Comparison of ZIC-HILIC and graphitized carbon-based analytical approaches combined with exoglycosidase digestions for analysis of glycans from monoclonal antibodies

Lea Mauko [a], Nathan A. Lacher [b], Matthias Pelzing [c], Anna Nordborg [a], Paul R. Haddad [a], Emily F. Hilder [a,*]

[a] *Pfizer Analytical Research Centre (PARC), Australian Centre for Research on Separation Science (ACROSS), School of Chemistry, University of Tasmania, Hobart, Tasmania, Australia*
[b] *Analytical R&D, Pfizer BioTherapeutics Pharmaceutical Sciences, Chesterfield, MO, USA*
[c] *Bruker Biosciences, Preston, Victoria, Australia*

ARTICLE INFO

ABSTRACT

Two LC approaches for analysis of therapeutic monoclonal antibodies (MAbs) are presented and compared. In the first approach, zwitterionic-type hydrophilic interaction chromatography (ZIC-HILIC) of 2-aminobenzamide-labelled glycans was coupled with fluorescence or electrospray ionisation mass spectrometric (ESI-MS) detection. The ZIC-HILIC method enabled relative quantification and identification of major glycan species. The sensitivity of fluorescence detection was higher compared to ESI-MS; however, MS detection enabled identification of co-eluted peaks. The new ZIC-HILIC approach was compared with porous graphitized carbon (PGC) separation of reduced glycans coupled with ESI-MS. Using PGC higher sensitivity was achieved compared to ZIC-HILIC due to the lower chemical background originating from the mobile phase and the derivatisation step, providing detailed information on minor glycan species. Furthermore, PGC exhibited excellent capability for separation of isobaric glycans with various degrees of mannosylation and galactosylation. The structures of glycans from MAbs used in this study were confirmed by exoglycosidase digestions. The two methods were applied to two monoclonal antibodies expressed in Chinese Hamster ovary cell lines and a monoclonal antibody expressed in a murine NS0 cell line. While the fluorescence-based approach is more suitable for routine glycan profiling due to the simplicity of data analysis, MS-based approaches were shown to provide detailed glycosylation analysis of complex glycoprotein samples.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Monoclonal antibodies (MAbs) are a rapidly growing group of pharmaceuticals, used primarily in anti-cancer and anti-rheumatic therapies [1–3]. Most currently used MAbs belong to the IgG structural class, containing two variable regions (Fv) with an antigen-binding specific site and a conserved region (Fc) that determines interaction with the effector molecules resulting in the elimination of the antigen [4]. Glycosylation in the Fc region plays an important role in the stability and therapeutic efficacy of the drugs [5,6]. The expression of therapeutic MAbs in non-human systems can also introduce different glycosylation patterns that can cause immune responses in patients [7,8]. Due to the structural complexity of glycans, glycosylation analysis remains a challenge and the main focus of current method development lies in the simplification of sample preparation, new separation approaches, and wide application of mass spectrometry (MS), either coupled with prior separation or on its own.

Common strategies in glycosylation analysis include liquid chromatography including anion-exchange chromatography, and capillary electrophoresis (CE), usually coupled with fluorescence or MS detection. Although fluorescence detection does not provide direct information on glycan structure or composition, it remains the most widely used approach for glycan profiling in the pharmaceutical industry. This is due to the straightforward data analysis, high sensitivity and capability of relative quantification of glycan species. Confirmation of the glycan structures usually requires chromatography of labelled standards, collection of fractions with offline MS analysis, or exoglycosidase digestions. In addition, the analysis may be extremely tedious and standards for less common glycans can be very expensive or are usually not available.

---

* Corresponding author. Tel.: +61 3 6226 7670; fax: +61 3 6226 2858.
E-mail address: Emily.Hilder@utas.edu.au (E.F. Hilder).

Furthermore, fluorescence detection is less suitable for the analysis of highly complex samples because overlapping of peaks can yield misleading results [9].

PNGase F released glycans are usually fluorescently labelled via reductive amination and the choice of the label depends on the separation technique used. Widely used fluorescent tags are 2-aminobenzamide (2-AB) and 2-aminobenzoic acid (2-AA) which are readily available in commercial kits and have been applied in combination with a range of techniques, including hydrophilic interaction liquid chromatography (HILIC), reversed phase liquid chromatography (RPLC) LC, anion-exchange chromatography, or CE [10–16]. Despite lower sensitivity and non-quantitative yields, 2-aminopyridine (2-AP) has been frequently used in the literature since it was introduced as a tag for sugar analysis much earlier than 2-AA and 2-AB [17,18]. Introducing tags with more charged groups, such as 8-aminopyrene-1,3,6-trisulfonic acid (APTS), is frequently used for CE analysis, both increasing resolution and reducing the time of the analysis [19]. In addition, sialylated glycans can be effectively separated by CE without prior labelling as demonstrated on recombinant erythropoietin [20].

HILIC columns with amide functionality have been used extensively for the separation of 2-AA and 2-AB labelled glycans coupled with fluorescence detection [11] and a database of 2-AB labelled glycans based on the HILIC retention properties has been established. Software has been developed that is linked to the database to automatically assign possible structures to a chromatographic peak [21]. Amide HILIC has also been coupled to ESI-MS. Since downscaling to capillary and nano format greatly enhances ESI-MS sensitivity, fluorescent labelling is no longer required and glycans can be analysed in the native form, thereby reducing the time and cost of sample preparation [22,23].

Recently, glycan profiling using HILIC columns with zwitterionic functionality (ZIC-HILIC, Merck SeQuant) has been introduced. This approach showed good capability for structural recognition of sialylated glycopeptides [24] and 2-aminopyridine derivatised glycans from human serum IgG [25]. Recently, we reported the separation of reduced glycans from monoclonal antibodies by ZIC-HILIC coupled with ESI-MS [26]. Similarly to amide HILIC, the sensitivity of the analysis was improved when a ZIC-HILIC nano column was employed. This allowed for the successful analysis of a range of neutral and sialylated glycans in the reduced form [27].

RPLC of glycans provides an orthogonal approach to HILIC, but requires a derivatisation step due to the poor retention of native glycans on C18 stationary phases. The same fluorescent tags as used in the HILIC mode can be employed in RPLC [15,16,28]. Since RPLC approaches allow the separation of permethylated glycans with or without fluorescent tag, permethylation may be employed to improve the quality of fragmentation data when the method is coupled to tandem MS [29]. Another stationary phase commonly employed in glycan separations is porous graphitized carbon (PGC), which exhibits an enhanced separation of isobaric species compared to HILIC and RPLC [30–32]. Glycans are commonly determined in the reduced form using PGC to avoid separation of anomers, which makes the method compatible with MS. The use of PGC has also been downscaled successfully to improve the sensitivity, either in nano column or microchip formats [33,34].

Due to the large number and complexity of glycan structures, various approaches for structural elucidation have been developed. One of the common approaches involves the HILIC separation of labelled glycans in combination with digestions using highly specific exoglycosidases, either simultaneously or sequentially [11,35]. Chromatography of standard glycans or MS is usually required for confirmation of the structures. The second approach for structural elucidation of glycans uses low-energy collision induced dissociation CID-MS/MS, either on its own or in combination with LC or other separation techniques [36,37]. The quality of structural

information obtained depends largely on the ions selected for fragmentation. Glycans can be fragmented in native, labelled, or permethylated forms. Permethylation increases sensitivity and the quality of fragmentation data and is highly suitable for high-energy MALDI-CID-MS/MS analysis of glycans without prior separation and can also involve exoglycosidase digestions [36,37]. However, when combined with PGC, permethylation of glycans can represent an additional analytical challenge due to the loss of chromatographic resolution [38,39].

In this paper we demonstrate the use of a ZIC-HILIC column for the separation of 2-AB labelled glycans released enzymatically from monoclonal antibodies. The ZIC-HILIC separation was coupled to fluorescence detection to obtain relative quantitative data and the method was optimised for direct coupling with ESI-MS for direct identification of glycans previously observed by fluorescence detection. The new method is compared to the PGC separation of reduced glycans coupled with ESI-MS detection, and the suitability of both methods for potential use in glycan profiling of MAbs is discussed. Three MAbs produced with recombinant technology were used in this study; MAbs denoted as MAb1 and MAb2 were expressed in Chinese Hamster ovary (CHO) cell lines and MAb3 was expressed in a murine NS0 cell line. Exoglycosidase digestions were used for the confirmation of the proposed structures.

2. Experimental

2.1. Reagents and chemicals

Unless otherwise noted, all chemicals were of analytical grade. Acetonitrile was obtained from VWR International (Poole, UK). Acetic acid, ethanol, sodium borohydride, 2-aminobenzamide labelling kit, PNGase F (from Elizabethkingia meningoseptica), α-galactosidase (from green coffee beans), $\alpha(2 \rightarrow 3,6,8,9)$ neuraminidase (from *Arthrobacter ureafaciens*) and α-mannosidase (from jack bean) were obtained from Sigma (St. Louis, MO, USA). β-N-acetylhexosaminidase (from jack bean) was purchased from Prozyme (CA, USA).

Samples of MAbs (denoted as MAb1, MAb2 and MAb3) prepared by recombinant DNA technology were kindly donated by Pfizer Inc. (Chesterfield, MO, USA).

2.2. Sample preparation

Glycoprotein samples (250–590 μg) were desalted prior to digestion using centrifugal filter units (Amicon Ultra, 10.000 MWCO, Millipore, Carrigtwohill, Ireland). Glycans were released from glycoproteins by digestion with 3 μL of PNGase F (500 units/mL) per 100 μL of glycoprotein solution in 50 mM ammonium bicarbonate buffer, pH 8.0, overnight at 37 °C. Proteins were removed by precipitation with 400 μL of ice-cold ethanol and the released glycans were dried under vacuum oven overnight at 40 °C. The supernatants were reduced with 30 μL of 0.5 M sodium borohydride in 0.025 mM sodium hydroxide in 50% ethanol (v/v) at room temperature overnight. The reaction was terminated by adding 5 μL of glacial acetic acid and samples were desalted by ion-exchange chromatography (Dowex MR-3 mixed bed, Sigma). The samples were then dried prior to the exoglycosidase treatment.

The exoglycosidase digestions were carried out using the following enzymes and conditions: α-galactosidase: 20 mU in 80 μL of 50 mM ammonium acetate pH 6.5, for 48 h, at room temperature; β-N-acetylhexosaminidase: 250 mU in 80 μL of 50 mM ammonium formate pH 4.8 for 48 h, at 37 °C; neuraminidase: 2.5 mU in 80 μL of 50 mM ammonium formate pH 4.8 for 48 h, at 37 °C; α-mannosidase: 680 mU in 80 μL of 250 mM ammonium formate pH

4.5 for 48 h, at 37 °C. The enzymes were removed prior to analysis by filtration using 10,000 MWCO centrifugal filters.

2.3. ZIC-HILIC coupled with fluorescence or ESI-MS detection

The glycans released from the MAbs were derivatised with 2-AB to enable fluorescence detection. Glycan profiling with fluorescence detection was performed on a Waters 2690 Separation Module equipped with a Waters 474 Scanning Fluorescence detector (Waters, Milford, MA, USA). The excitation wavelength was set at 330 nm and the emission wavelength at 420 nm. The column used was a ZIC-HILIC (2.1 mm × 150 mm, 3 μm) column from Merck SeQuant (Umeå, Sweden). Eluent A consisted of acetonitrile and Eluent B of 0.1% acetic acid (v/v) pH 3.25. The eluent flow-rate was 0.3 mL/min and the column oven temperature was thermostated to 30 °C. 2-AB labelled glycans were eluted using gradient elution, starting with a 10 min isocratic step at 20% B, followed by a linear gradient from 20% to 30% B in 50 min, followed by a wash step at 80% B for 5 min. Prior to the next injection, the system was equilibrated for 15 min at 20% B.

The ESI-MS experiments were performed using a MicrOTOF-Q (Bruker Daltonik, Bremen, Germany) with an Agilent 1200 series binary pump and autosampler (Agilent, Santa Clara, CA, USA). The ZIC-HILIC column was installed in a column oven (Thermosphere, TS-130, Phenomenex, USA) at 30 °C. The same gradient was applied as described for fluorescence detection. Data acquisition was performed in the positive mode. The ESI-MS conditions were: capillary voltage, 4.0 kV; drying temperature, 200 °C; drying gas flow, 8 L/min; nebulizer pressure, 1.5 bar; scan range m/z 50–3000.

2.4. Graphitized carbon column coupled with ESI-MS detection

Experiments were performed using a MicrOTOF-Q (Bruker Daltonik) with an Agilent 1200 series binary pump and autosampler (Agilent). Separations of reduced glycans were performed with a Hypercarb column (2.1 mm × 150 mm, 5 μm; Thermo Fisher Scientific) installed in a column oven (Thermosphere, TS-130, Phenomenex, USA) at 30 °C. The elution of glycans from MAbs was achieved using a binary gradient; eluent A consisted of acetonitrile and eluent B consisted of aqueous solution of 17 mM (0.1% (v/v)) acetic acid in water, unless otherwise noted. The flow-rate was set to 0.3 mL/min. Elution was achieved using a gradient from 7% to 30% A from 0 min to 40 min, followed by an isocratic wash step for 5 min at 60% A, and followed by an equilibration step for 15 min at 7% A. Data acquisition was performed in the positive mode and the same ESI-MS conditions were applied as described for 2-AB labelled glycans.

3. Results and discussion

3.1. Profiling of 2-AB labelled glycans by ZIC-HILIC coupled with fluorescence and ESI-MS detection

Despite fluorescence labelling being expensive and time consuming, fluorescence detection remains the most widely used detection mode in the quality control of biopharmaceuticals due to the high sensitivity and possibility to estimate the relative amounts of glycans in a glycoprotein. Since fluorescence detection does not allow for the direct structural elucidation, the identity of glycans must be confirmed either by MS or chromatography of labelled standards. However, the latter approach is limited by the number of commercially available standards.

In the present study a new method for the separation of 2-AB labelled glycans using standard-bore ZIC-HILIC coupled with fluorescence detection has been developed for the analysis of complex glycans from MAbs expressed in mammalian cell lines. A
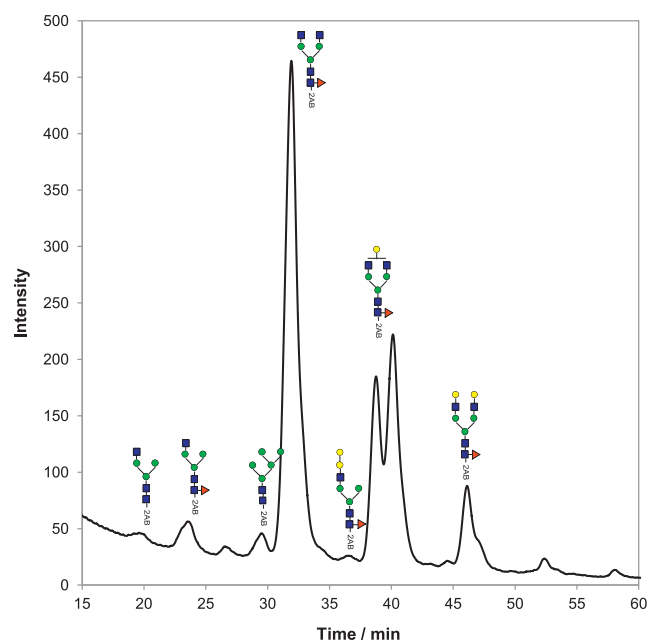

Fig. 1. Glycan profile of 2-AB labelled glycans from MAb3 obtained by ZIC-HILIC coupled with fluorescence detection. Gradient elution: mobile phase A – acetonitrile, mobile phase B – 0.1% (v/v) acetic acid. The glycan profile of the MAb3 expressed in the murine NS0 cell line exhibited greater heterogeneity with multiple peaks of low abundance. The relative peak areas are summarised in Table 1.

binary gradient consisting of acetonitrile and an aqueous solution of acetic acid was used for elution in order to make the method compatible for online coupling with ESI-MS. Three MAbs were used in this study: MAb1 and MAb2 were expressed in Chinese Hamster ovary (CHO) cell line, whereas MAb3 was expressed in murine NS0 cell line. MAb1 and MAb2 exhibited simple glycan profiles with dominating complex fucosylated glycans with low degree of galactosylation, as summarised in Table 1. On the other hand, the chromatogram of MAb3 (Fig. 1) shows a complex glycan profile with a wider range of minor glycan species and higher degree of galactosylation compared to MAb1 and MAb2. The peaks were assigned based on the ESI-MS results described below (Fig. 2). Several minor glycans were observed in the MAb samples when LC was coupled with fluorescence detection, and these could not be identified by ESI-MS due to the lower sensitivity. All 2-AB labelled glycans were identified as singly or doubly protonated and sodiated ions; the proportion of doubly charged species increased with the increasing molecular weight. Overlapping of major glycan species, G0 and G1-GlcNAc was observed, which indicated that relative quantification by fluorescence detection does not necessarily provide representative data for the identified glycans. New glycan species that were not previously identified by amide HILIC were also observed. The G0-Fuc glycan species was identified in MAb1 and MAb2 samples and two other previously unobserved glycan species were identified in MAb3, with the compositions $(Hex)_2(HexNAc)_1(Man)_3(GlcNAc)_2$ and $(Hex)_2(HexNAc)_1(dHex)_1(Man)_3(GlcNAc)_2$ (Table 1). Three possible structures could be assigned to both the nonfucosylated and the fucosylated forms; one complex structure with α-galactosylation on the 3-arm and two hybrid type structures with one or two mannose units on the 6-arm. For the m/z 1704.65, two peaks were found; however, it was not possible to elucidate the structures for the two peaks. For the early eluted peak only singly charged [M+H]⁺ ion was observed, whereas for the late eluted peak both singly and doubly protonated species were found. This suggested that the two observed peaks were likely to correspond to glycans with different compositions. All of the proposed structures

Table 1
ZIC-HILIC of 2-AB labelled glycans from MAbs coupled with fluorescence and ESI-MS detection.

Structure	Abbr.	$(m/z)_{theoretical}$, 2–AB $[M+H]^+$ $[M+2H]^{2+}$	Retention time/min	% Area		
				MAb1	MAb2	MAb3
⬛🟢🟢⬛⬛–2AB	G0-GlcNAc-Fuc	1234.4832 617.7452	19.9 24.9	<0.2	<0.2	0.4
🟢🟢⬛🔺⬛–2AB	G0-GlcNAc	1380.5411 690.7742	23.8	4.1	<0.2	2.9
Unknown			26.2	–	–	0.62
⬛🟢⬛⬛–2AB	G0-Fuc	1437.5626 719.2849	28.7	1.6	0.9	–
🟢🟢🟢⬛⬛–2AB	Man5	1355.5095 678.2584	30.0	1.3	<0.2	1.9
⬛🟢🔺⬛⬛–2AB	G0	1583.6205 792.3139	32.2	80.2	79.4	49.8
🟢🔺⬛🟡⬛–2AB	G1-GlcNAc	1542.5939 771.8006	33.2	–	–	ND
$(Hex)_2(HexNAc)_1 + (Man)_3(GlcNAc)_2$–2AB[a]		1558.5888 779.7981	37.7	–	–	0.4
🟡⬛🟢🔺🟢⬛⬛–2AB	G1	1745.6733 873.3403	39.1 40.5	3.4 7.0	7.3 10.9	13.7 21.0
$(Hex)_2(HexNAc)_1(Fuc)_1 + (Man)_3(GlcNAc)_2$–2AB[a]		1704.6467 852.8270	41.3	–	–	ND
Unknown			44.3	–	–	<0.2
🟡⬛🟢🔺🟡⬛🟢⬛⬛–2AB	G2	1907.7261 954.3667	46.8	0.7	1.0	8.0
Unknown			51.1	0.2	<0.2	–
Unknown			52.3	–	–	0.7
Unknown			57.5	–	–	0.4

⬛ : GlcNAc; 🟢 : Man; 🔺 : Fuc; 🟡 : Gal; ◇ : NeuGc. ND: not determined due to the co-elution with larger peaks.

[a] Structures not confirmed.

would be possible in MAbs expressed in murine NS0 cell line; however, the obtained MS data did not provide enough information to conclude which structure was correct.

The results obtained by ZIC-HILIC/fluorescence were compared to the standard procedure using amide HILIC for the separation of 2-AB labelled glycans as previously described [26]. Due to peak overlapping the relative peak areas of minor glycans were approximated and correlation between amide HILIC and ZIC-HILIC for the major glycan species was satisfactory. As expected, glycans from MAb1 and MAb2 were highly fucosylated with low levels of galactosylation, whereas higher galactosylation was observed in MAb3. Differences in relative quantification could be mainly contributed by co-elution of glycans. Due to the complexity of glycan samples the complete separation of all glycans may be extremely challenging. Fluorescence detection will generally only provide information on the major glycan species, the degree of galactosylation and fucosylation, and the presence of oligomannosidic and charged glycan species.

In the MAb1 and MAb2 samples, two peaks have been observed for the G0-GlcNAc-Fuc trace ($m/z = 1234.48$), which could be due to the separation of structural isomers. The two likely structures are GlcNAc attached to the 3- or 6-arm of the glycan core. For MAb3, only one G0-GlcNAc-Fuc peak was observed, suggesting that the difference in glycosylation pattern may be linked to the expression system of the MAbs.

The main advantage of the new ZIC-HILIC method compared to the standard HILIC procedure is shorter analysis time, which was reduced from 160 min to 85 min, including column equilibration
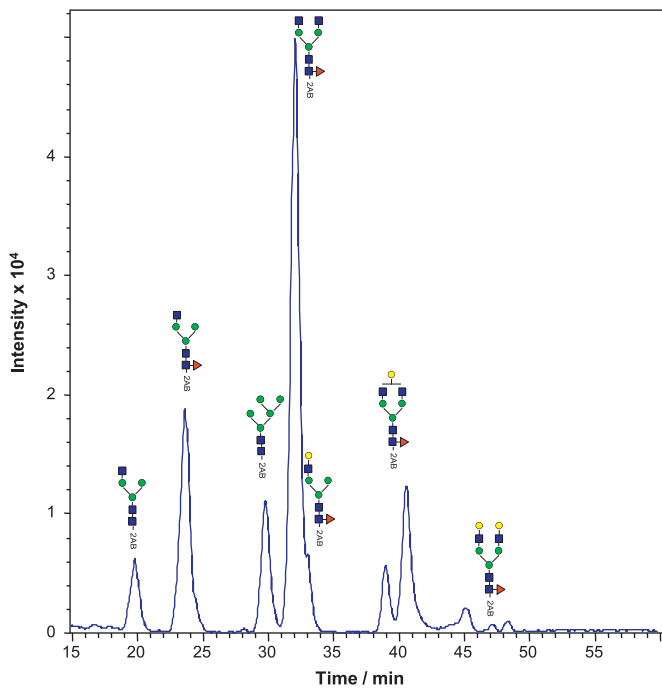
Fig. 2. BPC of 2-AB labelled glycans from MAb3 by ZIC-HILIC coupled with ESI-MS, positive ionisation mode. Gradient elution: mobile phase A – acetonitrile, mobile phase B – 0.1% (v/v) acetic acid.

time. Furthermore, the method was optimised for on-line coupling with ESI-MS using low concentrations of acetic acid as eluent. On the other hand, the standard procedure required high concentrations of ammonium formate buffer and was not suitable for the on-line ESI-MS detection of 2-AB labelled glycans due to the high ionisation suppression from this buffer resulting in the loss of sensitivity. A common approach for the identification of glycans using amide HILIC includes the use of 2-AB glycan standards and identification based on the retention time. This may require further collection of glycan fractions for confirmation of structures by MS. It is important to emphasise that not all the glycans that can be found in MAbs are commercially available and prices for purified and labelled glycans are often very high. The analysis of minor species is therefore extremely challenging and complete structural assignment of all peaks is not always possible using labelling and fluorescence detection. The new method proposed here could potentially be used for routine glycan profiling of therapeutic glycoproteins where stable glycosylation over time is expected. However, any change in glycan profile requires additional analysis employing MS or orthogonal separation techniques, often in combination with exoglycosidase digestions.

Despite the lower sensitivity there are some clear advantages of using ESI-MS instead of fluorescence detection. MS detection enables extraction of a single chromatogram trace, which is very useful when complex samples with co-eluted compounds are being analysed. As shown in Fig. 2, the glycan G1-GlcNAc is co-eluted with G0 glycan under the applied experimental conditions and hence it cannot be identified using fluorescence detection alone.

3.2. Glycan profiling by graphitized carbon LC with LC–MS combined with exoglycosidase digestions

Reduced glycans from the MAbs were further analysed using a PGC column coupled to ESI-MS. For MAb1 and MAb2 most of the glycans identified with the amide HILIC and ZIC-HILIC approaches could also be found in the samples using this method (Fig. 3).
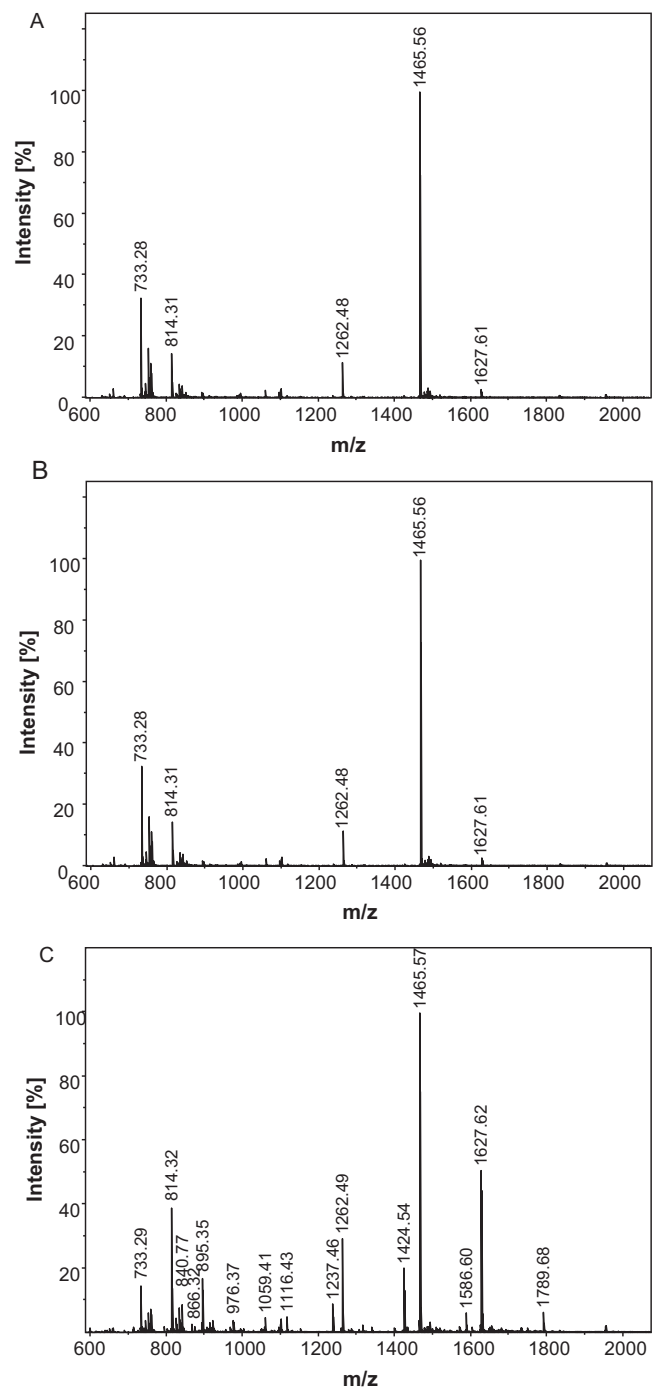

Fig. 3. Average mass spectra of reduced glycans from MAb1 (A), MAb2 (B) and MAb3 (C) over the entire elution range obtained by PGC, positive ion mode. Gradient elution: mobile phase A – acetonitrile, mobile phase B – 0.1% (v/v) acetic acid. MAb1 and MAb2 exhibited simple glycan profile with dominating G0 and G1 glycans. A wider range of low abundance complex and hybrid glycans was found in MAb3. Relative intensities in the mass spectra do not represent the actual relative amounts of glycans in MAbs due to the extensive in-source fragmentation.

The proposed structures discussed in this study were selected based on the glycan mass and previous reports of glycans found in the GlycoSuite database (http://glycosuitedb.expasy.org/glycosuite/glycodb). Accurate mass was used for the determination of monosaccharide composition and all the measured m/z were within 20 mDa error range, which allowed the confirmation of composition with high confidence. Due to the acidic mobile phase used in the PGC separation, the dominant ions were singly
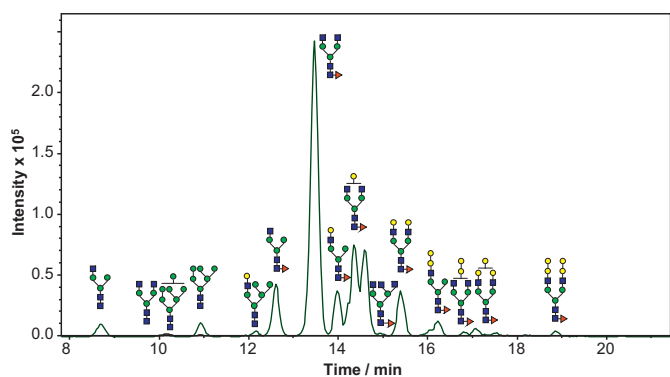
Fig. 4. PGC separation of neutral reduced glycans found in MAb3, positive ionisation mode. Gradient elution: mobile phase A – acetonitrile, mobile phase B – 0.1% (v/v) acetic acid. The sample was highly heterogeneous with multiple low abundant isobaric complex and hybrid species.

and doubly charged protonated ions. A preference to form doubly charged glycans was observed for larger glycans, as shown in Fig. 3. When isobaric species were present in the sample, a different ratio between the singly and doubly charged species was observed. This indicated different glycan composition, most likely due to various degrees of galactosylation and mannosylation.

The G1 glycans were partially separated under the conditions used. The separation of structural isomers was expected due to the stereo-selective properties of the PGC surface. For the G0-GlcNAc chromatogram trace several peaks were found, where the first peak belonged to the glycan and the later peaks were in-source fragments of G0 and G1 (not shown). Compared to ZIC-HILIC, the in-source fragmentation of both complex and high mannose glycans was significantly higher, most likely due to the higher acetic acid content in the mobile phase. The fragmentation pattern suggests the loss of terminal GlcNAc of nongalactosylated antenna in the G0 glycan, however the G1 fragmentation seems more complex with the loss of GlcNAc and GlcNAcGal. The loss of hexose (galactose) was not confirmed for any of the G1 isomers, and the glycosidic bond cleavage was observed only for GlcNAc of the antennae. For both G1 glycan isomers, the fragment peak of G0-GlcNAc with the same intensity was observed, indicating similar in-source stability of the 3- and 6-linked GlcNAcGal fragments. On the other hand, the loss of GlcNAc was more prominent for the early eluted G1 isomer. The results were not sufficient to conclude the terminal galactose position in G1 structural isomers, however they indicate a difference in stability of terminal GlcNAcs.

The in-source fragmentation was significantly reduced when 6 mM ammonium acetate was used as an aqueous mobile phase; however, sensitivity was lower due to ionisation suppression (data not shown). Since the focus of this study was on the analysis of minor glycan species, acetic acid was used in all the described experiments. The retention times and the proposed structures of all the glycans found in MAb1, MAb2 and MAb3 using the PGC column are summarised in Table 2. Consistent with the results seen in the HILIC chromatograms, the glycan profile of MAb3 exhibited greater complexity compared to the MAb1 and MAb2 with several previously unidentified glycans (Fig. 4). Single peaks were observed for G0 and Man5 glycans with single structural isomers. Several less common glycans were identified in the MAb3 sample and exoglycosidase digestions were used for confirmation of the proposed structures. For some m/z values in the control sample, multiple possible glycan structures can be assigned. Furthermore, for the same m/z chromatogram traces, several peaks were observed in the PGC chromatogram. Additional analysis was required to confirm whether the observed glycans were structural isomers or glycans with different monosaccharide composition. The high degree of

galactosylation and mannosylation observed in MAb3 allows for a large number of possible hybrid structures.

For the G2 chromatogram trace at m/z 895.34, three baseline separated peaks were observed. The first, and most intense, peak exhibited the same retention time as the G2 glycans from MAb1 and MAb2 and indicated the presence of the glycan with 2 β-linked galactose residues. The other two minor peaks corresponded to two α-galactosylated G2 glycans (Fig. 5A). No preference for either of the antennae was expected and similar peak intensity for both isomers was in agreement with the predicted structures. The α-galactosidase digestion confirmed the presence of an α-galactose epitope, which is potentially immunogenic in humans and is expected to be found in the MAb expressed in a murine cell line. Additionally, two peaks for m/z 976.37 and a peak for 1057.40 were found in MAb3 with confirmed terminal α-galactose. The similar peak intensity for G3 peaks suggested that there was no arm-preference for this structural motif as observed for α-galactosylated G2 glycan. In contrast, only one peak was observed for G0-GlcNAc(-Fuc), G1-GlcNAc(-Fuc) and G2-GlcNAc glycan species, indicating the arm-preference when only one arm is elongated by GlcNAc, which is in agreement with previously reported structures. It is suggested that GlcNAc is linked to the 3-arm of the core and the 6-arm is free or further mannosylated to form hybrid structures.

α-Galactosylation was confirmed in complex and hybrid structures; however, several peaks were found that could not be explained by α-galactosylation alone. The presence of isobaric hybrid species was also confirmed in the sample. Further N-acetylhexosaminidase and α-mannosidase digestions were performed to confirm the proposed hybrid structures.

Digestion of the G1 glycans with N-acetylhexosaminidase resulted in an additional intense peak for G1-GlcNAc with GlcNAc cleaved from the 3-arm (Fig. 5B). Similar results were observed for G2-GlcNAc, as a digestion product of α-galactosylated G2 glycan. These results confirmed that glycans from MAb3 formed only one structural isomer when there was only one complex-type antenna in the molecule, as previously suggested. By N-acetylhexosaminidase and α-mannosidase digestions it was possible to further distinguish between isobaric hybrid structures, as shown in Fig. 5B–D. Several isobaric hybrid species with various degrees of mannosylation and galactosylation were confirmed in the MAb3 sample. This practical example demonstrates the importance of separating glycan species with the same molecular weight to obtain complete information regarding the complexity of glycosylation in a given glycoprotein.

Digestion with α-mannosidase provided additional information on mannosylation in the hybrid structures. The α-mannosidase digestion was not complete since some mannosidic linkages are more resistant to α-mannosidase cleavage. It is suggested that α1 → 6 linkage is harder to digest than α1 → 3 and α1 → 2 [40]. The digestion of glycans with the proposed free 6-arm was very poor, indicating that core mannose was extremely resistant to α-mannosidase digestions. Similarly, the proposed glycans with one mannose residue on the 6-arm were only partially digested indicating the presence of α1 → 6 mannosidic linkage. On the other hand, proposed hybrid glycans with two mannose residues on the 6-arm were readily digested. The presence of additional closely eluted peaks in α-mannosidase digested samples suggests the possible presence of isomeric digestion products of higher mannosylated glycans. The presence of G2-GlcNAc and isobaric hybrid glycan in the sample is consistent with the results obtained by ZIC-HILIC where two peaks were observed for the corresponding m/z of 2-AB labelled glycan form. It is suggested that ZIC-HILIC has applicability for the separation of isobaric species; however, the MS sensitivity of this approach was lower compared to the PGC approach due to the higher content of acetonitrile in the mobile phase required for the

Table 2
Reduced glycans found in MAbs employing PGC coupled with ESI-MS detection.

Proposed structures	Abbr.	$M_{theor.}$/Da [M+H]$^+$ [M+2H]$^{2+}$	Retention time (min)	MAb1	MAb2	MAb3
	G0-GlcNAc-Fuc	1116.4301 558.7187	8.7	+	+	+
	G0-Fuc	1319.5095 660.2584	10.1	+	+	+
	Man7	1561.5620 781.2846	10.2 10.9	−	−	+
		1440.5357 720.7715	10.2	−	−	+
		1440.5357 720.7715	10.9	−	−	+
	G1-GlcNAc-Fuc	1278.4829 639.7451	10.9	−	−	+
	Man6	1399.5092 700.2582	10.9	−	−	+
		1602.5885 801.7979	12.2	−	−	+
	G0-GlcNAc	1262.4880 631.7476	12.6	+	+	+
	G0	1465.5674 733.28.73	13.5	+	+	+
		1602.5885 801.7979	13.1	−	−	+
		1586.5936 793.8005	13.2 14.9	−	−	+
		1440.5357 720.7715	13.7	−	−	+
	G1-GlcNAc	1424.5408 712.7740	14.0	−	−	+

Table 2 (*Continued*)
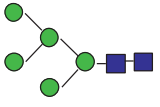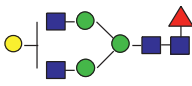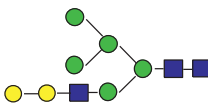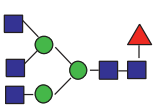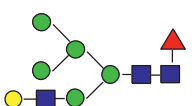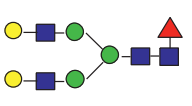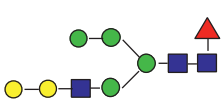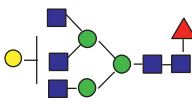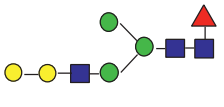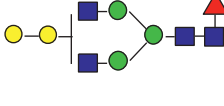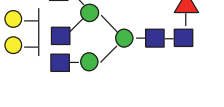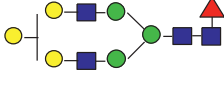
Proposed structures	Abbr.	$M_{theor.}$/Da [M+H]$^+$ [M+2H]$^{2+}$	Retention time (min)	MAb1	MAb2	MAb3
	Man5	1237.4564 619.2318	14.3	+	+	+
	G1	1627.6202 814.3137	14.4 14.6	+	+	+
		1764.6414 882.8243	14.7	–	–	+
		1668.6467 834.8270	15.0	–	–	+
		1748.6465 874.8269	15.0	–	–	+
	G2	1789.6730 895.3401	15.4	+	+	+
(Hex)$_1$(HexNAc)$_1$(dHex)$_2$ +(Man)$_3$(GlcNAc)$_2$ [a]		1570.60 785.80	15.5	–	–	+
		1748.6465 874.8269	15.5	–	–	+
		1830.6996 915.8534	15.9 17.2	–	–	+
(Hex)$_1$(HexNAc)$_2$(dHex)$_2$ +(Man)$_3$(GlcNAc)$_2$ [a]		1773.6781 887.3427	16.1 16.6	–	–	+
	G2-GlcNAc	1586.5936 793.8005	16.2	–	–	+
	G2	1789.6730 895.3401	16.1 16.8	–	–	+
		1992.7524 996.8798	16.7 17.6	–	–	+
(Hex)$_2$(HexNAc)$_2$(dHex)$_2$ +(Man)$_3$(GlcNAc)$_2$ [a]		1935.7309 968.3691	16.8 17.2	–	–	+
	G3	1951.7258 976.3666	17.1 17.5	–	–	+

Table 2 (*Continued*)

Proposed structures	Abbr.	$M_{theor.}$/Da $[M+H]^+$ $[M+2H]^{2+}$	Retention time (min)	MAb1	MAb2	MAb3
		1910.6993 955.8533	17.1	–	–	+
		2154.8052 1077.9062	18.2	–	–	+
(Hex)$_3$(HexNAc)$_2$(dHex)$_2$ + (Man)$_3$(GlcNAc)$_2$ [a]		2097.7837 1049.3955	18.2 18.8	–	–	+
	G4	2113.7787 1057.3930	18.8	–	–	+
		1909.6789 955.3431	27.1	–	–	+
		1893.6840 947.3456	28.4	–	–	+
	G1 + NeuGc	1934.7105 967.8589	29.8	–	–	+
		2055.7368 1028.3720	29.9	–	–	+
	G1 + NeuGc- GlcNAc	866.3192 1731.6311	30.2	–	–	+
	G2 + NeuGc	2096.7633 1048.8853	30.5	–	–	+
	G3 + NeuGc	2258.8162 1129.9117	31.9	–	–	+

■ : GlcNAc; ● : Man; ▲ : Fuc; ○ : Gal; ◇ : NeuGc.

[a] Structures not confirmed.

separation of 2-AB labelled glycans, which contributes more chemical background compared to the mobile phase with high water content used for PGC separation. Additional impurities were added to the sample by the derivatisation procedure, which increased the chemical noise in ZIC-HILIC-MS of 2-AB labelled glycans and identification of some peaks by MS detection was therefore more challenging.

All hybrid structures in the MAb3 sample were found in fucosylated and defucosylated form, whereas fucosylated species were dominant for complex glycans and only defucosylated mannose

glycans were found in the sample. The complex galactosylation pattern in MAb3 was not surprising, considering that the MAb3 was expressed in NS0 murine cell line which exhibits α-galactosyltransferase activity which is generally less likely to be found in CHO cells [7,41].

Sialylated species previously identified by nano ZIC-HILIC were observed in the PGC chromatogram of MAb3 glycans. As previously suggested, the charged glycans were much more strongly retained by PGC under the applied conditions, with broader peaks being observed compared to those of neutral glycans. Exoglycosidase
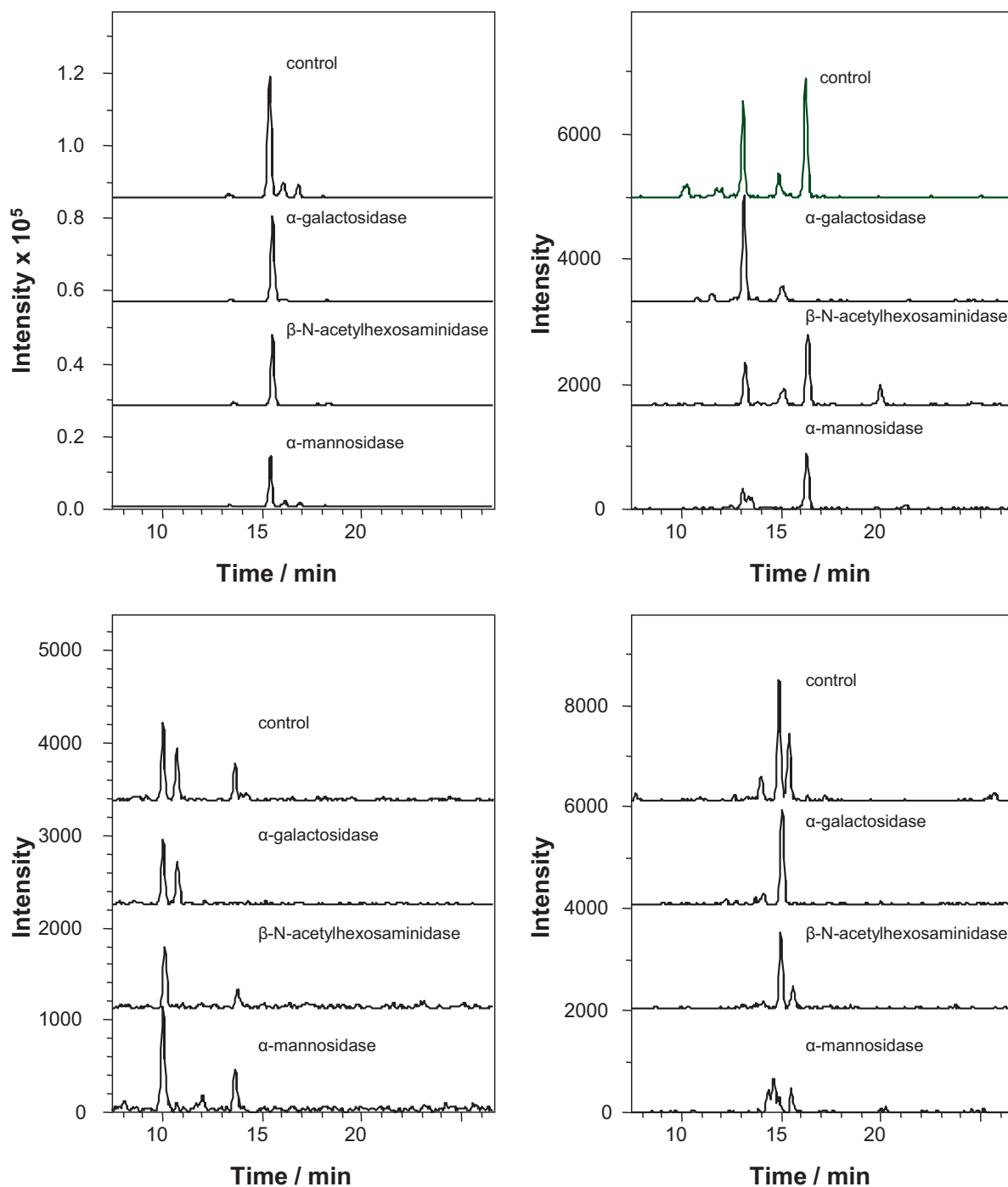
Fig. 5. Exoglycosidase digestions combined with PGC separation, positive ionisation mode. Gradient elution: mobile phase A – acetonitrile, mobile phase B – 0.1% (v/v) acetic acid. EICs of G2 glycan, $m/z = 895.34$ (A); $(Hex)_2(HexNAc)_1(dHex)_1 + (Man)_3(GlcNAc)_2$ glycan, $m/z = 793.80$ (B); $(Hex)_2(HexNAc)_1 + (Man)_3(GlcNAc)_2$ glycan, $m/z = 1440.54$ (C); $(Hex)_3(HexNAc)_1(dHex)_1 + (Man)_3(GlcNAc)_2$ glycan, $m/z = 874.83$ (D).

digestions confirmed the proposed structures, which were mainly core-fucosylated containing NeuGc rather than NeuAc. NeuGc was expected for the MAb expressed in murine system and consistent with previously reported data [27].

Using PGC, another series of complex glycans with additional GlcNAc was identified that was not observed by the ZIC–HILIC method due to the low abundance. There are two likely series of glycans with the same monosaccharide compositions, either with an additional bisecting GlcNAc or a GlcNAc attached to one of the arms forming triantennary glycans. Two glycans were confirmed as

triantennary; however, it is possible to deduce that all the observed glycans belong to the same structural group and contain none to three terminal galactose residues.

Using PGC, a series of glycans with additional fucose was observed in the MAb3 sample. Using the GlycoSuite database, two likely previously reported structures were found for most of the observed m/z values, one with the additional fucose linked to GlcNAc and the other one with fucose linked to terminal Gal. Since this glycosylation pattern is quite unusual, further digestions with linkage-specific fucosidase are required to elucidate this

structural motif. It is likely that all the observed glycans from the same glycoprotein would exhibit similar glycosylation pattern as evident in the case of the hybrid and complex glycans found in this MAb. It is assumed that all the additionally fucosylated glycans belong to the same structural class and differ only in the degree of galactosylation. Compared to the ZIC-HILIC of 2-AB labelled glycans, the PGC approach is less suitable for relative quantification due to the extensive in-source fragmentation and a large variety of charged species that depends on glycan size and structure. Another drawback is the different ionisation efficiency for neutral and sialylated glycans that greatly depends on the ionisation mode [20,27].

As demonstrated here, the use of PGC was extremely useful in the analysis of highly complex samples, such as the glycans released from MAb3. The previously described ZIC-HILIC approaches provided only limited information regarding the isobaric species present in the sample and the MS sensitivity was somewhat low due to a higher background originating from the higher acetonitrile content in the mobile phase. By using the ZIC-HILIC method for the separation of 2-AB labelled glycans no charged species were confirmed in any of the MAb samples and it is suggested that downscaling may be required to achieve the appropriate MS sensitivity [27]. On the other hand, the use of PGC was found to be less suitable for the analysis of sialylated glycans due to poor reproducibility of the retention times of charged glycans under the applied conditions. The observed properties of the column could be due to strong adsorption capabilities of the PGC surface which leads to rapid column fouling. Cleaning and regeneration of the PGC columns with strong acids and bases has been suggested to restore retention capabilities of the PGC surface and achieve reproducible retention times [42]. It was shown that PGC is superior for structural elucidation of neutral isobaric glycans compared to HILIC, with more detailed information being provided for hybrid structures. However, the PGC method is less suitable for glycan quantification and routine analysis in quality control of biopharmaceuticals. We believe that methods based on fluorescence detection will continue to develop due to the high sensitivity, amenability to quantitative analysis and simplicity of data interpretation.

4. Conclusions

In this work two analytical approaches for glycosylation analysis of MAbs have been compared. Dilute acetic acid was used as the aqueous eluent component in both approaches to achieve optimum ESI-MS sensitivity which allowed analysis of trace level glycans. A new approach employing ZIC-HILIC for 2-AB labelled glycans coupled with fluorescence and ESI-MS detection was demonstrated. While fluorescence detection exhibited higher sensitivity compared to ESI-MS and provided relative quantification data, MS detection was shown to be more useful when complex samples were being analysed due to the possible co-elution of peaks. The use of ZIC-HILIC was compared to the separation of reduced glycans employing graphitized carbon. PGC exhibited excellent capability for separation of isobaric glycan species and multiple glycans with various degrees of galactosylation and mannosylation were analysed and the proposed structures were confirmed by exoglycosidase digestions. The higher MS sensitivity of the PGC method due to the lower chemical background enabled identification of numerous trace glycans that have not been detected by HILIC analytical approaches. On the other hand, poor reproducibility of retention times for separation of sialylated glycans was achieved, indicating the need for development of suitable pre-treatment of the column surface to increase column stability. Due to its relatively simple data interpretation, we believe that fluorescence detection

will remain the most commonly used technique in routine glycosylation analysis of large sets of samples. However, the MS-based approaches exhibited clear advantages in providing direct information on glycan composition that can often be translated into structure. It was demonstrated that efficient separation with sensitive detection may be required to reveal the complexity within glycosylation of therapeutic glycoproteins.

References

[1] J. Griggs, K. Zinkewich-Peotti, Br. J. Cancer 101 (2009) 1807–1812.
[2] X.-R. Jiang, A. Song, S. Bergelson, T. Arroll, B. Parekh, K. May, S. Chung, R. Strouse, A. Mire-Sluis, M. Schenerman, Nat. Rev. Drug Discov. 10 (2011) 101–111.
[3] J. Wohlgemuth, M. Karas, W. Jiang, R. Hendriks, S. Andrecht, J. Sep. Sci. 33 (2010) 880–890.
[4] R. Jefferis, Nat. Rev. Drug Discov. 8 (2009) 226–234.
[5] K. Mori, S. Iida, N. Yamane-Ohnuki, Y. Kanda, R. Kuni-Kamochi, R. Nakano, H. Imai-Nishiya, A. Okazaki, T. Shinkawa, A. Natsume, R. Niwa, K. Shitara, M. Satoh, Cytotechnology 55 (2007) 109–114.
[6] Y. Kaneko, F. Nimmerjahn, E.V. Ravetch, Science 313 (2006) 670–673.
[7] K.N. Baker, M.H. Rendall, A.E. Hills, M. Hoare, R.B. Freedman, D.C. James, Biotechnol. Bioeng. 73 (2001) 188–202.
[8] D.M. Sheeley, B.M. Merrill, L.C.E. Taylor, Anal. Biochem. 247 (1997) 102–110.
[9] Y. Wada, P. Azadi, C.E. Costello, A. Dell, R.A. Dwek, H. Geyer, R. Geyer, K. Kakehi, N.G. Karlsson, K. Kato, N. Kawasaki, K.H. Khoo, S. Kim, A. Kondo, E. Lattova, Y. Mechref, E. Miyoshi, K. Nakamura, H. Narimatsu, M.V. Novotny, N.H. Packer, H. Perreault, J. Peter-Katalinic, G. Pohlentz, V.N. Reinhold, P.M. Rudd, A. Suzuki, N. Taniguchi, Glycobiology 17 (2007) 411–422.
[10] J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, R.B. Parekh, Anal. Biochem. 230 (1995) 229–238.
[11] G.R. Guile, P.M. Rudd, D.R. Wing, S.B. Prime, R.A. Dwek, Anal. Biochem. 240 (1996) 210–226.
[12] K.R. Anumula, S.T. Dhume, Glycobiology 8 (1998) 685–694.
[13] S. Kamoda, R. Ishikawa, K. Kakehi, J. Chromatogr. A 1133 (2006) 332–339.
[14] K. Deguchi, T. Keira, K. Yamada, H. Ito, Y. Takegawa, H. Nakagawa, S.I. Nishimura, J. Chromatogr. A 1189 (2008) 169–174.
[15] X. Chen, G.C. Flynn, Anal. Biochem. 370 (2007) 147–161.
[16] B.D. Prater, H.M. Connelly, Q. Qin, S.L. Cockrill, Anal. Biochem. 385 (2009) 69–79.
[17] K.R. Anumula, Anal. Biochem. 350 (2006) 1–23.
[18] S. Hase, T. Ikenaka, Y. Matsushima, Biochem. Biophys. Res. Commun. 85 (1978) 257–263.
[19] L.A. Gennaro, O. Salas-Solano, Anal. Chem. 80 (2008) 3838–3845.
[20] E. Balaguer, U. Demelbauer, M. Pelzing, V. Sanz-Nebot, J. Barbosa, C. Neusüß, Electrophoresis 27 (2006) 2638–2650.
[21] M.P. Campbell, L. Royle, C.M. Radcliffe, R.A. Dwek, P.M. Rudd, Bioinformatics 24 (2008) 1214–1216.
[22] M. Wuhrer, C.A.M. Koeleman, C.H. Hokke, A.M. Deelder, Int. J. Mass Spectrom. 232 (2004) 51–57.
[23] J. Zhao, W.L. Qiu, D.M. Simeone, D.M. Lubman, J. Proteome Res. 6 (2007) 1126–1138.
[24] Y. Takegawa, K. Deguchi, H. Ito, T. Keira, H. Nakagawa, S.I. Nishimura, J. Sep. Sci. 29 (2006) 2533–2540.
[25] Y. Takegawa, K. Deguchi, T. Keira, H. Ito, H. Nakagawa, S. Nishimura, J. Chromatogr. A 1113 (2006) 177–181.
[26] L. Mauko, A. Nordborg, J.P. Hutchinson, N.A. Lacher, E.F. Hilder, P.R. Haddad, Anal. Biochem. 408 (2011) 235–241.
[27] L. Mauko, M. Pelzing, S. Dolman, A. Nordborg, N.A. Lacher, E.F. Hilder, P.R. Haddad, J. Chromatogr. A 1218 (2011) 6419–6425.
[28] Y. Takegawa, K. Deguchi, S. Ito, S. Yoshioka, A. Sano, K. Yoshinari, K. Kobayashi, H. Nakagawa, K. Monde, S.-I. Nishimura, Anal. Chem. 76 (2004) 7294–7303.
[29] J. Delaney, P. Vouros, Rapid Commun. Mass Spectrom. 15 (2001) 325–334.
[30] M. Pabst, J.S. Bondili, J. Stadlmann, L. Mach, F. Altmann, Anal. Chem. 79 (2007) 5051–5057.
[31] N. Kawasaki, M. Ohta, S. Hyuga, O. Hashimoto, T. Hayakawa, Anal. Biochem. 269 (1999) 297–303.
[32] J. Stadlmann, M. Pabst, D. Kolarich, R. Kunert, F. Altmann, Proteomics 8 (2008) 2858–2871.
[33] N.G. Karlsson, N.L. Wilson, H.-J. Wirth, P. Dawes, H. Joshi, N.H. Packer, Rapid Commun. Mass Spectrom. 18 (2004) 2282–2292.
[34] C.S. Chu, M.R. Ninonuevo, B.H. Clowers, P.D. Perkins, H.J. An, H.F. Yin, K. Killeen, S. Miyamoto, R. Grimm, C.B. Lebrilla, Proteomics 9 (2009) 1939–1951.

[35] S. Prime, J. Dearnley, A.M. Ventom, R.B. Parekh, C.J. Edge, J. Chromatogr. A 720 (1996) 263–274.
[36] W. Morelle, J.-C. Michalski, Nat. Protoc. 2 (2007) 1585–1602.
[37] J. Qian, T. Liu, L. Yang, A. Daus, R. Crowley, Q.W. Zhou, Anal. Biochem. 364 (2007) 8–18.
[38] C. Costell, J. Contado-Miller, J. Cipollo, J. Am. Soc. Mass Spectrom. 18 (2007) 1799–1812.
[39] L.R. Ruhaak, A.M. Deelder, M. Wuhrer, Anal. Bioanal. Chem. 394 (2009) 163–174.
[40] B. Winchester, Glycobiology 15 (2005) 1R–15R.
[41] C.J. Bosques, B.E. Collins, J.W. Meador, H. Sarvaiya, J.L. Murphy, G. DelloRusso, D.A. Bulik, I.H. Hsu, N. Washburn, S.F. Sipsey, J.R. Myette, R. Raman, Z. Shriver, R. Sasisekharan, G. Venkataraman, Nat. Biotechnol. 28 (2010) 1153–1156.
[42] M. Pabst, F. Altmann, Anal. Chem. 80 (2008) 7534–7542.