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Discrimination between glycosylation patterns of therapeutic antibodies using a microfluidic platform, MALDI-MS and multivariate statistics

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ABSTRACT

Optimal glycosylation with respect to the efficacy, serum half-life time, and immunogenic properties is essential in the generation of therapeutic antibodies. The glycosylation pattern can be affected by several different parameters during the manufacture of antibodies and may change significantly over cultivation time. Fast and robust methods for determination of the glycosylation patterns of therapeutic antibodies are therefore needed. We have recently presented an efficient method for the determination of glycans on therapeutic antibodies using a microfluidic CD platform for sample preparation prior to matrix-assisted laser-desorption mass spectrometry analysis. In the present work, this method is applied to analyse the glycosylation patterns of three commercially available therapeutic antibodies and one intended for therapeutic use. Two of the antibodies produced in mouse myeloma cell line (SP2/0) and one produced in Chinese hamster ovary (CHO) cells exhibited similar glycosylation patterns but could still be readily differentiated from each other using multivariate statistical methods. The two antibodies with most similar glycosylation patterns were also studied in an assessment of the method's applicability for quality control of therapeutic antibodies. The method presented in this paper is highly automated and rapid. It can therefore efficiently generate data that helps to keep a production process within the desired design space or assess that an identical product is being produced after changes to the process.

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1. Introduction

Glycosylation of recombinant therapeutic antibodies mainly occurs at Asn297 in the Fc part of the γ -chain [1]. The glycosylation pattern affects both the residence time in the body and the efficacy of the drug as well as its immunogenicity [1–5]. Optimal glycosylation of the antibody is therefore desirable, and several strategies involving genetic engineering procedures have been developed to accomplish this. However, it may be difficult to achieve and maintain a high yield of a product having an optimal glycosylation pattern. It is therefore necessary to perform an analysis of glycosylation patterns of antibodies during both the development and manufacturing processes to monitor the quality of the product.

Large amounts of useful information can be generated by the implementation of a fast, reliable and cost-efficient technique for the analysis of antibody glycosylation patterns. The data obtained can be used to establish a robust production process and ensure that the production process is maintained within the desired design space [6]. This can aid the implementation of Process Analytical Technology (PAT) in the manufacturing of therapeutic antibodies.

Several methods have been reported for the determination of glycosylation in many different proteins [7–9]. These are generally fairly labour intensive and involve numerous manual or time consuming steps. Faster or simpler protocols have also been developed to determine the glycosylation patterns of single proteins [10–13]. Most of these latter methods also have important drawbacks, such as the need for manual handling steps or steps not amenable to parallel processing. We have recently presented a method for the determination of glycosylation patterns of therapeutic antibodies, whereby affinity capture of the antibody, enzymatic release, clean up, and co-crystallisation of the released glycans with a suitable MALDI-MS matrix is automatically performed on a microfluidic compact disc (CD) [14]. The total time required from putting the samples and CDs in the machine to being able to start the MALDI-MS analysis is less than 4 h, and up to 54 samples can be processed in parallel.

Multivariate analysis has been shown to be a useful tool for extracting important information necessary for successful biopharmaceutical production. De Alwis et al. described medium optimisation strategies for the growth of monoclonal antibodies from a specific cell line using principal component analysis (PCA) and Partial Least Square (PLS) [15]. Zamani et al. employed PCA for the analysis of nanoESI-TOF data to discriminate between antibodies produced in two different cell lines [16]. Selvarasu et al.

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utilised PCA and PLS to investigate the dependence of cell growth and antibody production on nutrient supplements and to predict an optimised cell medium design [17]. Monitoring of cell culture performance using FT-IR spectroscopy in combination with chemometric approaches has also been recently reported by Sellick et al. [18].

In the present work, the method which we presented earlier [14] was used to determine the glycosylation patterns of Erbitux, Remicade, Avastin (three commercially available therapeutic antibodies) and one antibody intended for therapeutic use (called IgG-CHO in this paper). Two of these, Erbitux and Remicade, are produced in mouse myeloma SP2/0 cells, whereas Avastin and IgG-CHO are produced in Chinese hamster ovary (CHO) cells.

Univariate and multivariate statistical methods were applied for the discrimination of the glycosylation patterns of the different antibodies. Successful discrimination between all the different therapeutic antibodies was achieved. Modelling of data and statistical analysis was also performed to assess the feasibility of the developed method for use in quality control of antibody therapeutics.

2. Materials and methods

2.1. Materials

Recombinant human monoclonal antibody IgG1 produced in a CHO cell line and affinity purified using Protein A and CHO cell culture supernatant after ultra-filtration was obtained from AstraZeneca AB, Sweden. 2,5-Dihydroxybenzoic acid (DHB), trifluoroacetic acid (TFA), dimethylaniline (DMA), as well as commercially available N-linked glycan standards NA2 ([Gal-GlcNAc)₂Man₃(GlcNAc)₂]) and Man₃ ([2Man₃(GlcNAc)₂]) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PNGase F enzyme (500,000 units/ml) for the release of N-linked glycans was obtained from New England Biolabs UK Ltd. Protein A-TSK 20 particles were prepared by coupling protein A to 10 μ m tressyl-5PW activated TSK beads (Tosoh). Graphitised carbon black 4 (GCB) with mesh >400 nm and surface area 210 m²/g was provided by the Laboratori Analitici di Ricerca Associati, Rome, Italy. All other common chemicals were purchased from Merck (Darmstadt, Germany). A prototype Gyrolab instrument (model PP 2.1) equipped with the same hardware as the commercially available instruments, microfluidic CDs and the therapeutic antibodies Remicade, Erbitux and Avastin were kindly supplied by Gyros AB. All solvents used were of analytical grade unless otherwise stated, and water was purified using a Millipore water purification system to a resistance >18 M Ω /cm. Antibodies were diluted in cell supernatant of non-transfected CHO cells to mimic a typical case for the determination of glycosylation patterns in, e.g. different production batches. The antibodies were prepared at concentrations of 0.25, 0.17, 0.17 and 0.35 mg/ml for Avastin, Erbitux, Remicade and IgG-CHO, respectively. These concentrations were within the concentration range of antibodies in a common production process.

2.2. Affinity capture and release of N-linked glycans performed in the CD

The microfluidic design and operation has been described in detail previously [14]. The microfluidic structure comprises two columns in series. Liquids can be dispensed to reservoirs in contact with the first or second column. The liquid reservoir that is in contact with the second column can be used to precisely define 200 nl of liquid [19]. A liquid router is placed in-between the first and second columns and can be used to direct liquid eluting from the first column to either waste or the second column depending

Table 1

Approximate running time for each step used in the method of glycan preparation on the CD platform for MALDI-MS analysis.

Step	Approximate running time/step (min)
Conditioning protein A columns (4) ^a	8
Selective capture of antibodies	8
Washing protein A columns (4) ^a	8
Conditioning outer columns (4) ^a	0.5
Cleaning outer columns 1st	0.5
Cleaning outer columns 2nd	0.5
Releasing glycans and recapturing them in outer columns	60
Cleaning outer columns (2) ^a	0.5
Elution	15

^a Number of repeated steps.

on the rotational frequency used to move the liquid. The first and second columns are packed with immobilised protein A and GCB particles, respectively. The design of the microstructure and the general workflow used is shown in Fig. 1. A detailed description of the workflow is given below.

The following method was used to capture the antibodies, release the glycans using PNGase F, capture the glycans, and finally clean-up, elute and co-crystallise the glycans with a suitable MALDI-MS matrix:

1. Conditioning the protein A columns with 700 nl phosphate buffered saline solution (PBS, pH 6.8), performed four times.
2. Selective capturing of antibodies from 500 nl of sample prepared in cell supernatant.
3. Washing protein A columns with 700 nl of PBS, performed 4 times.
4. Conditioning the outer columns 4 times with aqueous solutions containing 50% of ACN and washing twice with deionised water.
5. Enzymatic release of glycans in the inner columns using 350 nl of PNGase F solution with a concentration of 100,000 units/ml and capturing glycans on the outer columns.
6. Washing outer columns twice with deionised water.
7. Elution and co-crystallisation of glycans with an aqueous solution containing 50% ACN, 0.1% TFA and DHB modified with dimethylaniline with concentrations of 21.52 mg/ml DHB and 4.64 mg/ml DMA.

PBS, sample and PNGase F solutions were dispensed to the sample reservoir on the CD, which was positioned closer to the centre of the CD than the liquid router. 50% ACN aqueous solution, H₂O and matrix mixture were loaded in the inlets placed farther away from the centre than the liquid router. The spin programme was basically similar to that described previously [14] except for a few modifications to improve the robustness of the method. The time required for each of the steps in the automated process is shown in Table 1. A low rotational frequency of the CD was used to allow the capture of antibodies on the protein A column, whilst all other unwanted proteins were directed to waste. The rotational frequency was gradually increased from 700 rpm to 2200 rpm during this operation which resulted in a constant flow through the column of about 1.5 nl/s. Glycans were released on this column by enzymatic reaction using a low rotational frequency so that the liquid did not move (400 or 500 rpm). By employing rapid acceleration of the CD to a high rotation frequency (3500 rpm) a portion of the liquid can be directed by the liquid router to the second column packed with GCB material, and the released glycans can be subsequently captured. A rotational frequency of 3500 rpm corresponds to an initial flow rate of about 15 nl/s with a gradual decrease as the volume of liquid in the sample reservoir decreases. Purified glycans are eluted into an evaporation structure that is not covered by the lid of the

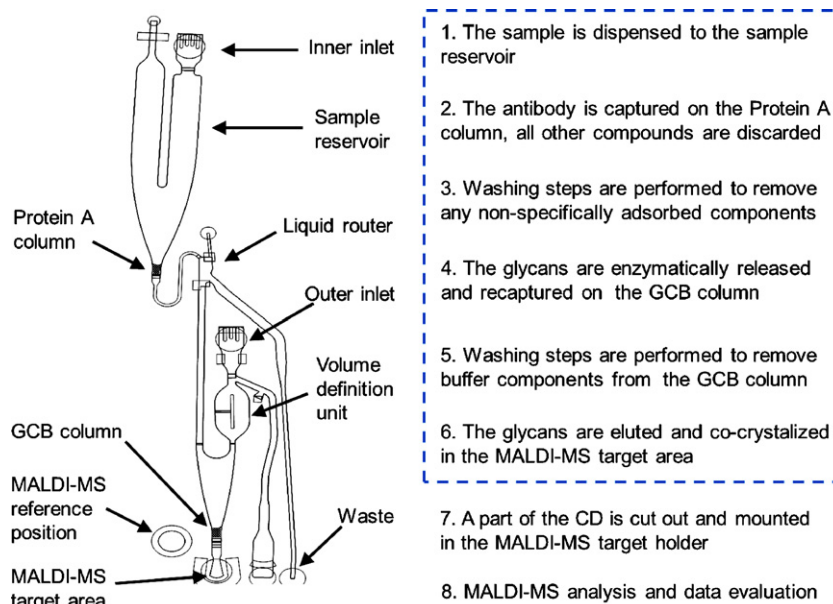


Fig. 1. CD microstructure and steps in the processing of samples for glycan profiling. The steps in the dashed box (1–6) are automatically performed in the Gyrolab instrument.

CD. A drop can be formed in this structure and held in place by balancing the centrifugal force moving the liquid out into the open and the capillary action pulling the liquid into the microfluidic channel. The rotational frequency used for this step was 700 rpm. The solvent evaporates leaving crystals containing the matrix and glycans. Reference structures are present in close proximity to each evaporation structure so that compounds suitable for calibration of MALDI can be co-crystallised in the MALDI matrix.

2.3. MALDI-TOF-MS analysis

The CD was cut in half and each piece was separately inserted into a dedicated sample holder. Mass spectra were acquired using an Ultraflex II MALDI-TOF/TOF (Bruker Daltonics) in positive reflector mode. The instrument was calibrated with Man3 and NA2 standard glycans. The calibration solution was dispensed to reference positions close to each evaporation well. A total of 500 shots were acquired for each sample.

2.4. Multivariate statistical analysis

The MS data were exported to ASCII files and Principal Component Analysis (PCA) was performed in MATLAB. The dataset was normalised to constant sum of all intensities of m/z of 1200 and above prior to multivariate analysis. Peaks were identified based on the maximum value within $\pm 1 m/z$ (Th) of the average mass of each detected glycan to prevent errors due to misalignment of the data. Whilst there are many commercial alignment softwares, this method was chosen for its simplicity and transparency. In this dataset the alignment problem is very simple because each peak is resolved very well. The main reason for the alignment is to acquire robust and simple peak integration. Peaks were then integrated by calculating the sum of the closest 50 data points corresponding to $1.2 m/z$ (Th) in each direction along the m/z . This integration includes the monoisotopic peak and the next peak in the isotopic pattern. To prevent human bias in where a peak starts and ends, a fixed amount of data points were used, and 50 was experimentally found to give the most stable value over the dataset. The reasons for choosing the criteria used in this study were: some peak-broadening of the monoisotopic peak, peak-splitting of low intensity peaks, and the extra variation in the data introduced

when peaks in the isotopic pattern close to the detection limit only sporadically were integrated. A different choice of integration criteria may be necessary for quality control of biomolecules having a more complex glycosylation pattern than antibodies produced using the cell types that has been studied here. This could involve for example shifting the m/z -region so that more of the isotopic pattern of the glycans and less of the m/z -region to the lower mass range of the monoisotopic peak is included. The integrated peaks were scaled to unit standard deviation. PCA was then applied to the scaled peak areas.

3. Results and discussion

3.1. MALDI-MS spectra

Of the four antibodies analysed in this work, two are produced in CHO cells and two in SP2/0 cells. These are two of three major cell types (CHO, NS0 and SP2/0) currently used in the production of monoclonal antibodies. Fig. 2 shows the MALDI-MS spectra of the commercially available antibodies Avastin, Erbitux and Remicade and one intended for therapeutic use, IgG-CHO. Identification of the peaks was made by submitting the monoisotopic m/z values of all the peaks into the GlycoMod database (<http://web.expasy.org/glycomod/>) with mass tolerance of 100 ppm as well as by comparison with data published in the literature. The identified glycans were amongst the glycoforms previously reported in human IgG molecules [20,21]. The main glycoforms in the studied antibodies were present at m/z of 1485 (H3N4F), 1647 (H4N4F) and 1809 (H5N4F) and are fucosylated biantennary glycans with 0, 1 and 2 galactose residues in the outer arms (also referred to as G0F, G1F and G2F). Non-fucosylated glycans, e.g. H3N4 (G0), H4N4 (G1), and H5N4 (G2) etc. were present at much lower levels. This was expected, as the cell lines currently used for the production of recombinant therapeutic antibodies generate glycans in the Fc part of the antibody that are fucosylated to a greater than 90% degree [22]. The high mannose glycan Man5 (H5N2) is generally present in IgGs produced with mammalian cell lines, typically at a level lower than 5% [23]. This glycan was also found to some extent in all four antibodies. Besides these common glycans, some others were detected with very low signal intensities, e.g. H3N3F, H4N3, H4N3F, H5N3, H6N2, H5N4, H6N3, H5N3F,

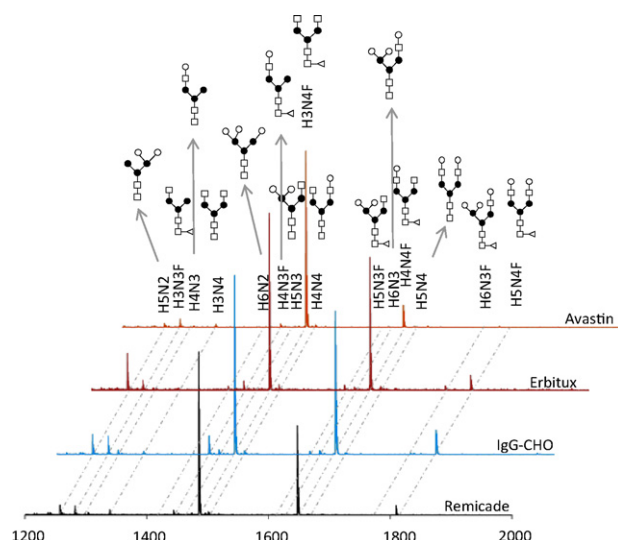


Fig. 2. MALDI-TOF-MS spectra of four antibodies, Avastin, Erbitux, Remicade and IgG-CHO. Empty squares, N-acetylglucosamine; filled circles, mannose; empty circles, galactose; empty triangles, fucose. Composition residues of glycans: H, hexose; N, N-acetylglucosamine; F, fucose. Representative *m/z* values of detected glycans: H5N2: 1257.4; H3N3F: 1282.4; H4N3: 1298.5; H3N4: 1339.4; H6N2: 1419.3; H4N3F: 1444.5; H5N3: 1460.4; H3N4F: 1485.6; H4N4: 1501.4; H5N3F: 1606.4; H6N3: 1622.4; H4N4F: 1647.5; H5N4: 1664.0; H6N3F: 1768.4; H5N4F: 1809.4.

H6N3F. These glycans have also been reported in recombinant IgG molecules [24–26].

Visual inspection of the spectra presented in Fig. 2 indicates that Avastin, produced in CHO cells, has the least variation in glycosylation amongst the four antibodies studied, with the H3N4F glycan giving the dominant signal and a relatively low signal corresponding to the H4N4F glycan. The other three antibodies (Remicade and Erbitux, produced in SP2/0 cells, IgG-CHO, produced in CHO cells) exhibited similar glycosylation patterns. Erbitux was also characterised by a higher signal due to the H5N2 glycan. Fig. 3 shows the relative abundances and standard deviations of the glycoforms of the four antibodies as area percentages. From this figure, it can be seen that the area percentage of the most abundant glycan, H3N4F, varies between the four antibodies with mean values of 75, 50, 45, and 39% for Avastin, Remicade, IgG-CHO, and Erbitux, respectively. The abundances of the other two major glycans, H4N4F and H5N4F,

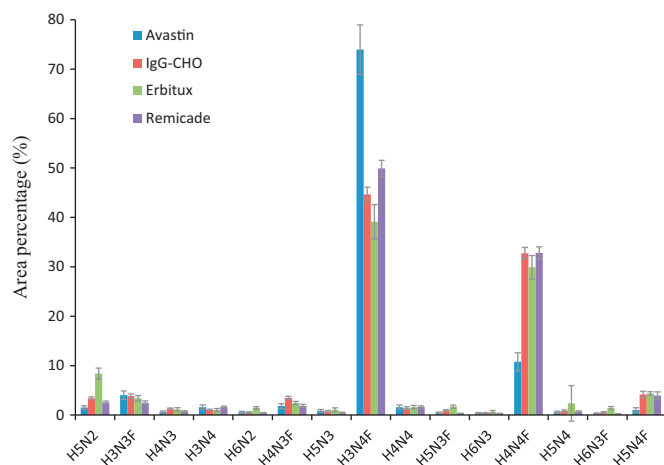


Fig. 3. Glycan distribution of four antibodies, Avastin, Erbitux, Remicade, and IgG-CHO. Means values were generated from a number of replicates, Avastin ($n=16$), IgG-CHO ($n=16$), Erbitux ($n=15$), Remicade ($n=15$). Error bars indicate standard deviations. Composition residues of glycans: H, hexose; N, N-acetylglucosamine; F, fucose.

were typically low for Avastin (about 11% and 1%, respectively) but were higher and similar in relative abundance for the other three antibodies. The area percentage of the high mannose glycan H5N2 ranged from 3 to 8% in the four antibodies. The proportion of these types of glycans reportedly depends on the clone selection and culture process [1,27,23,28] and strongly affects product efficacy [29]. H6N2 and H6N3F glycan levels were generally less than 2% but were highest in Erbitux. Other glycans such as H4N3, H3N4, H4N3F, H4N4, H5N3, H6N3 and H5N4 were generally less than 5%.

The relative standard deviation (RSD) of the H3N3F, H4N4F, H5N2 and H3N3F glycans were 5.3, 7.8, 14.0 and 15.8%, respectively. The less abundant glycans had larger RSD values (about 20%) whilst the H5N4 glycan exhibited a high variation of 53%. These values were calculated from 16 replicates of Avastin and IgG-CHO and 15 replicates of Remicade and Erbitux.

The mean abundances of each specific glycan in the different antibodies were compared using ANOVA followed by a Bonferroni correction. The five glycans that were present with the highest intensity in all four antibodies were evaluated: H5N2, H3N3F, H3N4F, H4N4F and H5N4F. The ANOVA showed that there was at least one mean for each glycan that was significantly different from the others. Individual pair comparison was analysed further using a Bonferroni correction. The test showed that the abundances of H5N2 and H3N4F glycans were significantly different between the four antibodies at confidence levels of 99.8 and 99.98%, respectively. The relative abundances of the H3N3F glycan of Remicade, Erbitux and Avastin were significantly different at the 99.2% confidence level. There was also a significant difference at the 99.98% level for the H4N4F glycan between the means of all groups except between those of CHO and Remicade. Only Avastin was significantly different concerning the relative abundance of the H5N4F glycan, and this was at the 99.98% level, whilst there was no significant difference for the other antibodies.

3.2. Discrimination of glycosylation patterns

PCA is a useful tool for dealing with multidimensional data and large datasets. It was therefore employed in the present study for the classification and discrimination of the glycosylation patterns of the different antibodies. The dataset contained 15 variables and 62 observations, including 16 analytical replicates of Avastin and IgG-CHO and 15 analytical replicates of Remicade and Erbitux. All variables were scaled to have an equal contribution in the data analysis, which resulted in better resolution for the differentiation of the groups. The score plot and loading plot of the PCA analysis with confidence levels of 95% are shown in Fig. 4a and b, respectively. The PCA interpretation shows that the dataset contains no strong outliers. The score plot indicates that the four antibodies are clearly separated by the first two principle components. Erbitux is mainly discriminated from the other antibodies along the first component, whereas Remicade, IgG-CHO and Avastin are only distinguished along the second component. The loading plot reveals that Erbitux is strongly separated from the other three antibodies because of higher relative abundances of several glycans, such as H6N2, H6N3F, H5N3F and H5N2, and lower relative abundances of H3N4 and H3N4F. On the other hand, Avastin, positioned in the lower left corner, has a higher relative signal of H3N4F and lower relative signals of other glycans, such as H5N2, H5N4F and H4N4F. Remicade is separated from IgG-CHO due to lower relative amounts of H5N2, H6N3F and H5N3F and higher relative amounts of H3N4 and H3N4F. It can also be seen that H5N4 has the weakest impact on the discrimination pattern due to the large standard deviation found in the analysis of this specific glycan in Erbitux. Moreover, the loading plot reveals a strong positive correlation between H4N4F, H5N4F, H4N3F and H4N3. Later principal components showed no

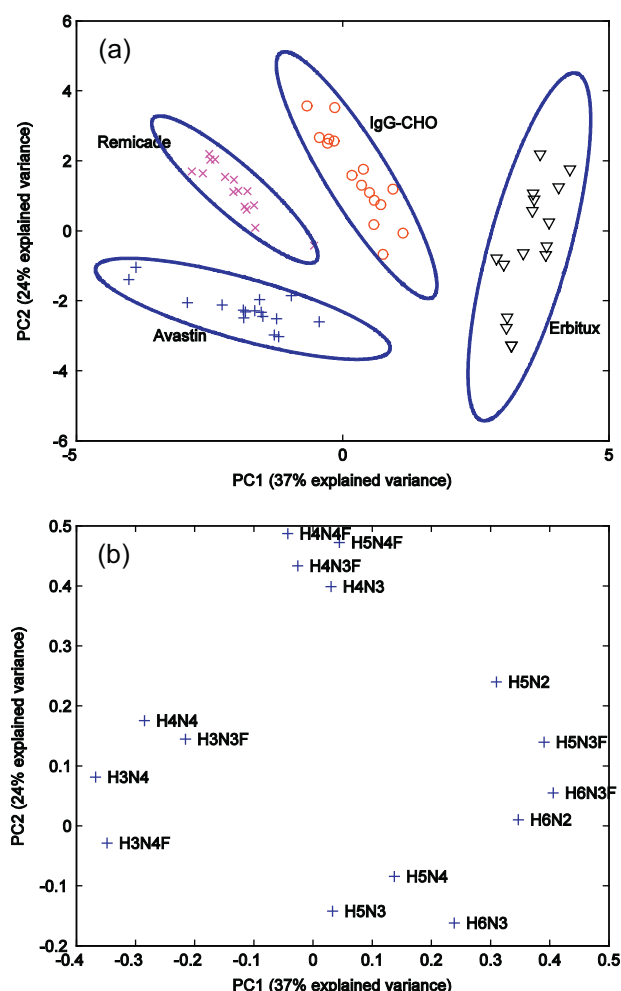


Fig. 4. (a) PCA score plot of glycosylation profiles derived from four antibodies, Avastin, Erbitux, Remicade, and IgG-CHO. The blue ellipses show 95% confidence intervals according to Hotelling's T^2 . (b) PCA loading plot of glycosylation profiles of four antibodies, Avastin, Erbitux, Remicade, and IgG-CHO. ("For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.")

ordered structure, and therefore most likely only describe random variation.

Random removal of replicates and the construction of new PCA-plots were performed to determine how many replicates were needed to produce group separation. The groups were clearly separated using as little as 10 data points per group. However, it is suggested that in a typical analysis more than 16 replicates would be used to define the normal distribution based on the glycosylation pattern for each antibody due to the very low material requirements for each replicate analysis. This would decrease the area of the confidence limits surrounding each group and thereby improve the definition of the normal distribution within one antibody.

3.3. SIMCA analysis for discrimination of glycosylation patterns

It is considerably difficult to acquire sufficient material to perform a real-case study of batch-to-batch or even lot-to-lot variation. Thus, mathematical modelling of the data generated in this study was performed in order to mimic quality control data. SIMCA analysis was used to test the feasibility of using the analytical procedure for quality control of therapeutic antibodies. Data from two antibodies were used to model a situation where a slight change in the glycosylation pattern occurred. The two antibodies used were

Table 2

The classification rates of the SIMCA models.

Model	True positive (%)	False positive (%)
Avastin	90.5	0.05
IgG-CHO	93.1	14.3
Erbitux	89.2	9.3
Remicade	93.2	9.0

Remicade and Erbitux as these are both produced in SP2/0 cells and have similar glycosylation patterns, except for a higher relative abundance of the high-mannose glycan H5N2 in Remicade. To simulate lot-to-lot variation, 22% of random noise was added to the generated data. This is equivalent to the variation reported in the literature [30]. Twelve replicates from each antibody were randomly selected and used to create SIMCA models. The remaining replicates from the same sample and replicates from the other sample were then used to estimate the model's ability to classify samples as belonging to the same or a different class. SIMCA analysis of Erbitux and Remicade gave a well defined group classification with an average false negative rate of 14% and an average false positive rate of 0.2%. False negative describes the samples of the model class but are not classified as the model class. False positive describes the samples of the other classes but are classified as the model class.

SIMCA analysis of the whole data set including all four antibodies was also performed the same way as above. The entire procedure, including the addition of noise, was repeated a thousand times for each sample to obtain an accurate estimation of the resolution between the classes. The classification rates of 4 different SIMCA models are summarised in Table 2. All four different antibodies were well classified with a typical result of approximately 10% false negatives meaning 90% true positives and 10% false positives. True positives are the samples which belong to the model class and are classified as the model class.

The dataset used was generated without optimisation of the MS parameters due to limited availability of the instrumentation. In addition, a relatively low number of shots were summed per sample and the number of replicates used to build the models was small considering that a multidimensional analysis was used. An improvement in one or all of these factors will most likely give better definition of the groups, and thereby an improvement in the ability to discriminate between different glycosylation patterns or identify production batches lacking a particular desired glycosylation pattern.

4. Conclusions

N-linked glycan profiling of four recombinant antibodies was performed using a CD platform in combination with MALDI-TOF-MS in a highly robust and time saving manner. The glycosylation profiles of the different antibodies could be separated using principal component analysis. Similar glycosylation patterns of two different antibodies could be reliably discriminated even under sub-optimal conditions, which demonstrates the potential of the method for monitoring and validating the glycosylation patterns of recombinant therapeutic antibodies during process optimisation, quality control or development of biosimilars.

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