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Reduction of high mannose glycoforms from monoclonal antibodies by affinity chromatography using a recombinant prokaryotic lectin

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ABSTRACT

Glycan profiles of monoclonal antibodies are critical quality attributes for their use as therapeutic agents. However, the control of glycosylation can prove difficult during large-scale manufacture. An on-going problem is the increase in high-mannose glycans (HM) that can occur unexpectedly under cell culture conditions that disrupt the glycosylation pathway. We have developed a method for removal of high-mannose glycans from a heterogeneous mixture of mAb glycoforms purified from a bioprocess. This involves the use of a prokaryotic lectin (RPL-Man2) bound to agarose beads to selectively bind reversibly to mAbs carrying HM-glycans. The unbound flow-through from this step contains the remainder of the mAb preparation with a reduced HM-glycan content. In this paper we demonstrate the application of this method using two distinct antibodies derived from Chinese hamster ovary (CHO) cells purposely grown under conditions that enhanced the content of HM-glycans. The presence of an elevated level of mannose in the culture of the producer cells as well as supplementation with a specific mannosidase inhibitor resulted in purified mAbs containing significantly higher levels of HM-glycans than would be expected normally under standard conditions. We showed that HM-glycans in the purified mAbs were reduced significantly by the application of lectin chromatography. Analysis by both UPLC using fluorescence detection as well as by mass spectrometry showed that the removal of the HM-glycoforms was selective and did not affect the profile of the remaining glycoforms. This method has potential for use in large-scale biomanufacturing for the reduction or elimination of HM-glycoforms of mAbs.

1. Introduction

The present global size of the biopharmaceutical market is around US $300 billion which is projected to increase to over US$550 billion by 2032 (Mikulic, 2024). Within this global market monoclonal antibodies (mAbs) continue to be dominant and represent > 50 % of all approved biopharmaceutical products (Walsh and Walsh, 2022). The value of mAbs as diagnostic molecules is that they are capable of binding to specific targets allowing the detection of specific compounds often at low concentrations. As therapeutic agents mAbs can be used to bind to specific compounds or viruses to neutralize their effects (Chen et al., 2021; Griffith and McCoy, 2021). mAbs not only bind to specific antigens but also have efficient immune effector functions that are capable of activating the immune system by recruiting innate lymphocytes such as Natural Killer cells for the destruction of target cells through antibody-dependent cellular cytotoxicity (ADCC) or through CDC (complement dependent cytotoxicity) (Dixon et al., 2021). This type of function is essential for their use in the treatment of serious diseases such as cancer (Zahavi and Weiner, 2020).

The immune effector function of mAbs is dependent upon their molecular structure and the binding of cellular receptors to the heavy chain CH2 domain at a position in which N-glycans are attached to a specific site in the immunoglobulin IgG (Spearman and Butler, 2015; Wada et al., 2019). The structure of the glycan at this position which is normally at asparagine 297 in IgG is an important factor in determining the affinity of the cellular receptors and therefore critical for the strength of the immune effector function (Boune et al., 2020). However, the glycan structures of antibodies produced both *in vivo* and *in vitro* through cellular bioprocesses are heterogeneous (Shipman et al., 2024). There is evidence that the heterogeneity and diversity of glycans can be controlled *in vivo* in response to specific physiological or pathological changes (Anthony et al., 2012).

Recombinant proteins including monoclonal antibodies are produced from mammalian cell cultures through bioprocesses under

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controlled conditions (O'Flaherty et al., 2020). However, many bioprocess parameters affect the heterogeneity of glycoforms. This includes the obvious physiochemical conditions for cell growth including composition of the nutrient medium, pH, dissolved oxygen, osmolality and temperature (Batra and Rathore, 2016; Bibila and Robinson, 1995; Xu et al., 2023). Although the predominant glycan structure of a mAb produced in a bioprocess is likely to be fucosylated and biantennary, glycan heterogeneity can show variability with respect to several structural features that include significant amounts of high-mannose (HM) structures and variable antennarity, galactosylation, fucosylation and sialylation (Spearman and Butler, 2015).

The biological function of a mAb including therapeutic efficacy also varies depending upon its glycosylation profile. The glycan profile of mAbs is particularly important for therapeutic applications in order to ensure clinical efficacy and safety (Luo and Zhang, 2024). Initially it might be thought important for the mAb profile to mimic the profile presented by natural antibodies. However, it is well documented that the profile *in vivo* can change in response to environmental and physiological condition (Lauc et al., 2014). It is also the case that therapeutic responses are enhanced by specific glycoforms and therefore there can be value in enrichment of mAbs with particular glycoforms. For example, it is known that highly sialylated mAbs confer anti-inflammatory activity (Ahmed et al., 2014; Park et al., 2023) and a high level of galactosylation or afucosylation increases antibody-dependent cell-mediated cytotoxicity (ADCC) (Kwiatkowski et al., 2020; Simonov et al., 2019). Thus there is value in determining the preferred glycan profile that maximises a desired bioactivity.

The control of glycosylation of recombinant proteins including mAbs during a bioprocess has been a major challenge in commercial production, because unlike protein or polynucleotide synthesis the pathway is not governed by a template but by the variable activities of enzymes within the cell as well as the changing substrate concentrations in the media (Spearman and Butler, 2015; Gupta and Shukla, 2018). The critical attributes of glycosylation profiles underly the importance of the control of mAb glycosylation during commercial bioprocesses. This is particularly important for the growing number of biosimilar products for which there is a need to match the glycan profile with that of the regulatory-approved originator molecule (Duivelshof et al., 2019). Understanding these relationships should then allow the control of conditions during a bioprocess so as to produce a protein with a preferred glycan profile. This can be done in one of several ways: (a) genetically modify the producer cell line, (b) modify the chemical and physical conditions within the bioreactor, (c) modify the glycoprotein derived from the bioreactor or (d) select the preferred glycoform from the heterogeneously glycosylated protein from the bioreactor (Edwards et al., 2022).

This paper focuses on the selective removal of high-mannose glycoforms from heterogeneously glycosylated mAbs isolated from cell culture. Endogenous human IgG *in vivo* contains a relatively low percentage of HM glycans (<1 %) (Flynn et al., 2010). However, in a commercial production bioprocess it is not uncommon to find this increase to 10 % or higher (Mastrangeli et al., 2020), despite the general recommendation for levels within the range of 3–10 % (Batra and Rathore, 2016). Significant increases in mannosylation are observed across batches of biotherapeutic IgG production. This change is associated with Endoplasmic Reticulum Stress and cell specific productivity (Mastrangeli et al., 2020). Repeated high yield bioprocessing runs can cause unpredictable changes to the glycan profile of the biotherapeutic product. The level of high-mannose (HM) structures is considered a critical quality attribute of IgG based biotherapeutics. Elevated levels of HM glycans have a potential impact on therapeutic functions of monoclonal antibodies including antibody-dependent cell cytotoxicity (ADCC) (Mimura et al., 2000) and pharmacokinetics (PK) (Welch et al., 2023) by decreasing serum half-life (Goetze et al., 2011). The unwanted HM glycoforms tend to accumulate if the steps through the glycosylation pathway are terminated early, typically from cells at high passage number or with an excessively high specific mAb productivity. The problem in bioprocessing is that these events may be unpredictable and the HM-glycan content of the mAb may drift to high levels above an acceptable range (Mastrangeli et al., 2020).

Fig. 1 shows the general cellular pathway of glycosylation. The consensus 14 oligomer chain is formed from nucleotide sugar precursors in the cytoplasm before addition to an asparagine (Asn) amino acid residue on the nascent protein chain that is being formed in the endoplasmic reticulum (ER). Further processing in the ER produces HM glycan structures that are normally transferred to the Golgi where a series of enzymes drive the formation of complex glycans. Disruption of the transfer from the ER to the Golgi or of mannose trimming in the Golgi can result in the accumulation of HM glycan structures.

The problem of controlling the high mannose content of mAb glycans in commercial bioprocesses has been recognised for some time (Mastrangeli et al., 2020). Methods are available to increase HM glycans (Huang et al., 2015) but methods to decrease the content have proved more difficult. Some reduction of the HM content can occur by controlling the conditions of cell culture or clone selection by using producer cells that produce low levels of HM (Shi and Goudar, 2014). Through controlled feeding Zalai et al. were able to vary the specific productivity (Qp) of cells and showed a strong correlation between increased Qp and the level of HM glycans (Zalai et al., 2016). This suggests that the elevated flux through the Golgi could saturate the glycosylation pathway and cause the accumulation of HM structures as intermediates in the process. Wu et al. found that the timing of supplementation of antioxidants during a late stage of culture could increase Qp without increasing HM glycans (Wu et al., 2025). Chai et al. showed that a high level of N-acetyl-D-mannose supplementation could reduce HM glycans in a mechanism that probably influenced the transition of intermediates between M5 and G0F in the glycosylation pathway (Chai et al., 2025). Alternatively, downstream approaches of modification have been attempted by chemoenzymatic glycoengineering that allow glycan remodelling by exposure of mAbs to selected enzymes to prepare a preferred glycan profile that can include elevated levels of galactosylated, agalactosylated or sialylated glycoforms (Mota et al., 2022; Tayi and Butler, 2023, 2019, 2018).

The objective of the experimental work described in this paper was to provide a mechanism for the selective reduction of HM glycoforms of IgG and thus facilitating the regulation of batch-to-batch HM glycans. We prepared mAbs with elevated levels of HM glycans by growing Chinese hamster ovary producer cells in media containing excess mannose as a substrate and the inhibitor Bis-Tris to block the enzyme, α-mannosidase 1 in an attempt to increase the levels Man5 to Man9 glycan content as shown in Fig. 1. We used a novel affinity matrix to selectively remove the HM from the mAbs isolated from these cultures. This method may be applied to the reduction or elimination of HM glycoforms, allowing the content to be controlled to within an acceptable range.

Lectins are carbohydrate-binding proteins that are highly specific for certain glycans (Tsaneva and Van Damme, 2020) and can be isolated from plants, microbial or animal sources and are capable of binding to specific groups carbohydrate groups. Examples are mannose-binding lectins, galactose-binding lectins, N-acetylglucosamine-binding lectins, fucose-binding lectins and N-acetylneuraminic acid-binding lectins (Gupta and Gupta, 2022). The recombinant versions of these lectins can be produced by genetic engineering in large quantities for use in commercial processes (Oliveira et al., 2013). A lectin can be covalently attached to an inert resin in order to form an affinity binding resin capable of binding to a specific glycan (Ongay et al., 2012). The lectin bound resin can then be loaded into a column to enable affinity chromatography which has been shown to separate or enrich proteins during analytical procedures (Fang and Zhang, 2008).

The research presented here demonstrates the application of lectin-based affinity chromatography for selective removal of HM glycans from purified mAbs that contain elevated levels of HM glycans.

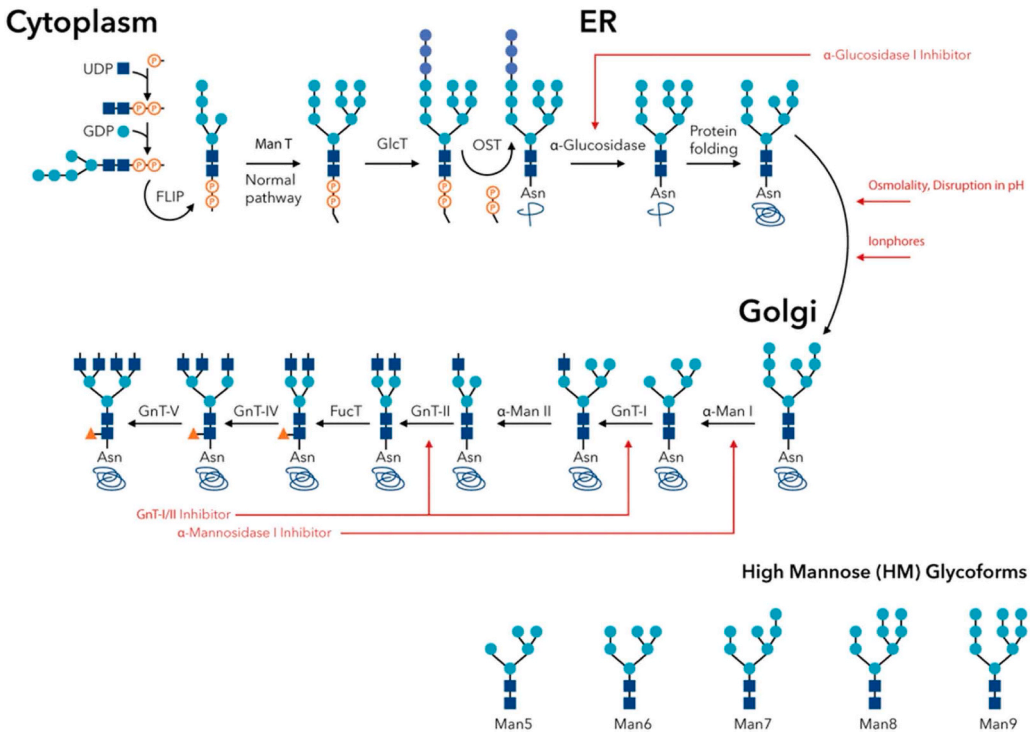


Fig. 1. Schematic of N-linked glycosylation pathway in cytoplasm, ER and Golgi. Inhibition of mannosidase may produce the key HM glycoforms shown at the bottom. The figure is modified and reproduced with permission from Rameez et al. (2021).

2. Materials and methods

2.1. Cell lines and culture

The first cell line (CHO-EG2) expressed a human-llama chimeric antibody (EG2-hFc) against EGFR and were kindly provided by Yves Durocher of the NRC, Canada (Zhang et al., 2009).

The GS-CHO cell line (CHO–S-RTX) expressing the chimeric mouse-human monoclonal antibody (designated rituximab) was kindly provided by Bjørn Voldborg of DTU Biosustain, Technical University of Denmark (Pristovšek et al., 2018).

Two pools of mAbs were produced from cell lines grown in 1 L stirred bench-top bioreactors (Applikon Biotechnology, Delft, Netherlands) at an inoculation density of 3×10^5 cells/mL under standard conditions of temperature 32 °C, dO_2 of, 30 % and stirring rate of 400 rpm. The cultures were maintained for 8–10 days at a controlled pH of 7.2 in BioGro-CHO serum free medium (BioGro Technologies Inc., Winnipeg, Canada) supplemented with 4 mM L-Glutamine.

In order to increase the content of HM glycans, the culture media was supplemented with a carbohydrate content of mannose/ glucose (96:4) and 10 mM Bis-Tris (Brantley et al., 2021). At the end of the culture period, the spent media was retained by centrifugation and then filtered through 0.2 μm PES filter (Fisher Scientific).

2.2. Cell viability measurement

Samples (5 mL) were taken under aseptic conditions using a sampling valve and sterile syringes, at defined time points. Cell numbers and viability were determined using the Trypan blue exclusion assay on a LUNA automated cell counter (Logos Biosystems, South Korea). Samples (10 μL) were incubated with 10 μL trypan blue. Duplicate samples were analysed by adding samples to the chambers of a LUNA cell counting chip of the counter.

2.3. Antibody purification

The mAbs were purified from harvested culture medium obtained from the bioreactors by Protein A affinity chromatography. The HiTrap ProteinA HP antibody purification columns (GE healthcare) were first equilibrated with binding buffer (25 mM sodium phosphate, pH 7.4) at a flow rate of 1.0 mL/min. Each filtered and neutralized sample of harvested spent media was loaded onto a protein A column and the column washed to remove unbound molecules. This was followed by elution at the same flow rate using elution buffer (0.1 M citric acid, pH 3.0). The collected fractions containing the target antibodies were neutralized by addition of 1 M Tris-HCl, pH 9.0, followed by buffer exchange against distilled water using Amicon ® Ultra centrifugal filter with molecular weight cut off of 30 kDa (Millipore, Bedford, MA, USA).

2.4. Antibody analysis

The antibodies were quantified by Cedex HiRes Analyzer (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) based on a turbidimetric immunoassay.

2.5. Coupling lectin to agarose beads

The RPL -MAN2 lectin (GlycoSelect) was coupled to two types of agarose beads, 4 Rapid Run™ (RR) and 4BCL (CL4B) (Agarose Bead Technologies) using glyoxal chemistry as described by the manufacturers. The resins were chosen because of their low content of aldehydes groups (15–25 μmoles Glyoxal /mL gel), which provides minimal distortion of the conjugated ligand.

2.6. Lectin chromatography

The prokaryotic lectin, RPL-MAN2 (GlycoSelect) was coupled to an agarose (Agarose Bead Technologies) solid phase for the affinity capture of HM glycoforms from heterogenous pools of IgG glycoforms. A high-mannose lectin affinity column was prepared by degassing the freshly

coupled matrix and manually packing a 2 mL disposable polystyrene column (Thermo Fisher Scientific). The beads in the column were stored in a storage buffer, 25 mM Sodium Phosphate buffer, 0.01 % Sodium azide, pH 7.4 (Fisher Scientific). Individual batches of resin were used to pack three identical columns which were run in parallel.

The columns were first equilibrated with 10 column volumes (1 mL) of binding buffer (20 mM Sodium Phosphate, 500 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4). Each column was incubated with the binding buffer for 30 min. Samples were applied to each column and incubated at 5° C for 30 min. The samples (1 mL) included glucose oxidase (1 mg/mL) and the two mAbs: EG2-hFc (1.7 mg/mL) and RTX (2.5 mg/mL).

Fractions (5 ×1 mL) containing flow-through material were collected and subsequently the column was washed with 10 mL of binding buffer. The bound protein was eluted into 6 × 1 mL fractions with elution buffer (300 mM Methyl-α-D-mannopyranoside, 20 mM Sodium Phosphate, 500 mM NaCl, pH 7). The column was then washed with 5 mL of elution buffer and 10 column volumes of water. The columns were prepared for storage with 10 column volumes of storage buffer (25 mM Sodium Phosphate, 0.01 % Sodium Azide, pH7) and stored at 4°C.

2.7. Protein analysis

The protein present in each fraction from the lectin affinity chromatography was quantified by colorimetric detection with bicinchoninic acid using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Absorbances at 562 nm were measured on a Bio-Tek Synergy H1 microplate reader (BioTek/ Agilent, Santa Clara, CA, USA).

2.8. Glycan analysis

The elution fractions from lectin affinity chromatography were analysed for N-glycan content. The fractions were buffer exchanged with water using a centrifugal device (Nanosep from Cytiva, Cork, Ireland) with 10 K omega membranes. N -glycans were released from these samples using Agilent Advance Bio Gly-X N-Glycan InstantPC labelling Kit (kindly provided by Agilent Technologies, Hayward, CA, USA). The released N-glycan samples were analysed by Hydrophilic interaction liquid chromatography (HILIC) on a Waters Cooperation Acquity UPLC system using a previously described method (Xie et al., 2021). Chromatograms were generated from a separation protocol that employed a Glycan BEH Amide Column 130 Å, 1.7 μm column (Waters Corporation) with a 73 % Acetonitrile 27 % sample gradient and 50 mM Ammonium Formate, pH 4.4 as the mobile phase. The N-glycan profile obtained was analysed, individual peaks integrated and marked based on retention times using Empower software.

2.9. Mass spectrometry

Structural identification was confirmed by mass spectrometry of chromatographic fractions. Analysis was performed by HILIC-UPLC-FLD-ESI-MS on a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a BEH Amide column (130 Å, 1.7 μm, 1.0 mm × 150 mm). Samples were stored at 5 °C before injection, with separations carried out at 60 °C using a 10 μL injection volume. Mass spectrometry was performed in positive ion mode with the following parameters: spray voltage 3.4 kV; capillary temperature 320 °C; auxiliary gas heater 300 °C; sheath gas 30 L/h; sweep gas 10 L/h; scan range m/z 450–2500; resolution 70,000. MS data was processed using Xcalibur software (Thermo Fisher Scientific).

3. Results

1. Production of mAbs with elevated levels of high-mannose glycan structures

   Pools of two specific monoclonal antibodies (EG2-hFc and RTX) were produced by growing genetically-engineered CHO cell lines in batch cultures (1 litre) under controlled conditions. Viable cell densities were monitored by daily sampling over 12 days when the cells grew from 3×10^5 cells/mL to a maximum density of $1.5–2.0 \times 10^6$ cells/mL. The secreted mAbs were purified from the resulting culture harvests at the end of the culture period by Protein A affinity chromatography. The pooled contents averaged 82 mg/L for EG2-hFc mAb and 53 mg/L for RTX mAb. The purity of each mAb sample was demonstrated by PAGE with single bands at 75 kDa for EG2-hFc and 150 kDa for RTX.

   In order to increase the level of HM glycans in mAbs, the carbohydrate content of the growth medium was adjusted to a high level of mannose (mannose/ glucose, 96:4) and supplemented with 10 mM Bis-Tris. The latter is a competitive inhibitor of mannosidase 1, an enzyme required as an integral part of the glycosylation pathway in cells. The effect of Bis-Tris is to prevent further processing of the high-mannose glycoproteins through the pathway in the Golgi in the production of complex glycans.

   Fig. 2 shows stacked glycan profiles of the isolated mAbs produced in cultures in the presence or absence of supplemented mannose and Bis-Tris. The mAbs were produced in the presence of Bis-Tris and high mannose concentration independently and in combination. For each mAb in Fig. 2 the profiles were derived from a standard culture (control), a culture supplemented with mannose and a culture supplemented with both a high mannose and Bis-Tris. Each profile shows the presence of the complex glycans, G0F, G1F (2 isomers), G2F, G2FS1 and G2FS2.

   In the case of the EG2-hFc mAb, the HM glycans M5 and M8 were present in the control profile but other HM structures were at a minimal level. The total HM glycan content in the presence of each of the two supplemented cultures increased to 13 % overall above the control content of 9 %. However, in the presence of both culture supplements (96 % mannose and 10 mM Bis-Tris) the value increased to 16 % overall of HM glycans.

   For the RTX mAb the increase in HM glycans was even higher in supplemented cultures increasing from a base level of 17–45 % of total glycans analysed. Two concentrations of Bis-Tris were used (10 and 15 mM) but there was no apparent significant increase in overall HM glycans in the presence of the higher Bis-Tris level. The greatest increase in HM glycans was for M6 to M9. In fact the M5 level decreased in the supplemented culture in the case of the RTX mAb.

   In summary, by adapting the growth media with a carbohydrate content of mannose/ glucose (96:4) and supplement of 10 mM Bis-Tris, HM glycan structures increased in EG2-hFc pools from 9 % to 16 % and in RTX pools from 17–45 %. These HM-enriched antibodies were purified from the cultures using Protein A affinity prior to the next stage of analysis.

2. Coupling of ligand to a resin

   A number of both solid phase resins and coupling chemistries were tested with the objective of producing a stable affinity ligand matrix. These included agarose, silica and magnetic beads from different suppliers using different coupling chemistries such as of N-hydroxy succinimide (NHS), cyanogen bromide (CNBr), amine activated and Ni affinity binding. Each was assessed for stability and efficiency of binding. Following these, it was decided to use agarose resins pre-activated with glyoxal aldehydes that covalently bound to the lectin via lysine residues. The attachment protocol through reductive amination provided yields of 92–98 % of conjugation of the lectin. It was shown that the coupling of the RPL-Man2 lectin to each of the two agarose resins (4BCL and 4RR) was stable for at least 6 months.

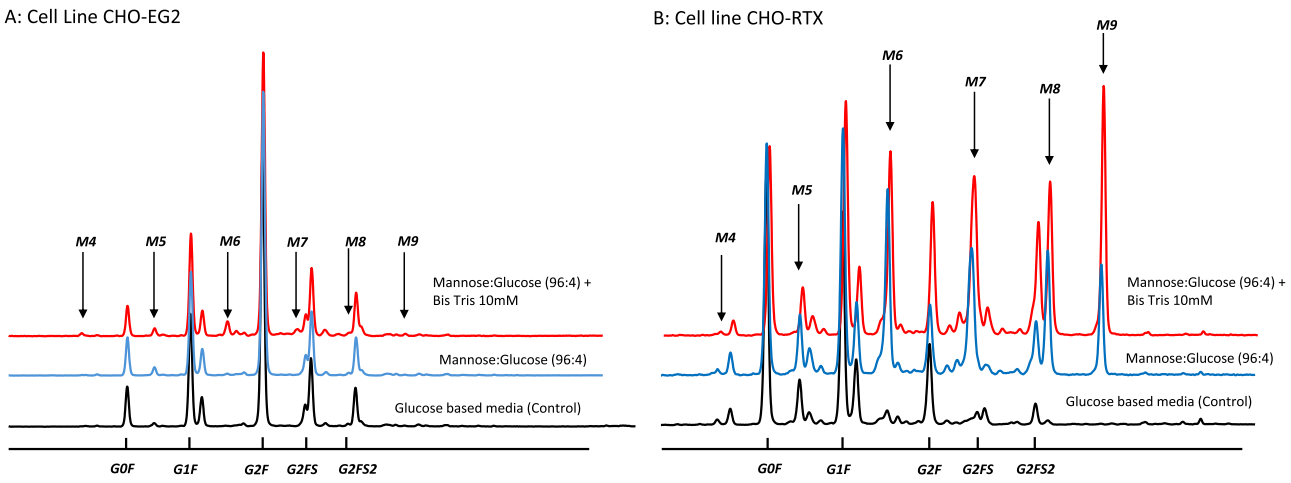


Fig. 2. Modulation of the Glycan profiles of two mAbs by media supplementation. Stacked chromatogram profiles are shown of glycans obtained by HILIC chromatography of fluorescently-labelled glycans from (A) EG2-hFc mAb and (B) RTX mAb. Isolated mAbs were produced from cultures independently supplemented with Bis-Tris (10 mM) and elevated mannose concentrations as well as with combined supplementation. The upper labels indicate the HM glycan peaks and the lower labels indicate the predominant complex glycans. The peak identities were assigned by the determination of GU values from a standard dextran ladder and confirmed by mass spectrometry.

3. Demonstration of high mannose binding with Glucose Oxidase

To assess binding efficiency, protein recovery, and matrix stability of the lectin columns we used glucose oxidase from *Aspergillus niger*, (Sigma Aldrich, Merck), which is a glycoprotein in which all glycans have a HM structure. The purpose of this was to determine the binding efficiency, protein recovery and matrix stability of each matrix using a glycoprotein standard with a known high HM-glycan content.

Fig. 3 shows the elution profiles of glucose oxidase using 2 prepared matrices (RPL-Man2 agarose CL4B and RPL-Man2 agarose 4RR). Protein recovery was measured over several fractions of the column eluent by absorbance at 280 nm. Each panel shows two phases. In the first phase the sample loaded onto the column was washed through with binding buffer at pH7.4. In the second phase the bound glycoprotein was released with binding buffer that contained free mannose. The protein recovery of unbound and bound pools were calculated based on absorbance readings carried out at 280 nm. These experiments were repeated in triplicate at Day 1, Day 30 and Day 60 following preparation of the matrix. The binding efficiency was determined as the % of the glycoprotein that was bound to the column and subsequently eluted with the elution buffer. The results indicate an initial binding efficiency of $>$ 90 % for both resin matrices and a decrease in efficiency from an average of 91.2–56.7 % at day 60 (CL2B resin) or from an average of 98.7–84.7 % at day 60 (4RR). Despite reduced binding over time, total protein recovery remained around 95 % across all conditions.

These results show the ability of both affinity matrices to bind and release glucose oxidase but with a superior performance by RPL–Man2 agarose 4RR. Consequently, all subsequent affinity chromatography experiments were performed with this matrix.

To determine the extent of any lectin leaching from the column, flow-through fractions were analysed by SDS-PAGE against standard RPL-Man2 lectin (0.25 µg). Whereas a clear single protein band was observed at 15 kDa for the lectin, there was no evidence of this band in the eluted fractions.

4. Identification of HM-glycans from monoclonal antibodies

The previously prepared pools of Protein A-purified HM glycan-enriched mAbs were then introduced into the RPL–Man2 agarose 4RR columns. The glycan content of both the human-llama chimeric antibody (EG2-hFc) and human Rituximab (RTX) had been enriched

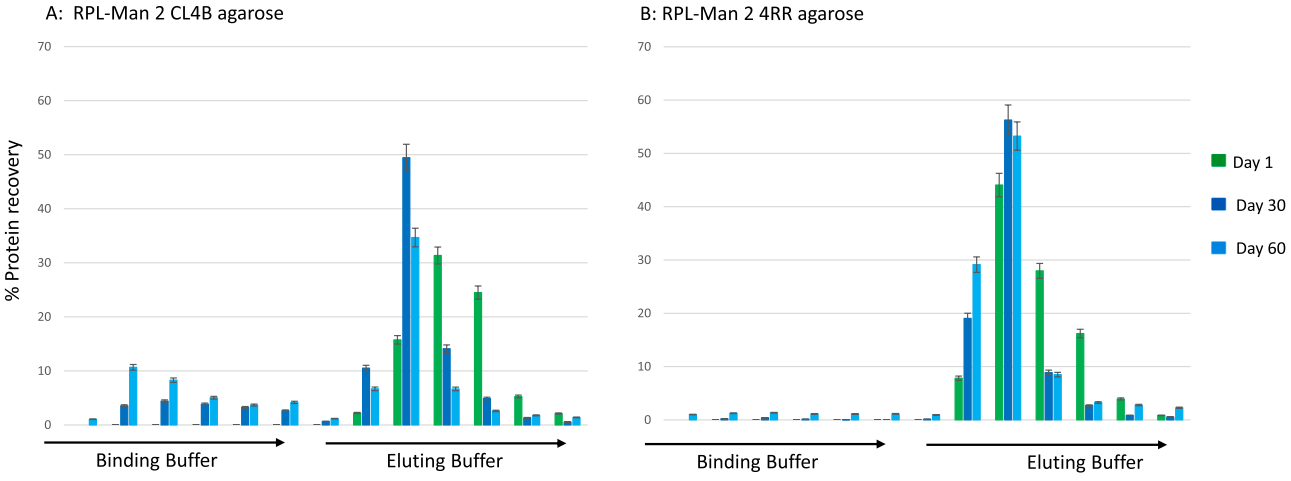


Fig. 3. Validating Low density RPL-Man2 affinity chromatography columns using glucose oxidase as a standard. The binding and elution profiles of glucose oxidase by affinity chromatography are shown for two column matrices. The matrices were prepared by coupling the ligand, RPL-Man2 to two different sources of agarose beads (CL4B and 4RR). Protein recovery was calculated as a % from absorbance readings taken @280 nm from three parallel runs (n = 3) conducted on each of day 1, day 30 and day 60 after preparation of the column.

with HM-glycans following their preparation in media containing mannose and supplementation with Bis-Tris. Lectin chromatography was performed using a similar protocol to that developed with the glucose oxidase standard. Each mAb sample (1 mL at ~2 mg/mL) was applied to 3 independent lectin affinity chromatography columns with triplicate runs. Fractions (5 ×1 mL) containing the unbound IgG (designated flow-through fraction) were collected for evaluation. The column was then washed with 10 mL of binding buffer. The bound protein was eluted (designated *bound and eluted fraction*) into 5 × 1 mL fractions with the mannose-containing elution buffer. The protein content of the fractions was determined by both absorbance at 280 nm and a BCA protein assay kit. The total protein recovery was > 90 % and binding efficiencies determined to be between 7 % and 12 %. The determined binding efficiencies were, as expected, lower than those determined for glucose oxidase separation and reflected the lower proportion of HM-glycans in the mAb samples.

The glycan content of each sample was assessed in three fractions: the input (antibody prior to column loading), the flow-through(flow-through with no column affinity), and the bound (eluted with buffer containing mannose after column retention). Glycans from these fractions were analysed by HILIC and LC-MS. Although structural assignments of the most prominent peaks could be made from standards based on relative retention times and glucose unit (GU) values of a dextran ladder, a more detailed analysis was performed by mass spectrometry. Fig. 4 shows individual HILIC profiles of glycans derived from *input, flow-through* and *bound* fractions of the affinity chromatography separation over 18 min and analysed by LC-MS. Thirty-three glycan peaks (GP1–GP33) were consistently detected and integrated across the chromatograms.

5. Selective removal of HM-glycans from monoclonal antibodies

The extent of removal of HM glycans by the lectin column was evaluated by determination of the relative abundance of these glycans in the separate fractions. Fig. 5 shows the identities of the predominant peaks in the glycan fractions obtained after lectin affinity chromatography. The structural assignments and calculation of relative abundances were based on reference to commercial standards. The profiles of the input samples prior to affinity chromatography show the predominance of the complex glycans. The unbound fractions show the change after the removal of the HM glycans that attach to the column. The bound/eluted fractions are those that were initially bound to the column and then subsequently removed by elution buffer. The profile of the bound/eluted fractions shows the predominant HM glycans (M4-M9), with M5 and M6 being the highest for both mAbs.

The relative abundances of each HM-glycan based upon integrated peak values are shown in Table 1. For EG2-hFc mAb, total HM-glycan content decreased from 16.4 % to 10.6 % after lectin chromatography, with 75.2 % of the bound and eluted fraction identified as HM-glycans. The most significant changes were that the Glycan peak 10 (Man 5) increased in relative abundance from 3.1 % in the Input pool to 17.5 % in the bound pool and peak 17 (Man6) increased in relative abundance from 4.0 % in the standard pool to 24.2 % in the bound pool.

The data for the RTX mAb demonstrate similar results except that in this case the proportion of HM-glycans in the original standard at 52 % was substantially higher than for the EG2-hFc mAb (Fig. 4 and Table 1). Undoubtedly this was due to the stronger effect of the modulating conditions of high mannose and supplementation with Bis-tris in the production cultures. Chromatography through the lectin column reduced the content of HM-glycans to a lower proportion of 32.4 %. This is explained by the higher content of HM-glycans in the bound fraction (74.4 %). In the case of the RTX mAb the most prominent HM-glycans were M5, M6, M7 and M8.

In summary, the data in Fig. 5 and Table 1 that for both mAbs there was a significant 36–38 % reduction in the overall following lectin chromatography with no apparent differences in the profile of glycoforms in the resulting mAb eluted from the columns.

In order to determine whether there was any selective removal of individual HM glycans by the lectin column, the relative abundance of each HM glycan was determined as a proportion of the overall HM glycan content in each fraction (Table 2). The data shows that the most predominant HM-glycan were M5, M6 and M7 in each of the fractions. However, there was no significant difference determined in the distribution of individual HM glycans between each of the fractions analysed (paired *t*-test; $p > 0.7$).

6. Detailed analysis of glycan elution profile using mass spectrometry

The HILIC analysis in Fig. 4 above assumes the structural assignment of the peaks based upon pre-characterised standards. Although this assumption is often made in glycan analysis, there may well be some overlapping structures and positioning of isomers that could confound some of the results. Because of this a more detailed structural analysis of the glycans was performed by LC-MS in order to confirm the HILIC analysis and obtain a full profile of glycans in each fraction obtained from the affinity columns.

The glycan structures in each peak were identified by mass spectrometry (Thermofisher Orbitrap in positive mode) with data collection

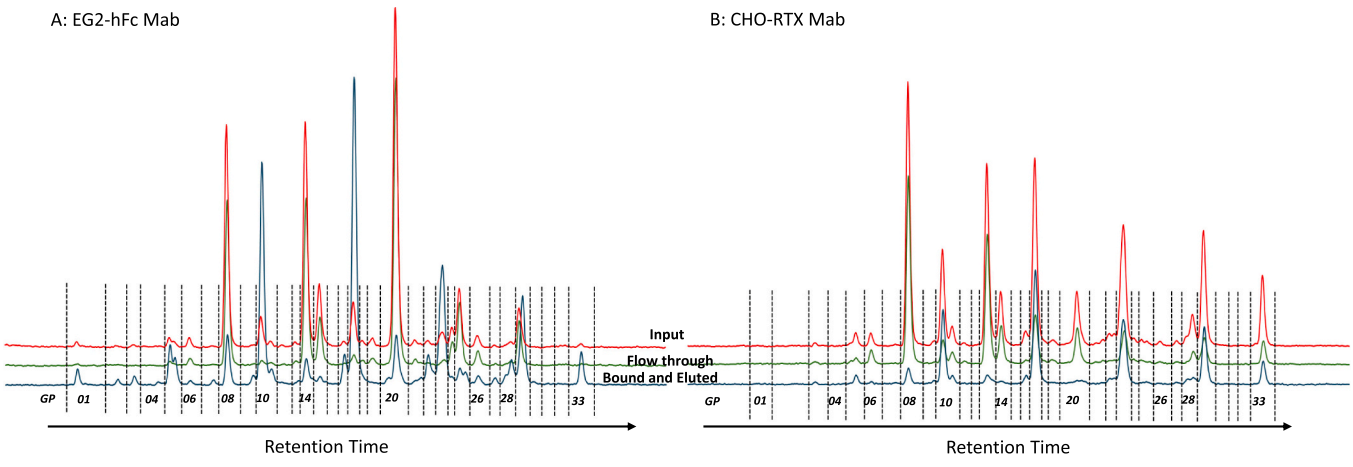


Fig. 4. Integrated Glycoprotein Peaks (GPs) from Pools of N glycans derived from two Cell lines. N-glycan profiles are shown of 3 fractions obtained from lectin chromatography. Glycan pools from Standard (red), Unbound (green) and Bound (blue) fractions pools of the glycans derived from (A) EG2-hFc mAb and (B) CHO-RTX mAb were obtained following HILIC separation. Thirty-three peaks (GP1 to GP33) were identified in each profile following HILIC chromatography over 18 min. Peak detection was by relative fluorescence.

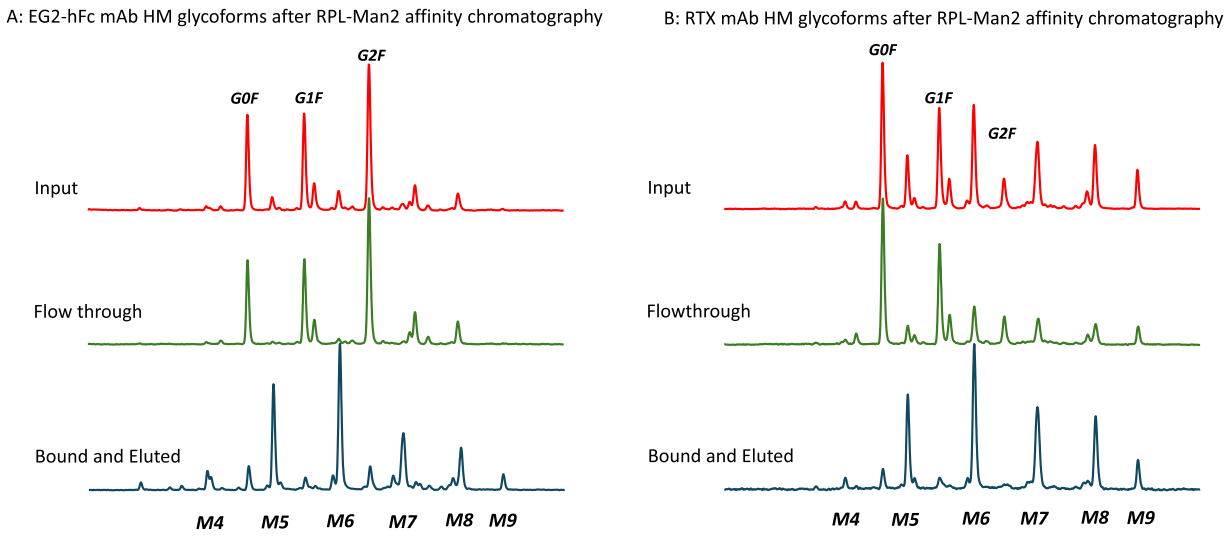


Fig. 5. The HM-glycan content of elution fractions derived from mAb glycoforms after RPL-Man2 affinity chromatography N-glycan profiles are of 3 fractions obtained from affinity chromatography of glycans derived from preparations of mAbs (A) EG2-hFc and (B) RTX. Structural assignment of the peaks was based on commercial standards. The upper labels show the location of the complex glycans G0F, G1F and G2F) which predominate in the standard samples before chromatography. The lower labels show the location of the HM glycans (Man 3-Man 9) that predominate in the bound/eluted fractions. based on standards. The relative abundances and standard errors were determined from three affinity runs conducted in parallel and the mean % content of each in relation to the total glycan pool are shown in Table 2. Peak detection was by relative fluorescence.

Table 1
The Percentage Abundance of HM-glycan glycoforms after RPL-Man2 affinity chromatography (n = 3). Glycan profiles of (A) EG2-hFc and (B) RTX mAbs were quantified as the percentage of each HM-glycan relative to the total glycans analysed. The values are based on peak integration measured in units of relative fluorescence. Values are presented as means (±SEM) from three biological replicates of mAb preparations, each subjected to lectin affinity chromatography. The glycan content of each of the three fractions (Input, Flow-through, and Eluted/Bound) was analysed by HILIC chromatography.

A: Cell Line CHO-EG2						
HM-Glycan	**Input**	**SE**	**Flow through**	**SE**	**Eluted**	**SE**
Man 3	0.60	0.06	0.41	0.03	1.21	0.01
Man 4	1.23	0.00	0.67	0.00	4.46	0.01
Man 5	3.11	0.01	1.05	0.01	17.45	0.05
Man 6	3.97	0.01	1.38	0.02	24.16	0.05
Man 7	2.71	0.01	1.26	0.01	14.92	0.03
Man 8	4.37	0.02	5.39	0.02	10.42	0.01
Man 9	0.40	0.00	0.47	0.00	2.60	0.01
Total HM%	**16.39**		**10.63**		**75.22**	
B: Cell line CHO-RTX						
HM-Glycan	**Input**	**SE**	**Flow through**	**SE**	**Eluted**	**SE**
Man 3	0.14	0.00	0.11	0.01	0.70	0.00
Man 4	1.27	0.00	1.50	0.02	2.42	0.00
Man 5	7.90	0.00	5.21	0.00	14.33	0.07
Man 6	13.46	0.08	7.71	0.00	20.94	0.09
Man 7	12.61	0.40	7.11	0.00	17.39	0.13
Man 8	11.66	0.01	6.98	0.10	13.68	0.02
Man 9	4.95	0.00	3.75	0.00	4.91	0.06
Total HM%	**51.99**		**32.37**		**74.37**	

Table 2
Abundance of individual HM-glycans as a proportion of the total HM glycan content of each pool (n = 3). The data obtained from the glycan analysis described in Table 1 was expressed as the relative content of individual HM-glycans as a proportion of the total HM-glycan content normalized to 100 % based on the major species (M3–M9). All values were determined as relative units of fluorescence. Minor glycans below the threshold of reliable integration were excluded from quantification.

A: Cell Line CHO-EG2			
HM-Glycan	**Input**	**Flowthrough**	**Bound**
M3	6.07	10.99	2.34
M4	9.52	11.20	3.57
M5	26.58	24.02	27.90
M6	31.70	19.18	31.88
M7	18.83	25.65	27.49
M8	4.08	3.13	6.11
M9	3.24	5.82	0.71
B: Cell line CHO-RTX			
HM-Glycan	**Input**	**Flowthrough**	**Bound**
M3	0.62	1.31	1.28
M4	2.17	4.17	1.39
M5	13.43	20.50	16.09
M6	29.25	28.85	30.14
M7	33.16	36.12	27.90
M8	13.22	13.23	16.09
M9	8.16	7.75	7.14

and analysis using Xcalibur and Glycoworkbench software packages. This detailed analysis included identification of structures within overlapping peaks and enabled confirmation of the position of structural isomers. The mass spectra generated showed a plot of intensity as a function of mass to charge ratios. The data obtained from this analysis confirm the main glycoforms identified in the flow through and bound material (Supplementary information).

The data shows that for the EG2-hFc mAb there was a 62 % decrease in the proportion of HM glycans in the unbound fraction following affinity chromatography. For the RTX mAb there was a 40 % decrease in the in HM abundance in the unbound fraction. This not only confirms the HILIC data but in the case of the EG2-hFc mAb the reduction of HM is considerably higher than shown in Section 3 above. This could be explained by the unusual chimeric structure of the EG2-hFc mAb. In conclusion the experiments described show that the lectin affinity chromatography described, resulted in a substantial reduction in the HM glycan content of the mAbs tested.

4. Discussion

There is no single method that deals with the problem of glycosylation heterogeneity or the variability of HM-glycans during a bioprocess.

The modification that we propose is a relatively simple method of selection and less expensive compared to chemoenzymatic glycoengineering for eliminating or reducing the content of a specific glycoform (Liu et al., 2024; Zeng et al., 2022). The technology facilitates the isolation of a recombinant protein as either a single glycoform or as a selected glycoform profile with the reduction or elimination of one type of glycosylation. The method utilises an affinity binding column to selectively eliminate or reduce the content of a specific glycoform from a heterogeneous mixture, thus creating a preferred glycan profile. The heterogenous mixture of glycoforms of the recombinant protein may be obtained directly from the culture harvest of a bioreactor or from a purified recombinant protein.

We used one of several possible prokaryotic lectins for selective affinity to a protein glycoform. Recombinant prokaryotic lectins display high affinity to different sugar residues, including mannose, galactose, fucose and sialic acid. The binding capacity for these groups is significantly higher than that of plant lectins (Fernandez-Poza et al., 2021). The prokaryotic lectin, RPL -MAN2 was coupled to an agarose solid phase for the affinity capture of HM glycoforms from heterogenous pools of IgG glycoforms.

To establish a high yield protocol for the generation of a multivalent, correctly orientated, stable affinity ligand matrix suitable for the removal of HM structures, a number of both solid phase resins and coupling chemistries were considered. Agaroses, silica resins and magnetic beads from different suppliers were tested alongside known coupling chemistries of N-hydroxy succinimide (NHS), Cyanogen bromide (CNBr), Amine activated and Ni affinity binding. Each was assessed for stability and efficiency of binding. Optimal coupling and affinity binding data was obtained from a Low Density Glyoxal chemistry with 4 Rapid Run™ (RR) and 4BCL (CL4B) agarose resin (Agarose Bead Technologies). The RR resin is designed to provide high flow rate in large scale processes at high pressure. The CL4b resin is suitable for batch or bench-scale column purification. Both resins have a low content of aldehydes groups (15–25 μmoles Glyoxal /mL gel), providing a minimum distortion of conjugated affinity ligand.

The solid phase that comprises the agarose beads was coupled to a recombinant prokaryotic lectin via activated aldehyde groups that bind lysine amino groups to correctly orientate the lectin. The lectins remain stable at variable pH and ionic strengths making them suitable for repeated cycles of chromatography. When used as a chromatography matrix the affinity ligand selectively binds to HM, as well as complex and hybrid structures that may have terminal mannose, from the heterogenous pools of IgG. This selection process is multivalent, reversable and density dependant. The efficiency of the prepared columns was tested by binding of glucose oxidase standard that is known to have a high level of HM structures. The data from this preliminary analysis showed that 98 % of the applied standard was retained by the preferred column (RPL-Man2 agarose 4RR) and then eluted with the appropriate buffer.

The two mAbs we used in this study differed structurally. The EG2-hFc mAb is a chimeric human-llama structure with reduced number of domains from the fully human form. This has been shown previously to have a high level of galactosylation and this was confirmed by our analysis (Liu et al., 2014). The RTX mAb is a fully humanised antibody that has been produced commercially and used as a therapeutic agent. The glycan profile we analysed was similar to many other humanised antibodies reported in the literature. Both antibodies were produced by genetically engineered CHO cell lines in media that was modified to maximise the HM-glycan content. Both the addition of excess mannose to the culture media and supplementation with a mannosidase inhibitor independently enhanced the level of HM-glycans in the resulting mAbs. The combined effect of these culture modifications was to produce mAbs with very high levels of HM glycans. The preparations of the isolated mAbs enabled us to fully test the effectiveness of the lectin affinity columns and our adopted protocol to selectively remove the HM glycoforms.

The extent of the glycan profile change following lectin affinity and removal of HM-glycoforms was analysed by UPLC chromatography. Structures were assigned to peaks based upon previously evaluated retention times and by glucose unit values in reference to a dextran ladder. Secondly, we analysed each peak by mass spectrometry. This involved more intense analysis and the ability to distinguish structure that overlapped between peaks. From this analysis we were able to show a reduction of 39 % in HM-glycoforms in the RTX mAb and a value of between 35 % and 58 % in the glycoforms of the EG2-hFc mAb. The two different values for the EG2-hFc analysis was a reflection of the difference between the methods used, but either one shows a significant decrease in HM-glycoforms.

Affinity chromatography is used routinely in antibody purification to capture mAbs from the culture harvest using Protein A. This is a bacteria-derived ligand protein that binds to the Fc region of a mAb. In our experiments, mAbs were subjected to lectin chromatography after Protein A purification. The possibility could be considered of switching the order and using lectin chromatography first. However, this could cause a problem of enrichment with host cell proteins containing HM-glycans that could be trapped by the lectin column. Host cell proteins are normally removed effectively from culture harvests by an appropriate washing stage during Protein A affinity purification (Shukla and Hinckley, 2008).

A significant problem in commercial production of mAbs is the possibility of leaching of Protein A from the chromatography column, a process enhanced by the presence of HCPs (Pilely et al., 2022). Further purification steps are generally required in the biomanufacture of mAbs to reduce residual Protein A to a low level of contamination following regulatory guidelines (Elder, 2017). During subsequent downstream processing several “polishing” steps such as ion exchange or hydrophobic interaction chromatography are available to ensure removal of such contamination (Hilbrig and Freitag, 2012).

As with Protein A, there is the potential for product contamination by leaching from a lectin column and so the polishing steps normally used in mAb purification would be important in the removal of any residual lectin. In our experiments we did perform SDS-PAGE on eluates from our lectin column and could not detect the presence of residual lectin. Nevertheless, conditions on scale-up could be different and also our test did not attempt to identify protein fragments that may occur from leaching (Carter-Franklin et al., 2007). Therefore, further purification using standard polishing steps would be important to ensure a product free of contamination.

In summary, the experimental work presented here is a proof of concept for the use of lectin affinity to reduce the HM-glycoform content of mAbs produced in a bioprocess. Although we performed this on a small laboratory-scale we believe there is potential for adoption at a higher commercial scale, where there is a need to ensure a %HM-glycan below a threshold level. Our experiments showed a significant reduction of HM-glycans through a single pass through the RPL-MAN2 lectin column. Lower levels of HM-glycans could be achieved through multiple passes or the use of longer columns that could be used at a larger scale.

CRediT authorship contribution statement

Neha Tushar Dalvi: Writing – review & editing, Methodology, Investigation. **Michael Butler:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Elizabeth Matthews:** Writing – review & editing, Methodology, Investigation, Conceptualization.

Declaration of Competing interest

The authors of this paper have no competing interests relating to the content of the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2025.09.008.

Data availability

Data will be made available on request.

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