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Chemoenzymatic glyco-engineering of monoclonal antibodies

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Summary

Monoclonal antibodies (mAbs) are an important class of therapeutic glycoproteins widely used for the treatment of cancer, inflammation, and infectious diseases. Compelling data have shown that the presence and fine structures of the conserved N-glycans at the Fc domain can profoundly affect the effector functions of antibodies. However, mAbs are usually produced as mixtures of Fc glycoforms and the control of glycosylation to a favorable, homogeneous status in various host expression systems is still a challenging task. In this chapter, we describe a detailed procedure of chemoenzymatic glyco-engineering of monoclonal antibodies, using rituximab (a therapeutic monoclonal antibody) as a model system. The protocol includes the deglycosylation of a mAb by an endoglycosidase (such as wild type EndoS) to remove the heterogeneous Fc N-glycans, leaving only the innermost GlcNAc or the core-fucosylated GlcNAc at the glycosylation site. Then the deglycosylated IgG serves as an acceptor for an endoglycosidase-catalyzed transglycosylation to add a desired N-glycan to the GlcNAc acceptor to reconstitute a defined, homogeneous natural glycoform of IgG, using a glycosynthase mutant as the enzyme and activated glycan oxazoline as the donor substrate. A semi-synthesis of sialylated and asialylated biantennary N-glycan oxazolines is also described. This detailed procedure can be used for the Fc glycosylation remodeling of other mAbs to provide homogeneous Fc glycoforms for various applications.

Keywords

monoclonal antibody; Fc glycosylation; glyco-engineering; chemoenzymatic synthesis; transglycosylation; ADCC

1. Introduction

Monoclonal antibodies (mAbs) are an important class of therapeutic proteins widely used for the treatment of cancer, autoimmune disorders, and infectious diseases (1,2). The IgG type mAbs consist of two light and two heavy chains that are covalently associated to form three domains, two identical Fab domains (responsible for antigen binding) and the Fc domain (responsible for downstream effector functions after antigen binding). The Fc domain is a homodimer bearing two conserved N-glycans, each of which is attached to the Asn-297 (the conserved N-glycosylation site). The Fc glycans are typical biantennary complex type N-glycans with significant structural heterogeneity due to terminal and core

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modifications (e.g., incomplete galactosylation and sialylation, addition of bisecting N-acetylglucosamine and a core fucose) (3). A minor fraction of IgG glycans can also bear hybrid or high mannose glycans causing further heterogeneity. The N-glycan has been shown to influence the binding of IgG to certain Fc receptors such as Fc γ RIIIa and complement component C1q (4). This influence over binding correlates directly to IgGs effector functions, including the antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Other studies have shown that the anti-inflammatory properties of IgG are dependent on the terminal sugars attached such as sialylation and/or full galactosylation (5–7).

The significant impact of fine structures of Fc N-glycans on the biological functions and therapeutic outcome of antibodies has stimulated tremendous interest in developing methods to control antibody's Fc glycosylation. A common approach involves the glycan biosynthetic pathway engineering in various host expression systems. These have been attempted in mammalian, yeast, and plant cell lines aiming to control the glycosylation profiles when producing mAbs in these host expression systems (8). This approach has resulted in the production of low-fucose or non-fucosylated monoclonal antibodies with improved ADCC activities and anti-cancer therapeutic efficacy. However, complete control of the Fc glycosylation to a given specific homogeneous glycoform is still a challenging task. We have recently described a chemoenzymatic approach for Fc glycan remodeling of antibodies using endoglycosidases such as EndoS from *Streptococcus pyogenes* and their mutants (9–11). EndoS is an endo- β -N-acetylglucosaminidase that is highly specific for the Fc N-glycans of IgG (12). Previously our lab and others have explored the use of endoglycosidase-based glycosynthases to transfer N-glycans to a core GlcNAc-containing peptide and protein including antibody Fc domain (13). This chemoenzymatic synthesis is dependent on the use of an activated glycan oxazoline as the donor substrate which mimics the transition state of the hydrolysis reaction. The glycosynthase mutant contains a point mutation in the active side of the glycosidase domain that inactivates the hydrolytic activity of the enzyme. We found that similar glycosynthase mutants of EndoS were highly efficient for the chemoenzymatic synthesis of various glycoforms of rituximab (9).

In this chapter, we describe a detailed procedure for the chemoenzymatic glyco-engineering of monoclonal antibodies, using rituximab, a therapeutic monoclonal antibody, as a model system (Fig. 1). The protocol includes the deglycosylation of rituximab by the wild type enzyme (EndoS WT) to remove the heterogeneous Fc N-glycans, leaving only the innermost GlcNAc or the core-fucosylated GlcNAc at the glycosylation site. Then the deglycosylated IgG serves as an acceptor for the endoglycosynthase-catalyzed transglycosylation to add the desired N-glycan to the GlcNAc acceptor from the glycan oxazoline substrate to reconstitute a defined, homogeneous natural glycoform of IgG. A semi-synthesis of the sialylated and asialylated biantennary N-glycan oxazolines from sialylglycopeptide (SGP) isolated from chicken egg yolks is also described (Fig. 2). The purity of the homogeneous glycoforms of rituximab remodeled by the chemoenzymatic was confirmed by LC-ESI-MS analysis.

2. Materials

All buffers and solutions were prepared by using Millipore water (ddH₂O) unless indicated otherwise. The NdoS gene can be purchased from addgene (plasmid 44655) or can be cloned from genomic DNA as described (14). The recombinant proteins EndoS WT-GST and EndoS D233Q-GST were prepared according to procedures described previously (9). The enzyme EndoS WT is also available from New England Biolabs (NEB). Sialylglycopeptide (SGP) was purified from chicken egg yolks according to procedures described previously (15). Alternatively SGP is also available from Tokyo Chemical Industry (TCI). EndoM WT was expressed according to the procedures described previously (16), which is also available from TCI.

2.1 Antibodies, Enzymes, and Substrates

1. Recombinant monoclonal antibody (mAb) Rituxan (rituximab) (Genetech, Inc., South San Francisco, CA) (*see* Note 1)
2. Deglycosylated rituximab (prepared as described in Subheading 3.2)
3. Recombinant EndoS WT (9)
4. Recombinant EndoM WT (16)
5. Recombinant EndoS D233Q (9)
6. Neuraminidase
7. Sialylglycopeptide (SGP) from chicken egg yolk (15)
8. Sialylated complex type glycan (SCT) (prepared as described in Subheading 3.4)
9. Sialylated complex type glycan oxazoline (SCTox) (prepared as described in Subheading 3.5)
10. Complex type glycan (CT) (prepared as described in Subheading 3.4)
11. Complex type glycan oxazoline (CTox) (prepared as described in Subheading 3.5)

2.2 Buffers, Columns, and Reagents

1. HiTrap Protein A column (GE Healthcare, Little Chalfont, United Kingdom)
2. HiTrap MonoQ XL column (GE Healthcare, Little Chalfont, United Kingdom)
3. Symmetry 300Å C18 Prep column 7 µm 19 × 300 mm
4. Sephadex-G15
5. CarboPac PA200 3 × 250 mm (Thermo Scientific, Waltham, MA)
6. CarboPac PA200 guard column 3 × 50 mm (Thermo Scientific, Waltham, MA)
7. Poroshell 300SB-C8 column 5 µm 1 × 75 mm (Agilent, Santa Clara, CA)

¹In the present study, we describe the detailed procedure of the chemoenzymatic glyco-engineering of monoclonal antibodies using rituximab as a model. This procedure is also applicable for glyco-engineering of other mAbs.

8. Amicon Ultra Centrifugal Filters Ultracel 30,000 NMWL (Millipore)
9. LC-MS Solvent A: 0.1% formic acid (v/v) in ddH₂O
10. LC-MS Solvent B: 0.1% formic acid (v/v) in acetonitrile (ACN)
11. HPAEC-PAD Eluent A: 100 mM NaOH
12. HPAEC-PAD Eluent B: 100 mM NaOH, 250 mM NaOAc
13. Protein A wash buffer: 1x Phosphate-buffered saline (PBS), pH 7.4
14. Protein A elution buffer: 100 mM Glycine-HCl, pH 2.8
15. Protein A neutralization buffer: 1.5 M Tris HCl, pH 8.8
16. 1 M Tris HCl pH 7.4
17. 300 mM NaCl
18. 10% sulfuric acid in ethanol: Slowly add 10 mL concentrated sulfuric acid to 90 mL ethanol in a wide mouth glass jar. The resulting solution will be hot especially if larger amounts are prepared.
19. Thin layer chromatography (TLC) silica gel 60 plate (Millipore, Billerica, MA)
20. Master ProHeat heat gun (Master Appliance, Racine, WI)
21. Tris(2-carboxyethyl)phosphine (TCEP) HCl
22. 1,3-Dimethyl-2-imidazolidinone (DMC)
23. Triethylamine (TEA)

2.3 Equipment and Software

1. Accela LC System attached to a LXQ linear ion trap mass spectrometer (Thermo Scientific, Waltham, MA)
2. ICS-5000 ion chromatography system consisting of an AS-AP autosampler, Electrochemical Detector, and SP pump (Thermo Scientific, Waltham, MA)
3. 626 HPLC instrument (Waters, Milford, MA)
4. 600 HPLC instrument (Waters, Milford, MA)
5. Akta FPLC with UPC-900 and P-920 (GE Healthcare, Little Chalfont, United Kingdom)
6. Micromass ZQ-4000 single quadrupole mass spectrometer (Waters, Milford, MA)
7. NanoDrop 2000c (Thermo Scientific, Waltham, MA)
8. MyCycler Thermal Cycler (Bio-Rad, Hercules, CA)
9. Xcalibur for raw MS data analysis (Thermo Scientific, Waltham, MA)
10. BioWorks for raw data deconvolution (Thermo Scientific, Waltham, MA)
11. Unicorn manager (GE Healthcare, Little Chalfont, United Kingdom)

12. Chromeleon 7 chromatography management system (Thermo Scientific, Waltham, MA)

2. Methods

2.1 LC-ESI-MS analysis of Rituximab

1. Buffer exchange of the commercial rituximab: Apply 20 mg of rituximab to the 30 kDa MWCO centrifugal filter unit and centrifuge at $6,000 \times g$. Wash three times with 4 mL of 1x PBS and resuspend the antibody into 1x PBS to a final concentration of 10 mg/mL.
2. Liquid chromatography-Mass spectrometry: Add 1 μ L of rituximab (10 μ g) into 17 μ L of ddH₂O with 0.1% formic acid (*v/v*) in a MS sample vial. Add 2 μ L of 500 mM TCEP (final concentration, 50 mM) and incubate mixture at 37 °C for 30 minutes.
3. Inject 10 μ L of the sample (*see* Note 2). LC conditions are 25% to 35% B in 6 minutes followed by a 4 minute 90% B wash and 4 minute 25%B equilibration. Under the following conditions the light chain of rituximab elutes in two peaks around 3 minutes and the heavy chain of rituximab elutes around 5 minutes.
4. Process and analyze the heavy chain data using Xcalibur qual browser. Process the respective raw data in BioWorkds to get the deconvoluted mass spectra of the heavy chain (Fig. 3a) (*see* Note 3).

3.2 Deglycosylation of Rituximab

1. Add 10 μ L of EndoS WT (100 μ g) to 1 mL of rituximab (10 mg). Incubate the mixture at 37°C for 1 hour (*see* Note 4).
2. Monitor the Fc deglycosylation process by LC-ESI-MS (*see* Subheading 3.1). Complete deglycosylation was achieved within 1 hour of incubation (Fig. 3b).
3. Purify the deglycosylated rituximab by Protein A affinity chromatography (*see* Subheading 3.3).

²Adjust injection volume to your MS system. Treatment with TCEP reduces the antibody to heavy and light chains which can be separated and analyzed separately. The reduction of the heavy chain to a monomeric form allows for better analysis.

³The retention times of the heavy and light chains depend on the column, temperature, glycosylation, and the amino acid sequence. When calculating the theoretical molecular weight it is important to consider the typical post translational modifications found on mAbs such as Gln to pyro-Glu conversion found on both the heavy and light chains as well as the cleavage of the C-terminal lysine on the heavy chain. Also the complete reduction of two intrachain disulfide from the light chain and of four intrachain disulfide bonds in the heavy chain leads to an expected mass of 23039 daltons for the light chain and 49067 daltons for the heavy chain. The addition of respective N-glycan to the Asn 297 of the heavy chain accounts for a mass of 50507 daltons for GOF, 50669 daltons for G1F, and 50831 daltons for G2F.

⁴The kinetics of EndoS deglycosylation could vary depending on the mAb used although under these conditions 100% deglycosylation has been observed for a variety of antibodies and Fc fragments. Deglycosylation can also decrease the stability of the antibody and lead to degradation or aggregation. We recommend using the deglycosylated antibodies for the next transglycosylation steps within a week, without longer period of storage to avoid potential degradation or aggregation due to deglycosylation.

3.3 Protein A Affinity Chromatography of the Fc Deglycosylated Rituximab

1. Prepare the Hitrap Protein A column: Wash the column with 2 mL of protein A elution buffer and equilibrate the column by washing with 10 mL of 1x PBS at a flow rate of 1 mL/min.
2. Load the deglycosylated rituximab onto the column (*see* Note 5).
3. Wash the column with 10 mL of 1x PBS pH 7.4
4. Elute the bound rituximab with 100 mM glycine-HCl pH 2.8. Immediately neutralize the eluted fractions by adding 1/10 volume neutralization buffer to the fraction tubes (*see* Note 6).
5. Exchange buffer into 1x PBS and concentrate the rituximab to 15 mg/mL using 30 kDa MWCO centrifugal filter as described in Subheading 3.1.

3.4 Purification of SCT and CT Glycans from the sialylglycopeptide (SGP)

1. Add 10 μ L of EndoM WT (100 μ g) to 5 mL of SGP (100 mg) in 100 mM phosphate buffer pH 6.5. Incubate the mixture at 30 °C overnight. Monitor the reaction progress by ESI-MS analysis (*see* Note 7).
2. Purify the released N-glycans away from the GlcNAc-peptide and enzyme using reversed-phase high-performance liquid chromatography (RP-HPLC). Load the reaction mixture onto the symmetry C18 column. Elute with the following LC conditions while collecting fractions: 100% of 0.1% TFA in ddH₂O for 10 minutes followed by a gradient elution of 0–90% 0.1% TFA in ACN for 20 minutes (*see* Note 8).
3. Check fractions for glycan using a sulphuric acid spot test on a TLC plate where 1 μ L of each fraction is spotted on a TLC plate and allowed to dry. The plate is then dipped in 10% sulfuric acid in ethanol and heated with a heat gun. The carbohydrates are charred to give grey or black spots. Analyze fractions that contain glycans with MS analysis.
4. Fractions with the biantennary complex type glycan are pooled and lyophilized
5. Dissolve the lyophilized solid in ddH₂O and load onto a Sephadex G-15 column eluting with ddH₂O. Analyze fractions with the sulphuric acid spot test (*see* Subheading 3.4.3). The free glycans will be eluted in the excluded volume (*see* Note 9).

⁵The flow through from the protein A column can be collected and the released glycans can be analyzed if desired.

⁶Antibodies can be eluted off a protein A column using a more gentle procedure if needed using 100 mM sodium citrate buffer pH 3.0–5.0.

⁷SGP can also be cleaved using other endoglycosidase such as EndoS WT, EndoF2, and EndoF3 using the appropriate pH conditions. Ensure that the pH of the reaction is checked as SGP is very acidic at this concentration. The mass of SGP is 2866 and ESI-MS analysis reveals an observed mass of 956 and 1434 daltons which corresponds to $[M+3H]^{3+}$ and $[M+2H]^{2+}$. The mass of the cleaved peptide is 863 daltons and the free glycan is 2020 daltons. ESI-MS analysis reveals an observed mass of 432 daltons which corresponds to $[M+2H]^{2+}$ of the peptide and 1011 daltons which corresponds to $[M+2H]^{2+}$ of the free glycan.

⁸Free glycan will not bind to the C18 column and elutes near the void volume. The peptide and enzyme will be absorbed on the column and eluted later. The fractions can be further analyzed using ESI-MS or HPAEC-PAD analysis.

⁹It is essential that all the salt is removed as the next step requires ion-exchange chromatography and low concentrations of NaCl can inhibit binding.

6. Wash a 1 mL HiTrap Mono Q FF column with 5 column volumes of 300 mM NaCl and equilibrate the column by washing with 10 column volumes of ddH₂O
7. Load glycan fractions over the column at 0.5 mL/min and elute the bound glycans with the following LC conditions while collecting fractions: 0 to 100% 300 mM NaCl over 60 minutes. Analyze fractions with the sulfuric acid spot test (*See* Subheading 3.4.3). Analyze fractions with glycan using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Dilute 1 µL of each fraction in 200 µL of ddH₂O and inject 10 µL over the CarboPAC PA200 column using a gradient of 0 to 100 mM NaOAc in 100 mM NaOH (*see* Note 10).
8. Combine and lyophilize pure disialylated (SCT) fractions. Dissolve the lyophilized solid into ddH₂O and load onto a Sephadex-G15 column eluting with ddH₂O.
9. Analyze fractions with the sulfuric acid spot test (*see* Subheading 3.4.3). Combine and lyophilize fractions containing the pure SCT glycan.
10. Dissolve 20 mg of SCT in 1 mL of 50 mM sodium citrate buffer, pH 6.0 and add 2 µL of Neuraminidase. Incubate the mixture for 16 hours at 37 °C. Monitor reaction completion by HPAEC-PAD (*see* Note 11).
11. Load the reaction mixture onto a Sephadex G15 column eluting with ddH₂O. Analyze fractions with the sulfuric acid spot test (*see* Subheading 3.4.3).
12. Combine and lyophilize fractions containing the pure CT glycan.

3.5 Conversion of CT and SCT into Oxazoline Sugars

The N-glycans are converted into oxazoline sugars and purified using a modified method described previously (17, 18).

1. Dissolve 20 mg of SCT in 200 µL of ddH₂O. Add 45 molar equivalents of triethylamine to the mixture and 20 molar equivalents of 2-chloro-1,3-dimethylimidazolinium chloride (DMC). Put the reaction mixture on ice with a stir bar for 45 minutes. Analyze product formation using HPAEC-PAD (*see* Note 12).
2. Load the reaction mixture onto a Sephadex-G15 column (pre-equilibrated with 0.1% TEA in ddH₂O) eluting with 0.1% TEA in ddH₂O to ensure basic conditions. Analyze fractions using the sulfuric acid spot test (*see* Subheading 3.4.3). Fractions containing glycan can be further analyzed using HPAEC-PAD.

¹⁰The monosialylated complex type glycan will elute before the disialylated complex type glycan. The disialylated typically elute around 50 mM NaCl under the conditions described. HPAEC-PAD analysis clearly shows the purification of SCT as the monosialylated complex type glycan elute at 12.9 minutes and the disialylated complex type glycan elutes at 19.1 minutes (Fig 4a).

¹¹Neuraminidase cleaves the sialic acids from SCT efficiently to give CT. HPAEC-PAD shows the product formation as the CT elutes at 6.9 minutes. Sialic acid does elute slightly before CT but after gel filtration the pure CT free glycan can be purified (Fig 4b).

¹²Dilute the oxazoline reaction 1/200 in 20 mM NaOH as the oxazoline sugar is unstable under neutral or acidic conditions. Oxazoline formation reduces the retention time as the SCT oxazoline sugar elutes at 15.6 minutes using program A on the CarboPac PA200 and the CT oxazoline sugar elutes at 4.2 minutes (Fig. 4c,d).

3. Combine and lyophilize fractions containing the SCTox. Dissolve the lyophilized solid SCTox (19 mg) in 20 mM NaOH to a concentration of 100 µg/µL (*see* Note 13).
4. Dissolve 15 mg of the desialylated glycan (CT) in 120 µL of ddH₂O. Add 45 molar equivalents of triethylamine to the mixture and 20 molar equivalents of DMC. Store the reaction on ice with a stir bar for 45 minutes. Analyze oxazoline formation using HPAEC-PAD (*see* Note 12).
5. Load the reaction mixture onto a Sephadex-G15 column (pre-equilibrated into 0.1% TEA in ddH₂O) eluting with 0.1% TEA in ddH₂O to ensure basic conditions. Analyze fractions using the sulfuric acid spot test (*See* Subheading 3.4.3).
6. Combine and lyophilize fractions containing the CTox. Dissolve the solid CTox (14 mg) in 20 mM NaOH to a concentration of 100 µg/µL (*see* Note 13).

3.6 Enzymatic transfer of defined glycans to the deglycosylated rituximab

1. Transfer of SCT to the GlcNAc residue attached to Asn 297 of rituximab: Add 335 µL of the deglycosylated rituximab solution (15 mg/mL), 14µL of the mutant enzyme EndoS D233Q (10 mg/mL), 40 µL of 1M Tris HCl pH 7.4, 7.5 µL of ddH₂O, and 3.5 µL of the SCTox solution (100 mg/mL).
2. Transfer of CT to the GlcNAc residue attached to Asn 297 of rituximab: Add 335 µL of the deglycosylated rituximab solution (15 mg/mL), 14µL of the mutant enzyme EndoS D233Q (10 mg/mL), 40 µL of 1M Tris HCl pH 7.4, 7.5 µL of ddH₂O, and 3.5 µL of the CTox solution (100 mg/mL). Incubate the reaction mixture at 37 °C for 90 minutes (*See* Note 14).
3. Monitor the reactions using LC-ESI-MS analysis (*see* Subheading 3.1). Complete transfer of the SCT and CT to rituximab is achieved in 90 minutes (*see* Note 15).
4. Purify the S2G2F and G2F rituximab glycoforms using a protein A column following the previously stated conditions (*see* Subheading 3.2) (*see* Note 16).
5. Exchange the buffer of the fractions containing the respective glycoengineered rituximab glycoform into 1x PBS using an Amicon Ultra centrifugal filter membrane 30 kDa to give the pure, homogeneous rituximab glycoforms (4.9 mg of S2G2F rituximab and 4.8 mg of the G2F rituximab).

¹³Due to the instability of the oxazoline sugar at neutral and acidic pH, addition of a small amount of NaOH in the solution ensures an alkaline environment. SCTox and CTox should be stored –20°C.

¹⁴Here we describe the chemoenzymatic synthesis of S2G2F and G2F rituximab glycoforms but the acceptor rituximab could be treated with a fucosidase prior to the transfer of the SCT and CT to make S2G2 and G2 rituximab glycoforms if desired. These glycoforms have been shown to have increased binding to FcγRIIIa and increased ADCC activity. The final concentration of IgG is important and concentrations greater than 5 mg/mL allow for efficient transfer although concentrations greater than 10 mg/mL tend to give the best results. The molar ratio of glycan oxazoline to rituximab is an important consideration as best results are obtained when the ratio is around 50:1 (25:1 when both sites are considered). The excess glycan oxazolines are usually recovered as free glycans (after hydrolysis) from the flow through of the protein A affinity column, and can be readily converted back to the glycan oxazoline for further use in glyco-engineering. The calculated theoretical molecular weight of S2G2F rituximab is 51426 daltons and the calculated theoretical molecular weight of G2F rituximab is 50832 daltons (Fig. 5a,b).

¹⁵Endotoxin contamination is likely as the EndoS enzymes are expressed in *E. coli*. The potential endotoxin contaminant should be cleaned up (usually done with an endotoxin removal kit) if *in vivo* or cell-based studies are to be performed.

6. Confirm the identity and purity of the glycoengineered rituximab through LC-ESI-MS analysis (Fig. 5) (*see* Subheading 3.1 and Note 17).

4. Notes

¹In the present study, we describe the detailed procedure of the chemoenzymatic glyco-engineering of monoclonal antibodies using rituximab as a model. This procedure is also applicable for glyco-engineering of other mAbs.

²Adjust injection volume to your MS system. Treatment with TCEP reduces the antibody to heavy and light chains which can be separated and analyzed separately. The reduction of the heavy chain to a monomeric form allows for better analysis.

³The retention times of the heavy and light chains depend on the column, temperature, glycosylation, and the amino acid sequence. When calculating the theoretical molecular weight it is important to consider the typical post translational modifications found on mAbs such as Gln to pyro-Glu conversion found on both the heavy and light chains as well as the cleavage of the C-terminal lysine on the heavy chain. Also the complete reduction of two intrachain disulfide from the light chain and of four intrachain disulfide bonds in the heavy chain leads to an expected mass of 23039 daltons for the light chain and 49067 daltons for the heavy chain. The addition of respective N-glycan to the Asn 297 of the heavy chain accounts for a mass of 50507 daltons for GOF, 50669 daltons for G1F, and 50831 daltons for G2F.

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⁵The flow through from the protein A column can be collected and the released glycans can be analyzed if desired.

⁶Antibodies can be eluted off a protein A column using a more gentle procedure if needed using 100 mM sodium citrate buffer pH 3.0–5.0.

⁷SGP can also be cleaved using other endoglycosidase such as EndoS WT, EndoF2, and EndoF3 using the appropriate pH conditions. Ensure that the pH of the reaction is checked as SGP is very acidic at this concentration. The mass of SGP is 2866 and ESI-MS analysis reveals an observed mass of 956 and 1434 daltons which corresponds to $[M+3H]^{3+}$ and $[M+2H]^{2+}$. The mass of the cleaved peptide is 863 daltons and the free glycan is 2020 daltons. ESI-MS analysis reveals an observed mass of 432 daltons which corresponds to $[M+2H]^{2+}$ of the peptide and 1011 daltons which corresponds to $[M+2H]^{2+}$ of the free glycan.

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¹²Dilute the oxazoline reaction 1/200 in 20 mM NaOH as the oxazoline sugar is unstable under neutral or acidic conditions. Oxazoline formation reduces the retention time as the SCT oxazoline sugar elutes at 15.6 minutes using program A on the CarboPac PA200 and the CT oxazoline sugar elutes at 4.2 minutes (Fig. 4c,d).

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Figure 1.
Glycosylation remodeling of rituximab to provide homogeneous glycoforms

Figure 2.
Semi-synthesis of sialylated complex type glycan oxazoline (SCTox) and the asialylated glycan oxazoline (CTox)

Figure 3.
LC-ESI-MS analysis of the heavy chain of rituximab: a) commercial rituximab b)
deglycosylated rituximab

Figure 4. HPAEC-PAD analysis of the synthetic glycans. a) SCT free glycan b) CT free glycan c) SCT oxazoline; d) CT oxazoline

Figure 5.
LC-ESI-MS analysis of the heavy chain of the glycoengineered rituximab: a) S2G2F
rituximab b) G2F rituximab.