to complete galactosylation
3. Modification of specific sialic acid linkages can be controlled by ST6 or ST3
4. Effectiveness in altering the glycosylation profile varies with the protein. Acting alone, ST6 and ST3 show a modest increase in sialylation. But in combination with GalT, ST can dramatically increase sialylation
5. The ability to modify the glycosylation profile to closely resemble the original was demonstrated
6. A range of glycovariants can be generated to support SAR studies and QbD experiments
7. Potential exists for using transferases to generate biosimilar as well as glyco-optimized "biobetter" therapeutics

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