Analytical Biochemistry 623 (2021) 114205

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

High-throughput and high-sensitivity N-Glycan profiling: A platform for biopharmaceutical development and disease biomarker discovery

Yongjing Xie [a,**], Letícia Martins Mota [a], Adam Bergin [a], Roisin O'Flaherty [a], Aled Jones [b], Bethan Morgan [c], Michael Butler [a,*]

[a] National Institute for Bioprocessing Research and Training, Foster Avenue, Mount Merrion, Blackrock, Co. Dublin, Ireland
[b] Agilent Technologies Inc, 3832 Bay Center Pl, Hayward, CA, 94545, USA
[c] Agilent Technologies LDA UK Ltd, Essex Rd, Church Stretton, SY6 6AX, UK

ARTICLE INFO

Keywords:
Glycosylation
Glycomics
Glycan profiling
Antibody
Human serum
Biomarker discovery
HILIC-FLD
Exoglycosidase

ABSTRACT

Protein glycosylation contributes to critical biological function of glycoproteins. Glycan analysis is essential for the production of biopharmaceuticals as well as for the identification of disease biomarkers. However, glycans are highly heterogeneous, which has considerably hampered the progress of glycomics. Here, we present an improved 96-well plate format platform for streamlined glycan profiling that takes advantage of rapid glycoprotein denaturation, deglycosylation, fluorescent derivatization, and on-matrix glycan clean-up. This approach offers high sensitivity with consistent identification and quantification of diverse N-glycans across multiple samples on a high-throughput scale. We demonstrate its capability for N-glycan profiling of glycoproteins from various sources, including two recombinant monoclonal antibodies produced from Chinese Hamster Ovary cells, EG2-hFc and rituximab, polyclonal antibodies purified from human serum, and total glycoproteins from human serum. Combined with the complementary information obtained by sequential digestion from exoglycosidase arrays, this approach allows the detection and identification of multiple N-glycans in these complex biological samples. The reagents, workflow, and Hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FLD), are simple enough to be implemented into a straightforward user-friendly setup. This improved technology provides a powerful tool in support of rapid advancement of glycan analysis for biopharmaceutical development and biomarker discovery for clinical disease diagnosis.

1. Introduction

Post-translational modifications (PTMs) refer to the covalent and enzymatic biochemical modifications of proteins following biosynthesis in a time- and signal-dependent manner [1]. PTMs have been well acknowledged to play a critical role in regulation and diversification of the cellular proteome due to their broad scope in various biological processes, such as gene regulation, cell proliferation, differentiation and apoptosis, tissue development, disease progression, and drug resistance [2,3]. Currently, over 650 unique PTMs have been experimentally identified (http://www.uniprot.org/docs/ptmlist.txt) [4], and the most common PTMs include phosphorylation, glycosylation, methylation, ubiquitination and acetylation. As one of the most prominent and abundant PTMs, protein glycosylation has attracted extensive interest due to the diversity and complexity of multiple glycoforms that may be associated with protein isoforms.

Protein glycosylation has significant impacts on protein structure and function, by influencing stability, solubility, protein folding, and mediating protein-protein interactions. Additionally, glycosylation contributes to numerous crucial biological functions, such as pathogen binding, cell recognition and adhesion, molecular trafficking and clearance, receptor activation, signal transduction and endocytosis [5–7]. Increased understanding of molecular details of protein glycosylation has facilitated the alignment between glycosylation and human physiological conditions. It has been recognized that aberrant protein glycosylation associates with many diseases [6,8,9]. Consequently, the discovery and detailed characterization of protein glycosylation as a diagnostic and prognostic biomarker has significant biomedical implications, especially in the field of cancer, neurodegenerative disease, inflammatory disease, and liver disease, etc. [10–13]. Additionally,

* Corresponding author.
** Corresponding author.
E-mail addresses: yongjing.xie@nibrt.ie (Y. Xie), michael.butler@nibrt.ie (M. Butler).

https://doi.org/10.1016/j.ab.2021.114205
Received 17 November 2020; Received in revised form 11 March 2021; Accepted 8 April 2021
Available online 20 April 2021

protein glycosylation of biopharmaceutical drugs plays a vital role in assuring product quality, safety, and potency. This is because glycosylation contributes to modulating a variety of biological properties, such as immunogenicity, serum clearance, *in vivo* circulating half-life, and anti-inflammatory activity [14,15]. Identification, description, and mapping of protein glycosylation are of great importance for determining its functional role in a biological context. Therefore, a comprehensive knowledge of protein glycosylation is necessary to ensure the biomanufacturing of efficacious therapeutics as well as for disease diagnosis and prognosis. To this end, developing sensitive and reliable strategies for high throughput glycomic analysis is decidedly necessary.

However, due to their complex biosynthetic pathways, glycans are highly heterogeneous, varying in site occupancy, monosaccharide composition, glycosidic linkages, and length [16]. This complexity has considerably hampered the progress in glycosylation related disease biomarker discovery and biopharmaceutical development. Therefore, detailed structural and quantitative analysis of aberrant glycoproteins in a fast and sensitive way has become critical. There are a variety of techniques available to profile glycoprotein glycans, including high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), isoelectric focusing (IEF), and lectin-based microarray [17–20].

High-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAE-PAD) was the first established chromatographic technique for carbohydrates analysis [21]. This method takes advantage of the weakly acidic nature of carbohydrates and ionizes the hydroxyl groups at high pH for highly selective separations by using strong anion exchange stationary phases. The separation is based on oligosaccharide charge, size, composition, and linkage. HPAE-PAD does not require any derivatization before carbohydrates analysis, and is routinely used for determination of monosaccharides, sialic acids, mannose-6-phosphate (M-6-P), sugar alcohol, and oligosaccharides etc [22–24].

MS instrumentation, combined with glycopeptide/protein fragmentation and glycan enrichment techniques, has increasingly been used for mapping protein glycosylation and detailed glycan structural determination [25–28]. Whereas linkage information and monosaccharide identification are usually deduced by gas chromatography (GC) [29] and solution state nuclear magnetic resonance spectroscopy (NMR) [30–33] or by the HPAE-PAD technique mentioned above [24,34–36]. However, the availability of these sophisticated instrumentations remains a major hurdle in structural glycobiology. Hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FLD) has been well recognized as a standard method for the separation and quantitation of released N-glycans after conventional 2-aminobenzamide (2-AB) labelling [37] or other fluorescent dyes [38]. However, the whole glycan preparation procedure is time consuming, usually taking several hours or even days for glycan sample preparation, with poor reproducibility and low sensitivity.

In this study, we present data on applications of an improved high-throughput, high-sensitivity platform for fast glycan profiling that takes advantage of a streamlined 96-well plate format for rapid glycoprotein denaturation, deglycosylation, fluorescent derivatization, and glycan purification. With the introduction of an innovative fluorescent derivatizing reagent, 2-(diethylamino) ethyl 4-({[(2,5-dioxopyrrolidin-1-yl) oxy] carbonyl} amino) benzoate (named: InstantPC), the released glycosylamine intermediate is fluorescently labelled within only 1 min at 50 °C using activated carbamate chemistry. This is compared to the commonly utilized reductive amination derivatization method using either 2-AB or procainamide, where 2- or 3-h reaction times at 60 °C are routinely used. The total glycan preparation time of our current improved workflow has been shortened to less than 10 min, which is a significant improvement on alternative methods. A 96 well-plate format technique for glycan sample preparation that included glycan release from monoclonal antibodies, derivatization with 8-Aminopyrene-1,3,6-trisulfonic-acid (APTS) and purification has been reported previously [39]. However, the total glycan sample preparation took approximately 60 min prior to analysis by capillary electrophoresis.

We compared our current workflow using InstantPC with previously used procedures using 2-AB and procainamide as the derivatizing reagent. Procainamide serves as a "bridging molecule" in this comparison because it shares structural similarity to InstantPC, whilst derivatizes glycans via reductive amination in an identical mechanism to that of 2-AB. Comparisons of the chemical properties and glycan derivatization mechanisms for InstantPC, procainamide and 2-AB are summarised in Table 1. We first demonstrated its application in N-glycan profiling of recombinant monoclonal antibodies with different molecular sizes produced from Chinese Hamster Ovary (CHO) cells, and polyclonal antibodies purified from human serum in a fast and reproducible fashion. We then applied this methodology to a total N-linked glycan analysis of healthy human serum glycoproteins. This demonstrated the applicability of the method to N-glycan analysis of glycoproteins from complex biological samples. This improved 96-well plate format for N-glycan profiling showed good reproducibility. InstantPC exhibited comparable fluorescence sensitivity to procainamide, whilst significantly higher fluorescent sensitivity to the commonly used 2-AB. The method described in this paper offers high-throughput and high-sensitivity for N-glycan detection and quantification as required for multiple sample analysis in biopharmaceutical development and disease diagnosis by mass screening of clinical samples.

2. Experimental section

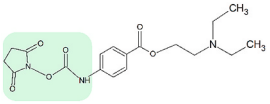
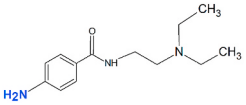
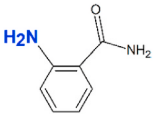
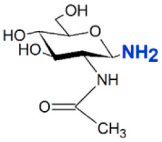
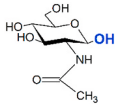
Materials and Reagents The AdvanceBio Gly-X N-Glycan Prep with InstantPC kit, 96-ct (GX96-IPC) consisting of three modules, including Gly-X deglycosylation module (GX96-100), Gly-X InstantPC labelling module (GX96-101), and Gly-X InstantPC cleanup module (GX96-102), was donated by Agilent Technologies. The AdvanceBio Gly-X N-Glycan Prep with 2-AB express kit, 96-ct (GX96-2AB) consisting of three modules, including Gly-X deglycosylation module (GX96-100), Gly-X 2-AB express labelling module (GX96-401), and Gly-X 2-AB express cleanup module (GX96-402), were donated by Agilent Technologies. AdvanceBio exoglycosidases of Sialidase A (GK80040), Sialidase C (GK80030), β (1–3,4)-Galactosidase (bovine testis, GKX5013), β (1–4)-Galactosidase (*Streptococcus pneumoniae*), GKX5014), β-N-acetylhexosaminidase (GK80050), α (1–2,3,6)-Mannosidase (jack bean) (GKX-5010), and α (1–2,3,4,6)-Fucosidase (bovine kidney) (GKX-5006) were donated by Agilent Technologies. Procainamide (10733211) was purchased from Thermo Fisher scientific. Human serum from human male AB plasma (H4522) was purchased from Sigma Aldrich. HPLC-grade acetonitrile was purchased from Sigma and Milli-Q water was used in all preparations. All the common chemicals were purchased from Sigma Aldrich.

Monoclonal antibodies of EG2 and Rituximab expression The stably transfected Chinese Hamster Ovary (CHO) cell line (DUXB II) expressing chimeric human-llama heavy chain monoclonal antibody (designated EG2) was kindly provided by Yves Durocher, NRC, Canada [40]. The GS-CHO cell line (CHO–S-RTX) expressing the chimeric mouse-human monoclonal antibody (designated rituximab) was kindly provided by the Technical University of Denmark [41].

The cells were inoculated at an initial cell density of 0.5×10^6 cells/ml with a working volume of 2 L and grown in suspension under serum- and protein-free conditions in the chemically defined Biogro-CHO medium (Biogro Technologies Inc, Winnipeg, Canada) containing 25 mM glucose, 4 mM glutamine. Viable cell density and viability measurements were performed by Trypan blue exclusion method as described previously [42]. The cells were grown until the desired cell density was attained, followed by cell harvesting by centrifugation at 1500 rpm for 5 min. The supernatant which contained the EG2 and Rituximab antibodies was then filtered through 0.45 μm pore sized Supor ® hydrophilic polyethersulfone (PES) membrane (Pall Corporation, Port Washington, NY) for clarification, and stored at −20 °C until further use.

Monoclonal and polyclonal Antibodies purification and

Table 1
Chemistry properties of the studied fluorescent dyes for glycan derivatization.

Name	InstantPC	Procainamide	2-Aminobenzamide
Chemical structure			
IUPAC name	2-(diethylamino)ethyl 4-({[(2,5-dioxopyrrolidin-1-yl)oxy]carbonyl} amino)benzoate	4-amino-*N*-[2-(diethylamino)ethyl] benzamide	2-Aminobenzamide
Molecular formula	$C_{18}H_{23}N_3O_6$	$C_{13}H_{21}N_3O$	$C_7H_8N_2O$
Formula weight	377.3917	235.3253	136.1512
Monoisotopic Mass	377.1587	235.1685	136.0637
Reaction mechanism	activated carbamate chemistry	Reductive amination	
Targeting glycan functional group	 Glycosylamine	 Glycan	
Glycan molecular weight addition	261.1477	219.1736	120.0688
FLD	λex = 285 nm, λem = 345 nm	λex = 310 nm, λem = 370 nm	λex = 260 nm, λem = 430 nm

quantification The purification of EG2 and Rituximab monoclonal antibodies and human serum polyclonal antibodies was performed with protein A affinity chromatography [43,44] using an AKTA Avant System (GE healthcare). The HiTrap Protein A HP antibody purification column (GE healthcare) was first equilibrated with binding buffer (25 mM sodium phosphate, pH 7.4) at a flow rate of 1.0 mL/min. After sample loading and column washing to remove unbound molecules, elution was performed at the same flow rate using 0.1 M citric acid, pH 3.0. The collected fraction containing the target antibodies was neutralized by adding suitable amount 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). The antibodies were quantified by Cedex HiRes Analyzer (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) based on the turbidimeric immunoassay [45,46], then stored at −20 °C for subsequent analysis.

Rapid in-solution glycoprotein denaturation and enzymatic deglycosylation The in-solution enzymatic deglycosylation of human serum or isolated glycoproteins and antibodies was carried out according to the manufacturer's instruction of Agilent AdvanceBio Gly-X N-Glycan Prep with InstantPC kit or 2-AB express kit, respectively. Human serum (5 μl) or glycoprotein samples (40 μg) were diluted with a suitable amount of 50 mM NH_4HCO_3 or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0) to make a final volume of 20 μl. Gly-X denaturant (2 μl) was added to the 20 μl of glycoprotein solution, mixed thoroughly and incubated at 90 °C for 3 min. After leaving at room temperature for 2 min, 2 μl of N-Glycanase working solution was added, mixed thoroughly and incubated at 50 °C for 5 min. For glycan labelling with 2-AB or procainamide, 2 μl of finishing reagent was added to the glycoprotein solution, mixed thoroughly and incubated at 50 °C for another 10 min. For glycan labelling with InstantPC the finishing reagent step is not required.

N-glycan labelling with InstantPC and purification. InstantPC dye solution was prepared by dissolving one vial of InstantPC dye with 150 μl of the accompanying solvent and mixed well. The dye solution (5 μl) was added to the above-prepared sample, and incubated at 50 °C for 1 min. The Load/Wash solution (150 μl of 2.5% formic acid/97.5% acetonitrile) was added to each sample, and then the entire sample (179 μl) was transferred to each well of the Gly-X Cleanup plate containing 400 μl of Load/Wash solution. After passing the solution through the cleanup plate by applying a vacuum, samples were washed with 600 μl of Load/Wash solution three times. InstantPC labelled N-glycans were eluted with 100 μl of Gly-X InstantPC eluent (160 mM ammonium formate/10% (v/v) acetonitrile, pH 4.4). The time of this entire procedure for glycan labelling was reduced to less than an hour. The collected N-glycan solutions were analysed immediately without the need for further concentration, or alternatively stored at −20 °C for future analysis.

N-glycan labelling with procainamide and purification Acetonitrile (150 μl) was added to each sample, centrifuged for 1 min, the supernatant collected and dried by speedVac Vacuum Concentrator (Thermo Fisher Scientific). Procainamide labelling solution was prepared by dissolving procainamide 40 mg and sodium cyanborohydride 25 mg with 500 μl of DMSO: acetic acid: H_2O (280: 120: 100 v/v/v) solution, and mixed well. Procainamide labelling solution (25 μl) was added to each sample, and incubated at 65 °C for 1 h. Acetonitrile (150 μl) was added to each sample, and then the entire sample (175 μl) was transferred to each well of the Gly-X Cleanup plate containing 400 μl of acetonitrile. After removing the solution, the sample was washed with 600 μl of acetonitrile three times. The procainamide labelled N-glycans were eluted with 100 μl of H_2O. The entire procedure for glycan sample preparation took approximately 3 h. The collected N-glycan solutions were analysed immediately without the need for further concentration, or alternatively stored at −20 °C for future analysis.

On-matrix N-glycan labelling with 2-AB and purification Acetonitrile (150 μl) was added to each sample, and then the entire sample (176 μl) transferred to each well of the Gly-X Cleanup plate containing 450 μl of acetonitrile. After removing the solution, the sample was washed with 600 μl of acetonitrile once. 2-AB labelling solution (80 μl of 2-AB solution: 2-AB reductant: 2-AB catalyst: acetonitrile at the ratio of 1: 1: 2: 44 v/v/v/v) was added to each well, and incubated at 65 °C for 1 h. After washing with 600 μl acetonitrile three times, the 2-AB labelled N-glycans were eluted with 100 μl distilled water three times. The entire procedure for sample preparation took approximately 2 h. The collected N-glycan solution was dried by speedVac Vacuum Concentrator (Thermo Fisher Scientific), and then stored at −20 °C for future analysis.

N-Glycan profiling by HILIC-FLD The profiles of fluorescently labelled N-glycan were determined by Hydrophilic Interaction Liquid Chromatography with fluorescence detection (HILIC-FLD) using two chromatography systems. The first was an Acquity I class ultra-performance liquid chromatography system (UPLC) equipped with Acquity UPLC Glycan BEH Amide Column (130 Å, 1.7 μm,

2.1 mm × 150 mm, SKU: 186004742) under the control of Empower software (Waters Corporation). The second was a 1260 Infinity high performance liquid chromatography system (HPLC) equipped with AdvanceBio Glycan Mapping column (120 Å, 2.7 μm, 2.1 mm × 150 mm, Part NO: 683775–913) under the control of OpenLAB CDS ChemStation software (Agilent Technologies). Both systems consist of a quaternary solvent pump, autosampler, and a fluorescence detector. A comparison of the chromatographic conditions used to investigate the glycan separation is available in the supporting information (Tables S1–S3, and Figs. S1–S4). The detector was set with excitation and emission wavelengths of 260 and 430 nm for 2-AB, 310 and 370 nm for procainamide, and 285 and 345 nm for InstantPC, respectively. Prior to injection, the previously 2-AB labelled glycan samples were solubilized in 5 μl of H_2O and 15 μl of acetonitrile, and the injection volume was 5 μl. The procainamide and InstantPC labelled glycan samples were injected at a volume of 1 μl without any further treatment prior to injection. The N-glycans were separated with 50 mM ammonium formate (pH 4.4) as solvent A and acetonitrile as solvent B. The separation method was by a linear gradient of 70–60% acetonitrile (v/v) at a flow rate of 0.5 ml/min in a 35 min analytical run. Samples were maintained at 5 °C before injection and the separation temperature was 60 °C. The systems were calibrated using Agilent AdvanceBio InstantPC Maltodextrin ladder (GKPC-503), Ludger procainamide labelled glucose homopolymer ladder (CPROC-GHP-30) and Waters 2-AB dextran calibration ladder (186006841). The correlation between glucose unit (GU) value and retention time T (min) was fitted to 5th order polynomial curve to obtain the standard curve, as shown in supporting information Tables S4 and S5, and Figs. S5 and S6.

N-glycan exoglycosidases sequential digestion The N-glycan exoglycosidases sequential digestion was accomplished by the addition of 2.0 μl of α (2–3,6,8,9) Sialidase A, 2.0 μL of β (1–4)-Galactosidase, 2.0 μL of β-N-Acetyl-hexosaminidase, and 2.0 μL of α (1–2,3,4,6)-Fucosidase to 10 μL of labelled N-glycan samples containing reaction mixture (released from equivalent of 40 μg glycoprotein). For multiple exoglycosidases digestion, 10x reaction buffer of 0.5 M ammonium acetate pH 5.5 was utilized. The reactions were incubated at 37 °C for 24 h without shaking. For the complete physiochemical properties of the exoglycosidases, and full exoglycosidases sequential digestion panel, please refer to supporting information Tables S6 and S7, respectively.

Data Processing and Analysis The chromatographic glycan peaks resulting from the HILIC-FLD analysis were processed with either Empower or OpenLAB CDS ChemStation software using an automated method with a traditional integration. Individual glycan peaks were analysed on the basis of the correlation between measured retention time and glucose units, and subsequently compared to reference values in GlycoStore database (https://glycostore.org/) for glycan structure assignment [47]. Total area normalization was performed, where the area under the curve (AUC) of each major labelled N-linked glycans were divided by the total area of all the major glycans to calculate the relative abundances (AUC %). Repeatability was estimated using replicates of the same sample calculating the coefficient of variation (CV, the ratio of the standard deviation to the mean).

The signal-to-noise (S/N) ratios were calculated using noise from within the same chromatogram as follows: $S/N = 2H/h$, where H is the height of the peak measured from the peak apex to a baseline extrapolated over a distance ≥ 5 times the peak width at its half-height, and h is the differences between the largest and smallest baseline (noise) values observed over ≥ 5 times the width at the half-height of the peak.

3. Results

(1) Qualitative and quantitative analysis of the glycosylation pattern of monoclonal antibodies

EG2-hFc is a humanized chimeric single domain monoclonal antibody produced from Chinese hamster ovary cells (CHO-DUXB), targeting the epidermal growth factor receptor (EGFR) that is most commonly over-expressed in non-small cell lung cancer (NSCLC). EG2-hFc is a camelid-type antibody devoid of light chains, reducing its molecular size to approximately 80 kDa with the potential to increase tissue penetration and reduce dosage requirements [48–50]. To test the capability of the developed high-throughput, high-sensitivity workflow for qualitative and quantitative N-glycan analysis, we first carried out glycan profiling of EG2-hFc. The released N-glycans were derivatized with 2-AB, procainamide, and InstantPC, respectively, followed by HILIC separation and fluorescence (FLR) detection. As shown in Fig. 1A, it is clear that HILIC separation of EG2-hFc N-glycans labelled with InstantPC resulted in well resolved peaks for all major N-glycan species with the 30 min method used. This N-glycan profile is typical of monoclonal antibodies produced in Chinese hamster ovary cells: predominantly neutral biantennary complex N-glycans with core fucose, and a relatively low proportion of glycans without core fucose.

As shown in the insert of Fig. 1A, the glycan fluorescence profiles for both InstantPC and procainamide are comparable to those obtained for 2-AB, although the HILIC retention times of InstantPC and procainamide labelled N-glycans are longer than 2-AB labelled N-glycans due to the presence of non-reactive alkyl chain and tertiary amine group. As shown in Fig. 1B and supporting information Table S8, InstantPC gave approximately twice-higher fluorescence intensities than procainamide, while both dyes gave significantly higher fluorescence intensities than 2-AB, x149, and x67 fold enhancement, respectively. These findings are in good agreement to previous report, where 2-AB, procainamide and another derivatizing agent of Rapi-Fluor-MS were investigated [51,52].

We used the FA2 peak in the EG2 glycan profiles to compare the signal-to-noise ratios (SNR). The noise values were measured as 0.038, 0.045 and 0.050 μv for InstantPC, procainamide and 2-aminobenzamide, respectively, and were comparable for the three different fluorescent dyes although different excitation and emission wavelengths were employed. However, the SNR determined for InstantPC was calculated to be approximately x1.8 and x71.5 higher than for procainamide and 2-AB, respectively (data not shown). In other words, InstantPC and procainamide showed comparable limits of fluorescent detection and quantification, while 2-AB exhibited a significantly lower sensitivity.

The higher sensitivity of InstantPC and procainamide labels enabled certain peaks to be detected that were absent in the 2-AB chromatograms. As shown in Fig. 1 and the scaled EG2 glycan profiling chromatograms in supporting information Fig. S8, low abundant glycan species were detectable with either InstantPC or procainamide derivatization at a retention time >20 min, but apparently absent with the 2-AB label, even at 25 times concentrated sample injection for chromatographic detection as stated in Materials and Methods. Therefore, it is reasonable to deduce that labelling with either InstantPC or procainamide may hold the potential to increase the capability to identify minor N-glycan species that may have significant biological activity. As shown in Fig. 1C, the relative abundance of each major glycan species quantified by relative percentage of area under the peak demonstrated that the N-glycan profiles for InstantPC, procainamide, and 2-AB are in reasonable agreement with very little deviation. Additionally, as shown in Supporting information Fig. S7 and Table S9, the reproducibility of the developed workflow for N-glycan analysis was high, with coefficient of variation (CV) at 0.083, 0.011 and 0.012 for 2-AB, procainamide and InstantPC label, respectively.

We further tested the developed workflow by carrying out N-glycan profiling of rituximab, another genetically engineered chimeric monoclonal antibody containing murine light and heavy chain variable region sequences and human constant region sequences, with a molecular size of approximately 145 kDa. Rituximab targets and attaches to the CD20 antigen primarily found on the surface of both normal and malignant immune system B cells and is routinely used to treat adults with Non-Hodgkin's Lymphoma (NHL) or Chronic Lymphocytic Leukemia (CCL) [53,54]. As shown in Fig. 2A and supporting information Table S7,

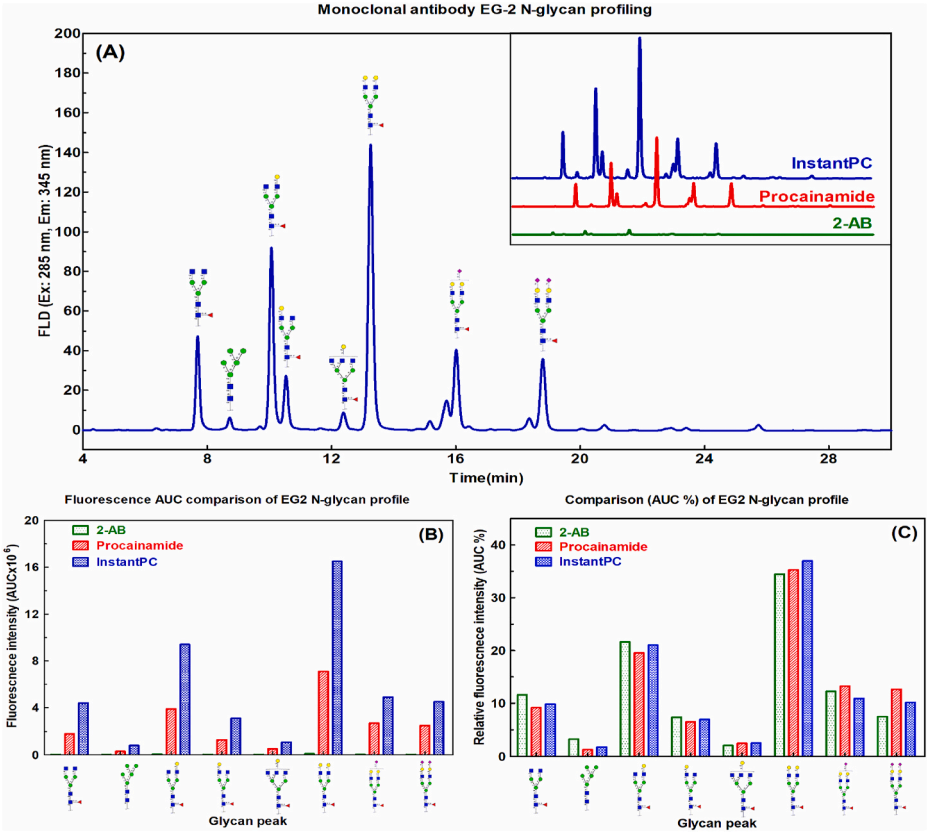


Fig. 1. N-glycan profiling from humanized chimeric heavy chain monoclonal antibody (EG2-hFc). (A) N-glycan profiles from EG2-hFc labelled with InstantPC, and the insert shows glycans labelled with InstantPC (blue), procainamide (red), and 2-Aminobenzamide (green), respectively on the same scale of fluorescent intensity. (B) Comparison of the fluorescence intensity area under the curve (AUC) of major N-glycans from EG2-hFc labelled with 2-Aminobenzamide, procainamide, and InstantPC, respectively. (C) Comparison of the relative fluorescence intensity abundance (AUC %) of major N-glycans from EG2-hFc labelled with 2-Aminobenzamide, procainamide, and InstantPC, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

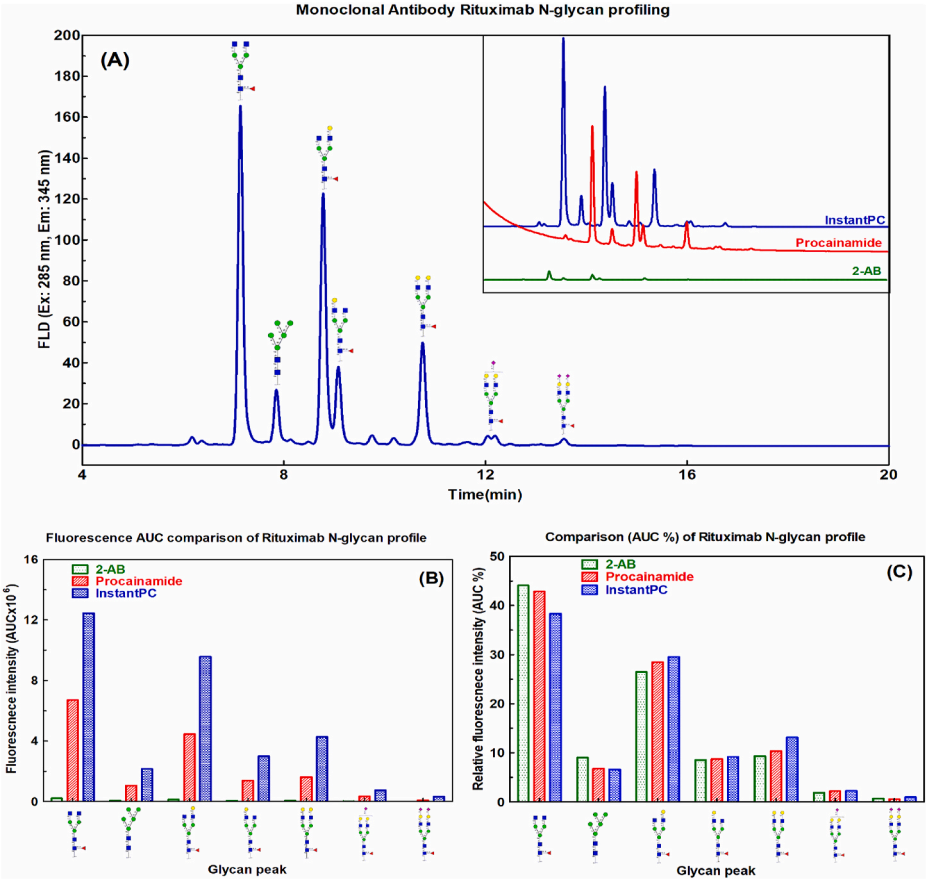


Fig. 2. N-glycan profiling from rituximab monoclonal antibody. (A) N-glycan profiles from rituximab labelled with InstantPC, and the insert shows glycans labelled with InstantPC (blue), procainamide (red), and 2-Aminobenzamide (green), respectively on the same scale of fluorescent intensity. (B) Comparison of the fluorescence intensity area under the curve (AUC) of major N-glycans from rituximab labelled with 2-Aminobenzamide, procainamide, and InstantPC, respectively. (C) Comparison of the relative fluorescence intensity abundance (AUC %) of major N-glycans from rituximab labelled with 2-Aminobenzamide, procainamide, and InstantPC, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

similarly, the N-glycan profile for rituximab is typical for monoclonal antibodies produced in Chinese hamster ovary cells: predominantly neutral biantennary complex N-glycans with core fucose, and a relatively low proportion of glycans without core fucose. However, a noticeable difference from the EG2-hFc monoclonal antibody is that rituximab shows a lower level of galactosylation and a negligible quantity of sialic acid containing N-glycans. Additionally, as in the earlier analysis of EG2-hFc, the N-glycans of rituximab labelled with InstantPC, procainamide and 2-AB resulted in comparable well-resolved profiles, but with slightly different HILIC retention times and significantly different fluorescence intensities. InstantPC gave approximately x1.3 fold higher fluorescence intensities than procainamide, while both dyes gave significantly higher fluorescence intensities than 2-AB, x100 and x31 fold enhancement, respectively. The relative abundance of each major glycan species quantified by relative percentage of area under the peak demonstrated that the N-glycan profiles for InstantPC, procainamide, and 2-AB are in reasonable agreement (Fig. 2A, B and 2C, and supporting information Table S10).

(2) Qualitative and quantitative analysis of the glycosylation pattern of polyclonal antibodies

Subsequently, we extended this workflow to more complex polyclonal antibodies, immunoglobulin G (IgG) subclass purified from human serum. As shown in Fig. 3A and B, and supporting information Table S11, the HILIC separation of IgG N-glycans labelled with InstantPC, procainamide, and 2-AB resulted in well resolved peaks for all major glycan species. The glycan fluorescence profiles of InstantPC and procainamide showed comparable glycan profiles to 2-AB but gave significantly higher fluorescence intensity than 2-AB, approximately 113 and 59 times, respectively. Additionally, as shown in Fig. 3C, the relative abundance of each major N-glycan species quantified by relative percentage of area under the peak demonstrated that the N-glycan profiles for both InstantPC and 2-AB are in reasonable agreement, with only slight deviations.

As shown in Fig. 3C and supporting information Table S11, the N-glycan profiles labelled with 2-AB, procainamide, and InstantPC consist of predominantly high fucose (93.43, 94.67, and 94.42%, respectively), intermediate levels of galactose (43.61, 39.75, and 45.26%, respectively) and sialic acid (33.43, 41.59 and 33.30%, respectively) and a low level of GlcNAc-bisected glycan (16.17, 18.09, and 14.10%, respectively). This result was similar from that reported previously, where the levels of fucose, galactose, sialic acid, and bisected glycan are 95%, 45%, 10% and 15%, respectively [55].

We noted a significantly higher level of sialylated glycans following InstantPC derivatization. Terminal sialic acid is vulnerable to hydrolysis, and conventional 2-AB method for glycan derivatization usually takes up to 3 days for N-glycan sample preparation. It has been reported previously that reductive amination conditions can potentially lead to oligosaccharide desialylation, and this was especially significant for highly sialylated oligosaccharides [56]. With the introduction of the current improved high-throughput, high-sensitivity workflow, the time spent for N-glycan preparation was shortened significantly, and it is likely that this shortened timeframe reduces the likelihood of sialic acid degradation. However, sialylated glycans labelled with procainamide demonstrated a slightly higher relative percentage compared to the counterparts labelled with InstantPC and 2-AB. It is possible that procainamide selectivity enriches derivatization of sialylated glycans to influence the observed fluorescent profile. This is currently under investigation and will be addressed in a separate paper.

To further verify the N-glycan profile assignment of IgG purified from human serum, the 2-AB labelled N-glycans were subjected to

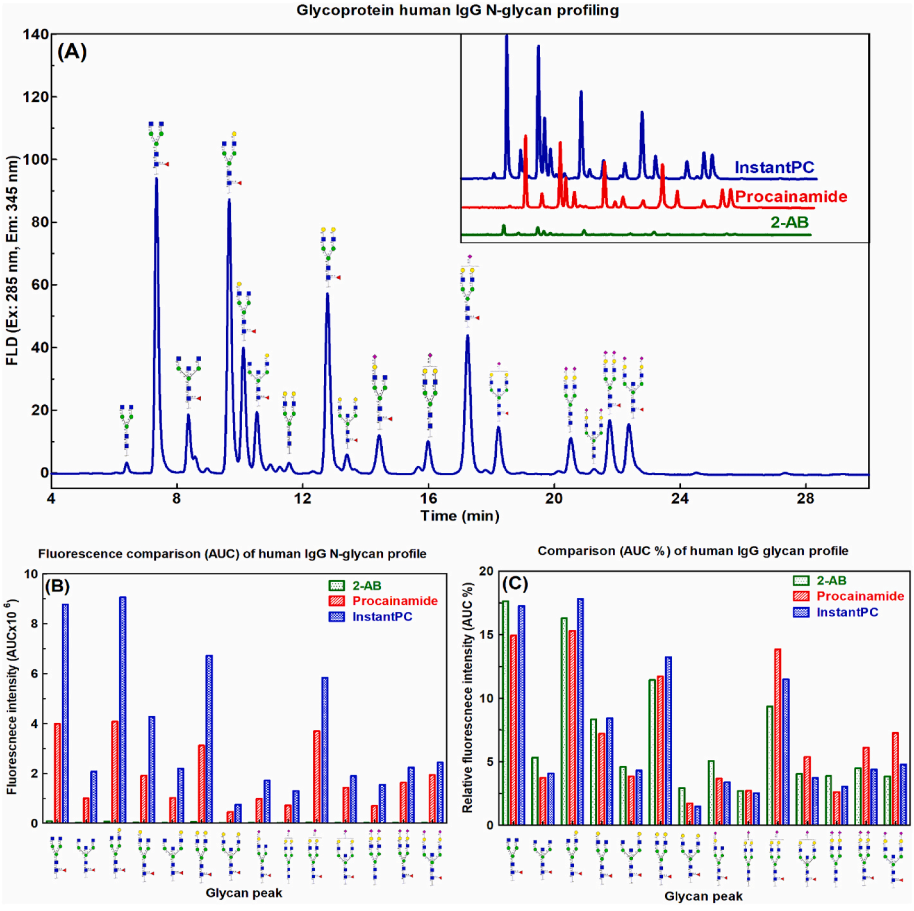


Fig. 3. N-glycan profiling from polyclonal antibodies purified from human serum. (A) N-glycan profiles from human serum IgG labelled with InstantPC, and the insert shows glycans labelled with InstantPC (blue), procainamide (red), and 2-Aminobenzamide (green), respectively on the same scale of fluorescent intensity. (B) Comparison of the fluorescence intensity area under the curve (AUC) of major N-glycans from human serum IgG labelled with 2-Aminobenzamide, procainamide, and InstantPC, respectively. (C) Comparison of the relative fluorescence intensity abundance (AUC %) of major N-glycans from human serum IgG labelled with 2-Aminobenzamide, procainamide, and InstantPC, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

exoglycosidases sequential digestion (supporting information Tables S3 and S4). As shown in Fig. 4, a variety of enzymes of α (2–3,6,8,9) Sialidase A, β (1–4)-Galactosidase, β (1–2,3,4,6)-N-acetylhexosaminidase and α (1–2,3,4,6)-Fucosidase were employed to remove N-acetylneuraminic acid (NANA), nonreducing terminal galactose, nonreducing terminal N- acetylglucosamine (GlcNAc) and fucose, respectively. The enzymatic arrays shifted the peaks in each chromatogram predictably and in accordance with the assigned glycan structures. Therefore, it is reasonable to deduce that the N-glycan peak assignment for the human serum IgG is correct. The high-throughput experimental method used here is straightforward and user-friendly, can be adapted to any analytical labs without the requirement of high level of expertise and special instrumentation. However, as shown in supporting information Fig. S4, the removal of core α 1-6-linked fucose from N-glycans labelled with the reactive N-hydroxysuccinimide carbamate fluorescent dye of InstantPC is severely impeded, with M3 N-glycans with and without core fucose coexisting even after incubation with α (1–2,3,4,6)-Fucosidase for an extended period. This finding is in reasonably good agreement to the previous report, where another aminoquinoline fluorescent label was investigated. It has been suggested that the rigidity of the aminoquinoline label contributed to steric clashes within the fucosidase active site, considering the molecular size of the enzyme is around 210–220 kDa [57]. It was reported that a novel broad specificity Omnitrophica α-L-fucosidase is capable of removing core α 1-6-linked fucose from N-glycans labelled with urea-linked fluorescent dye of such as InstantPC [58]. However, this is beyond the scope of this study, and will be addressed in more detail in a separate paper.

(3) Qualitative and quantitative glycosylation analysis of complex biological human serum

Many glycomics studies have focused on understanding disease mechanisms and identifying non-invasive human serum biomarkers for a wide range of diseases by monitoring alterations in protein glycosylation patterns. High-throughput, high-sensitivity glycan sample preparation is a prerequisite for this to be used in serum screening and identification of minor peaks. To this end, we extended the developed workflow to human serum, which is an even more complex biological sample, in order to test its robustness and feasibility. Individual N-glycan levels in total human serum protein were identified and quantified from small serum samples; 1 μl with InstantPC and procainamide as the derivative dyes and slightly higher (5 μl) with 2-AB. As shown in Fig. 5 and Table 2, the HILIC separation of human serum N-glycans labelled with InstantPC resulted in well resolved peaks for all major N-glycan species, and the glycan fluorescence profiles for both InstantPC and procainamide show comparable N-glycan profiles to those obtained for 2-AB, but gave significantly higher fluorescence intensity than 2-AB, 101 and 64 times, respectively. The relative abundance of each major glycan species quantified by relative percentage of area under the peak demonstrated that the N-glycan profiles for InstantPC, procainamide, and 2-AB are in reasonable agreement.

As reported previously, in a typical human serum from healthy individuals, around 165 N-glycan peaks have been identified, including 22 sialic acids linkage isomers with different GU values from the original structures [59,60]. In this paper, only 28 of the most predominant N-glycan peaks were shown in Fig. 5. As shown in Fig. 5C and Table 1, it was clear that the most abundant N-glycan was A2G2S2 with biantennary structure; accounting to 49.50, 48.56, and 54.83% of the total relative abundance when being labelled with 2-AB, procainamide and InstantPC respectively. It was followed by A2G2S1 with the relative abundance at 9.70, 8.75, and 6.74% of the total relative abundance when being labelled with 2-AB, procainamide and InstantPC respectively. Whereas the major triantennary structure, A3G3S3, accounted for 6.4, 6.61 and 7.76% of the total relative abundance when being

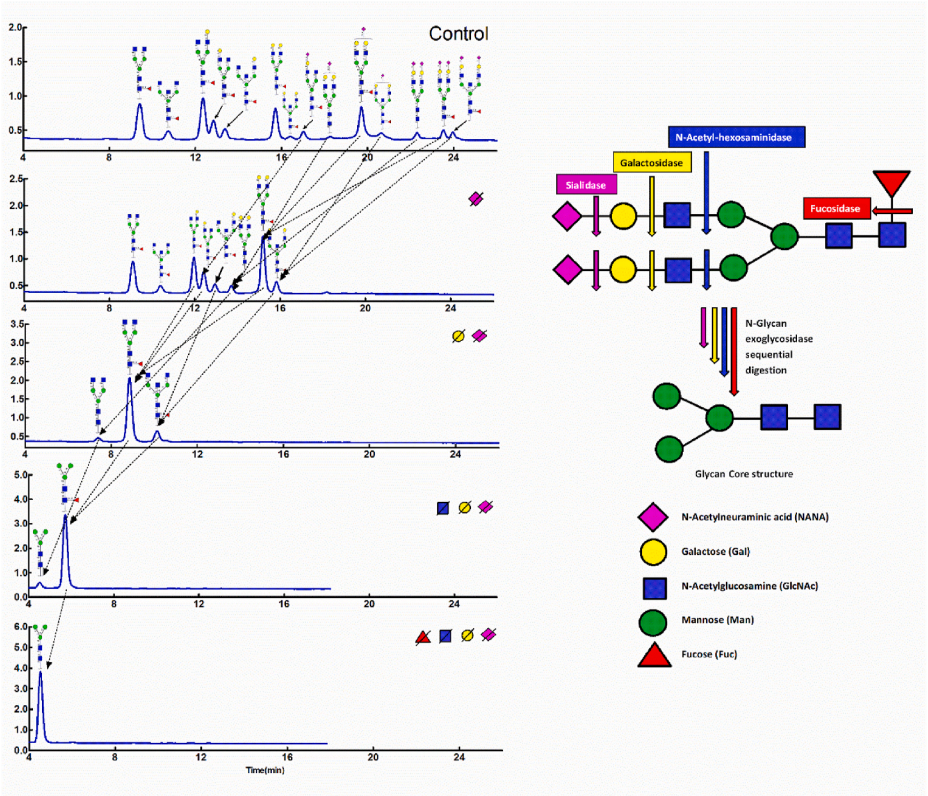


Fig. 4. Exoglycosydases sequential digestions of human serum IgG N-glycans labelled with 2-AB by using enzymes of α(2–3,6,8,9) Sialidase A, β(1–4)-Galactosidase, β(1–2,3,4,6)-N-acetylhexosaminidase and α(1–2,3,4,6)-Fucosidase for the removal of N-acetylneuraminic acid (NANA), nonreducing terminal galactose, nonreducing terminal N- acetylglucosamine (GlcNAc) and fucose, respectively.

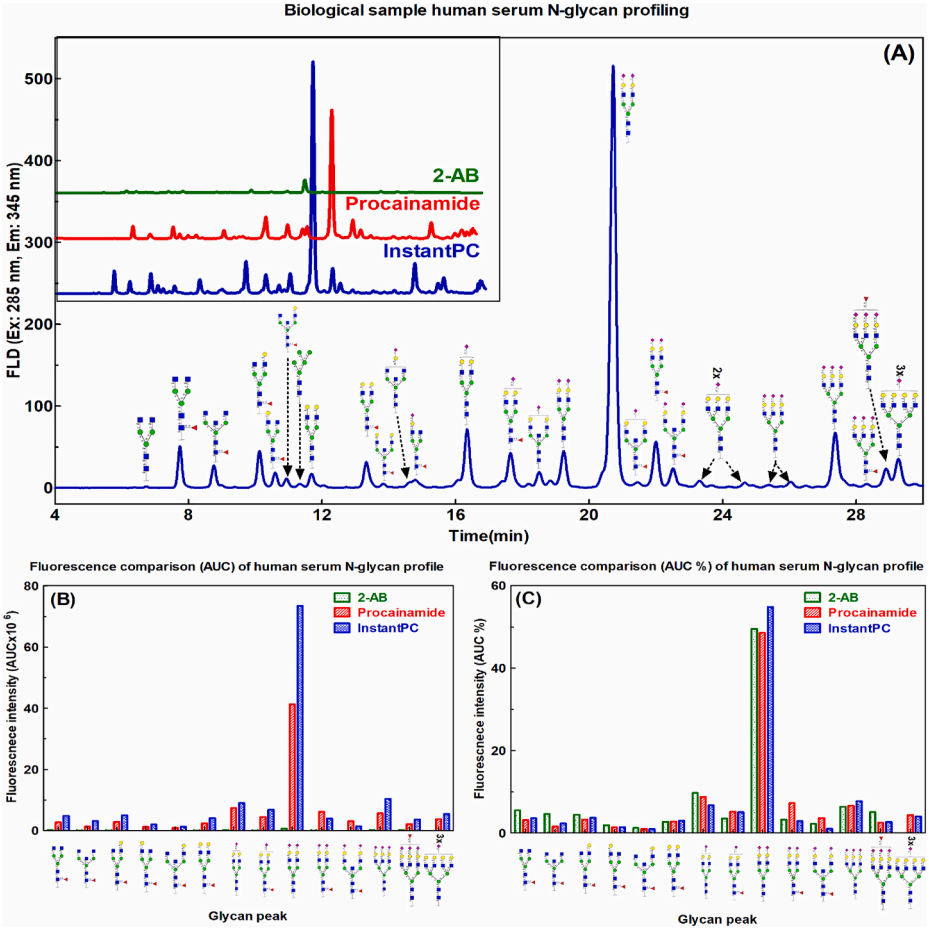


Fig. 5. Representative HILIC chromatogram of human serum N-glycan composition profiling with the predominant glycan structures. (A) N-glycan profiles from human serum labelled with InstantPC, and the insert shows glycans labelled with InstantPC (blue), procainamide (red), and 2-Aminobenzamide (green), respectively on the same scale of fluorescent intensity. (B) Comparison of the fluorescence intensity area under the curve (AUC) of major N-glycans from human serum labelled with 2-Aminobenzamide, procainamide, and InstantPC, respectively. (C) Comparison of the relative fluorescence intensity abundance (AUC %) of major N-glycans from human serum labelled with 2-Aminobenzamide, procainamide, and InstantPC, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

labelled with 2-AB, procainamide and InstantPC respectively. Additionally, it is noteworthy to state that with significantly enhanced fluorescence sensitivity, it becomes realistic to identify and investigate N-glycans at very low abundance with very little human serum required (as little as 1 μl) with the introduction of InstantPC and procainamide. Consequently, it is highly possible to extend this high-throughput and high-sensitive N-glycan profiling to human serum or plasma to link significant deviations of the normal serum N-glycome to be used as biomarkers of specific diseases.

4. Discussion

(1) Streamlined high-throughput glycan sample handling in Glycomics

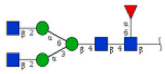
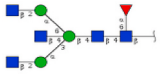
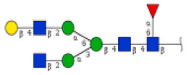
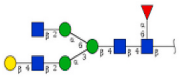
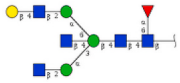
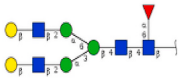
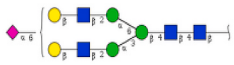
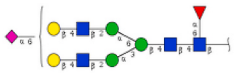
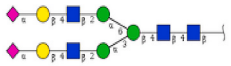
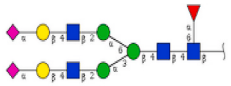
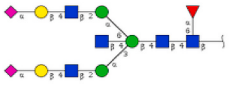
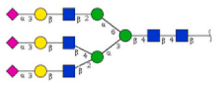
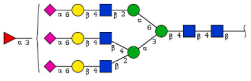
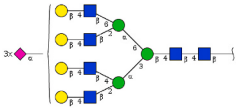
For quantitative glycomics analysis, an efficient, reliable method for the release of glycans from glycoprotein or glycopeptide backbones is a prerequisite for their detection. In the present study, an improved 96-well plate format workflow for streamlined N-glycan sample preparation is presented. As shown in Fig. 6 left panel, with the improvement in rapid in-solution glycoprotein denaturation and deglycosylation, fluorescent dye derivatization and 96-well plate format N-glycan purification and collection, the total N-glycan preparation time has been shortened significantly to less than 10 min. Compared to conventional N-glycan sample preparation followed by 2-AB label via reductive amination, which usually takes several hours or even days, this developed workflow is high-throughput, robust with good reproducibility, and suitable for automation.

The first key improvement was the development of fast glycoprotein denaturation in 3 min. It is well known that the efficiency of glycosidase enzymatic digestion is much higher for pre-denatured glycoproteins, with the direct benefits of accelerated enzymatic digestion, reduced enzyme consumption and complete release of all glycans [61]. Conventional denaturant cocktails, that include sulfhydryl reducing reagent such as dithiothreitol or β-mercaptoethanol, ionic detergent such as sodium dodecyl sulphate, and alkylating reagent such as iodoacetamide, possess several technical drawbacks. For example, ionic detergent is a potent inhibitor of glycanase and its removal can be difficult, although non-ionic detergent is not inhibitory and can be used in approximately 5-fold excess to counteract the inhibitory effects of ionic detergent. Here the glycoprotein substrate was denatured at 90 °C for 3 min in the presence of a mild anionic surfactant as denaturant, which significantly enhanced both the rate and extent of enzymatic deglycosylation of glycoprotein without suppressing the exoglycosidase activity.

The second key improvement was the development of novel Glycanse (PNGase F) to allow complete and unbiased enzymatic cleavage of N-glycans from target glycoproteins in just 5 min with 50 °C incubation. This was tested with a variety of glycoproteins in the current study, ranging from small molecular size monoclonal antibody of EG2-hFc (80 kDa), to normal molecular size monoclonal antibody of rituximab (145 KDa), and then extending to the more complex polyclonal antibody of human serum IgG and total glycoproteins from human serum. This was compared to conventional deglycosylation protocol of incubating glycoprotein with PNGase F for an extended period of time, normally overnight to 24 h under 37 °C [62]. The higher sialic acid content that we observe with the rapid deglycosylation protocol is attributed to the shortened time during which the sialic acid suffers significantly less thermal degradation [63], thus ensuring a complete quantitative analysis.

Finishing Reagent converts the glycosylamine produced by N-

Table 2
Human serum N-glycan profiling labelled with different fluorescent dyes.

N-glycan	2-AB label FLR		Procainamide label FLR		InstantPC label FLR	
	AUC (x10^6)	AUC (%)	AUC (x10^6)	AUC (%)	AUC (x10^6)	AUC (%)
	0.07	5.5	2.67	3.14	4.78	3.57
	0.06	4.6	1.33	1.57	3.10	2.32
	0.06	4.4	2.79	3.28	4.93	3.68
	0.03	1.9	1.17	1.38	1.97	1.47
	0.02	1.2	0.85	1.00	1.27	0.95
	0.04	2.7	2.39	2.81	4.05	3.02
	0.13	9.7	7.44	8.75	9.03	6.74
	0.05	3.5	4.42	5.19	6.80	5.08
	0.65	49.5	41.31	48.56	73.44	54.83
	0.04	3.3	6.14	7.22	3.87	2.89
	0.03	2.2	3.08	3.62	1.37	1.02
	0.08	6.4	5.63	6.61	10.39	7.76
	0.07	5.1	2.13	2.50	3.56	2.66
	0.00	0.0	3.72	4.37	5.37	4.01
Total FLR AUC	1.32	100.00	85.07	100.00	133.93	100.00
A2G2S2	0.65	49.5	41.31	48.56	73.44	54.83
A2G2S1	0.13	9.7	7.44	8.75	9.03	6.74
A3G3S3	0.08	6.4	5.63	6.61	10.39	7.76

The relative abundance of each N-glycan was calculated with the following equation:

$$\text{FLR AUC (\%)} = FLR\ AUC_{Glycan\ 1} / sum(FLR\ AUC_{Glycans}) \times 100\ \%$$

Glycanase digestion to a glycan with a free reducing end, ready for labelling with 2-Aminobenzamide or procainamide via Schiff-base condensation [64]. The incubation time for finishing is 10 min at 50 °C. The other major function of the finishing reagent is for efficient surfactant removal since the denaturant can be easily broken down by the acidic condition of the finishing reagent. InstantPC fluorescent derivation directly targets the functional group of glycosylamine, thus making the finishing reagent step unnecessary and consequently the experimental procedure is simplified significantly.

The third key improvement was the introduction of InstantPC as a label for N-glycan fluorescent derivatization, which can achieve complete N-glycan derivatization in only 1 min under 50 °C incubation. InstantPC targets the transient hydrolysed glycosylamine moiety on the N-acetylglucosamine (GlcNAc) and can achieve 'instant' N-glycan labelling due to the introduction of an N-hydroxysuccinimide carbamate group by activated carbamate chemistry [65,66]. However, amine buffer components, such as Tris or amino acids, should be avoided to prevent reaction with the InstantPC dye.

The final key improvement was the development of high-throughput on-matrix solid phase extraction glycan immobilisation in a 96-well plate format for non-stop glycan purification and derivatization. After enzymatic deglycosylation and derivatization, the released glycans are in solution containing a mixture of proteins, peptides, salts, excess fluorescent dyes, and possibly detergents. Therefore, it is necessary to carry out glycan purification to eliminate interference with the sample. Dialysis or microfiltration methods based on the molecular weight

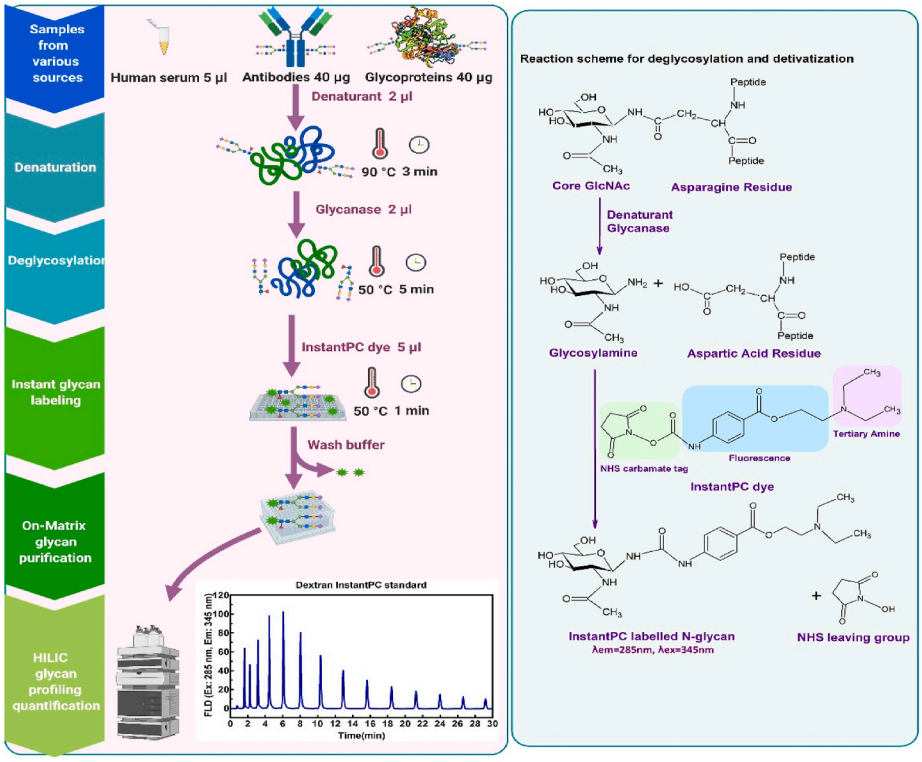


Fig. 6. Schematic workflow for 96-well plate format for N-glycan preparation and analysis incorporating express glycoprotein denaturation and deglycosylation, instant glycan fluorescent derivatization, and on-matrix glycan purification. Reaction scheme for glycoprotein deglycosylation and N-Hydroxysuccinimide (NHS) functionalized fluorephore derivatization using activated carbamate chemistry. (Created with ACD/ChemSketch and BioRender.com).

difference between glycans and their contaminants separate glycans from protein, buffer salts, or detergents. However, the major technical drawback is potential glycan sample loss in a time-consuming step. Protein precipitation by adding high concentration methanol, ethanol, or acetonitrile is commonly utilized as well to remove proteins from glycan sample mixtures. Solid phase extraction (SPE) is another efficient

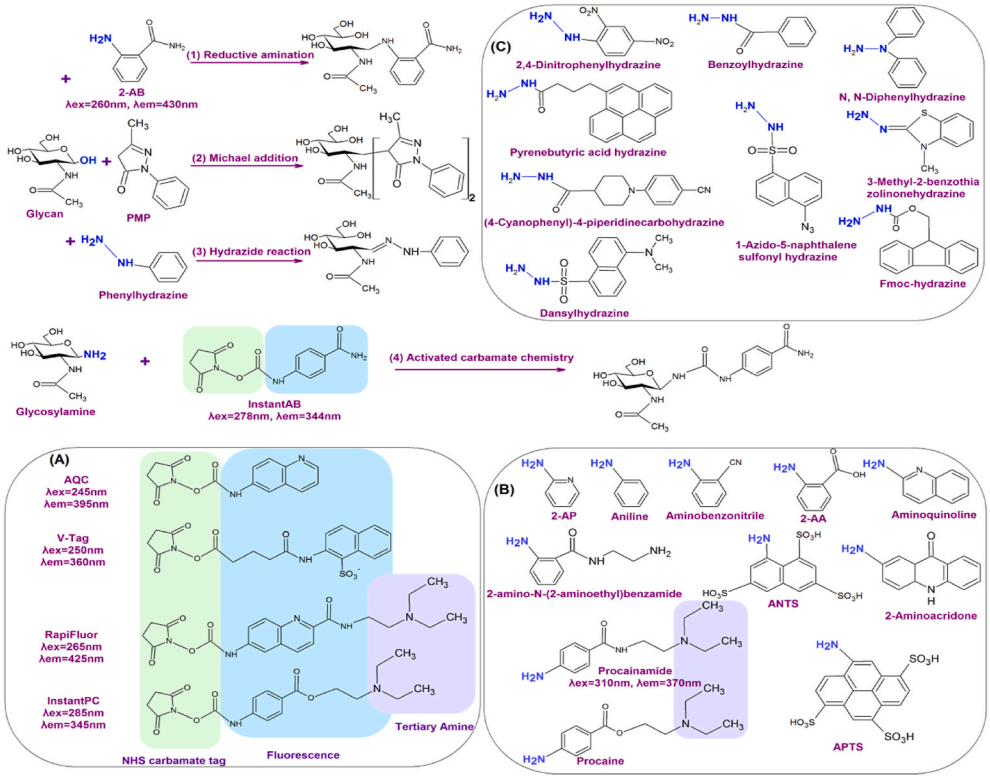


Fig. 7. Commonly utilized reaction schemes for N-Glycan derivatization using (1) reductive amination, (2) Michael addition, (3) hydrazide reaction and (4) activated carbamate chemistry, respectively. (A) Selection of N-Hydroxysuccinimide (NHS) carbamate functionalized fluorophores frequently used for activated carbamate chemistry for instant glycan labeling. (B) Selection of amine functionalized fluorophores frequently used for reductive amination. (C) Selection of hydrazine derivatives frequently used for hydrazine reaction. (Created with ACD/ChemSketch).

method for extracting glycans from digested mixtures according to differences in hydrophobicity [67,68]. In the present study, addition of the wash/load buffer of formic acid and acetonitrile precipitated the protein and allowed the released glycans to be eluted for the subsequent fluorescent labelling. Thus, the step of protein removal by centrifugation filter with molecular weight cut off is not necessary. As a result, this process eliminates the need for glycan drying prior to the fluorescent dye labelling step, thereby reducing total sample preparation time significantly.

(2) **Rational screening high-sensitivity glycan derivatization in Glycomics**

The released native glycans lack chromophores or fluorophores and are not suitable for optical detection in liquid chromatography profiling. Therefore, it is essential to carry out glycan derivatization with suitable chromophores or fluorophores to make them detectable by ultraviolet (UV) or fluorescence detection (FLD). Recent advances in glycan labelling chemistry have yielded significant improvements in both labelling speed and analytical sensitivity. As shown in Fig. 7, a variety of strategies have been developed for glycan derivatization and detection by various types of liquid chromatography coupled with fluorescence detector (LC-FLD). The most commonly employed reaction is reductive amination or Schiff base condensation, followed by Michael addition and hydrazide reaction, which targets the reducing end of the glycans. Another approach is activated carbamate chemistry, which targets the glycosylamine moiety on the N-acetylglucosamine (GlcNAc) instead [38]. Of course, permethylation, which increases hydrophobicity, stability, and ionization efficiency of glycans without introduction of either chromophores or fluorophores, was specifically developed for mass spectroscopy (MS) based glycan analysis [19,69,70]. This requires a higher level of expertise and dedicated instrumentation, is beyond our current research scope and will be addressed in a separate paper.

Michael addition can be performed under alkaline conditions using reagents such as 1-phenyl-3-methyl-5-pyrazolone (PMP), avoiding the risk of loss of sialic acids under acidic conditions which is intrinsic to reductive amination reactions. However, as two PMP label molecules are added per glycan, the chromatographic properties of the derivatized glycans are dominated by the tags, which may be disadvantageous in isomer separation. Hydrazide reaction is another widely applied approach for glycan derivatization, which can achieve 100% labelling yield, demonstrating its efficiency and reliability for glycan analysis [38,71–74].

Currently, N-glycan derivatization with amine functionalized fluorophores, such as 2-Aminobenzamide (2-AB), using reductive amination chemistry is still the gold standard approach in glycan profiling. The major advantage is that 2-AB lacks negative charges and the ratio of its stoichiometric attachment to glycan is 1:1, allowing direct quantitation based on fluorescence or UV-absorbance intensity [75,76]. Additionally, an extensive database is available which uses the standardized elution positions of 2-AB labelled glycans in hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FLD) for structural assignment [47]. Procainamide utilises the same reaction scheme of reductive amination, but shows significantly increased fluorescence performance, which makes it an attractive alternative dye for glycan derivatization without the need for extensive workflow optimization [51,52], yet at an affordable price. Additionally, a database for structural assignments of procainamide labelled glycans based on standardized elution positions has also been developed [47].

InstantPC was rationally designed to combine the strong fluorescence intensity of 6-Aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC), procaine, or procainamide and the ionization benefits of a tertiary amine [77–80], as well as the rapid (or “instant”) derivatization provided by activated carbamate chemistry [66,81]. Additionally, the entire workflow of using InstantPC is simple and user-friendly. For a glycobiological laboratory already well established and equipped to use reductive amination technology, there is no extensive work to be carried out for protocol optimization and cross-validation to switch to these technologies. Furthermore, albeit via different chemical reaction mechanism and targeting different glycan functional groups, InstantPC and procainamide share structural similarity, both possessing the tertiary amine group which contribute to improved ionization during mass spec and consequently increased MS signal intensity in positive mode (as shown in Table 1 and Figs. 6 and 7). Therefore, InstantPC should hold the potential of enhanced MS intensity at similar magnitude as procainamide. It has been shown that InstantPC demonstrated comparable MS response to Waters RapiFluor and superior MS response to procainamide (2.4–5 fold enhancement) [81,82].

Of course, there are some drawbacks for using InstantPC. Since the rapid labelling chemistry only works with released glycosylamine which is an unstable intermediate, longer glycoprotein deglycosylation and labelling step may compromise the labelling efficiency. At the same time, the present limited commercial availability of InstantPC dye labelled glycan standards make it difficult for glycan assignment and quantification without a detailed database for the labelled glycans. However, due to its great potential for high-throughput, high-sensitivity N-glycan profiling, the developed workflow featuring InstantPC glycan derivatization, we believe will attract significant future interest for both pharmaceutical quality control and biomedical research.

5. Conclusion

In the current study, we present glycosylation data from the use of a high-throughput, high-sensitivity platform for N-glycan composition analysis. This features streamlined express glycoprotein denaturation and deglycosylation, and instant N-glycan derivatization and purification, all in a 96-well plate format. It is highly sensitive and reliable, and has been successfully used to carry out N-glycan composition analysis for recombinant monoclonal antibodies of EG2-hFc and rituximab produced from Chinese hamster ovary cells, polyclonal antibodies immunoglobulin G (IgG) subclass purified from human serum, as well as more complicated biological human serum samples. This workflow is simple enough to be implemented into a straightforward user-friendly setup without the requirement of complicated instrumentation like mass spectrometer (MS). Additionally, it is flexible and potentially suitable for automated handling systems if required. This platform holds the potential to be used for rapid glycan analysis of biopharmaceuticals as well as for disease diagnosis in a clinical setting where there are considerable advantages for sensitive and reliable strategies for high throughput glycomics analysis.

CRediT authorship contribution statement

Yongjing Xie: Conceptualization, Methodology, Investigation, Writing – review & editing. **Letícia Martins Mota:** Investigation, Conceptualization. **Adam Bergin:** Conceptualization. **Roisin O’Flaherty:** Conceptualization. **Aled Jones:** Conceptualization, Resources, Writing – review & editing. **Bethan Morgan:** Conceptualization, Resources. **Michael Butler:** Validation, Writing – review & editing.

Declaration of competing interest

A.Jones and B. Morgan are employees of Agilent Inc, who provided materials for the methods described in this paper. The other co-authors disclose no conflict of interest.

Acknowledgement

This work was supported by Science Foundation Ireland/Enterprise Ireland Technology Innovation Development Award (18/TIDA/6064). The Agilent® AdvanceBio Gly-X Glycan sample preparation kit and related exoglycosidase enzymes, and AdvanceBio Glycan mapping

column were kindly provided by Agilent Technologies, which is deeply appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2021.114205.

References

[1] M.G. Zavala-Cerna, E.A. Martínez-García, O. Torres-Bugarín, B. Rubio-Jurado, C. Riebeling, A. Nava, The clinical significance of posttranslational modification of autoantigens, Clin. Rev. Allergy Immunol. 47 (2014) 73–90.

[2] H. Lu, G. Li, C. Zhou, W. Jin, X. Qian, Z. Wang, H. Pan, H. Jin, X. Wang, Regulation and role of post-translational modifications of enhancer of zeste homologue 2 in cancer development, Am J Cancer Res 6 (2016) 2737–2754.

[3] T.A. Dar, L.R. Singh, Protein Modificomics : from Modifications to Clinical Perspectives, first ed., Academic Press, London, U. K., 2019.

[4] U. Consortium, Reorganizing the protein space at the universal protein resource (UniProt), Nucleic Acids Res. 40 (2012) D71–D75.

[5] M.A. Kukuruzinska, K. Lennon, Protein N-glycosylation: molecular genetics and functional significance, Crit. Rev. Oral Biol. Med. 9 (1998) 415–448.

[6] K. Ohtsubo, J.D. Marth, Glycosylation in cellular mechanisms of health and disease, Cell 126 (2006) 855–867.

[7] K.W. Moremen, M. Tiemeyer, A.V. Nairn, Vertebrate protein glycosylation: diversity, synthesis and function, Nat. Rev. Mol. Cell Biol. 13 (2012) 448–462.

[8] G.W. Hart, R.J. Copeland, Glycomics hits the big time, Cell 143 (2010) 672–676.

[9] C. Reily, T.J. Stewart, M.B. Renfrow, J. Novak, Glycosylation in health and disease, Nat. Rev. Nephrol. 15 (2019) 346–366.

[10] M.J. Kailemia, D. Park, C.B. Lebrilla, Glycans and glycoproteins as specific biomarkers for cancer, Anal. Bioanal. Chem. 409 (2017) 395–410.

[11] P.M. Drake, W. Cho, B. Li, A. Prakobphol, E. Johansen, N.L. Anderson, F.E. Regnier, B.W. Gibson, S.J. Fisher, Sweetening the pot: adding glycosylation to the biomarker discovery equation, Clin. Chem. 56 (2010) 223–236.

[12] H. Hwang, J. Zhang, K.A. Chung, J.B. Leverenz, C.P. Zabetian, E.R. Peskind, J. Jankovic, Z. Su, A.M. Hancock, C. Pan, T.J. Montine, S. Pan, J. Nutt, R. Albin, M. Gearing, R.P. Beyer, M. Shi, Glycoproteomics in neurodegenerative diseases, Mass Spectrom. Rev. 29 (2010) 79–125.

[13] X. Verhelst, A.M. Dias, J.F. Colombel, S. Vermeire, H. Van Vlierberghe, N. Callewaert, S.S. Pinho, Protein glycosylation as a diagnostic and prognostic marker of chronic inflammatory gastrointestinal and liver diseases, Gastroenterology 158 (2020) 95–110.

[14] P. Zhang, S. Woen, T. Wang, B. Liau, S. Zhao, C. Chen, Y. Yang, Z. Song, M. R. Wormald, C. Yu, P.M. Rudd, Challenges of glycosylation analysis and control: an integrated approach to producing optimal and consistent therapeutic drugs, Drug Discov. Today 21 (2016) 740–765.

[15] N. Kawasaki, S. Itoh, N. Hashii, D. Takakura, Y. Qin, X. Huang, T. Yamaguchi, The significance of glycosylation analysis in development of biopharmaceuticals, Biol. Pharm. Bull. 32 (2009) 796–800.

[16] H.J. An, P. Gip, J. Kim, S. Wu, K.W. Park, C.T. McVaugh, D.V. Schaffer, C. R. Bertozzi, C.B. Lebrilla, Extensive determination of glycan heterogeneity reveals an unusual abundance of high mannose glycans in enriched plasma membranes of human embryonic stem cells, Mol. Cell. Proteomics 11 (2012) M111, 010660.

[17] X. Yang, M.G. Bartlett, Glycan analysis for protein therapeutics, J Chromatogr B Analyt Technol Biomed Life Sci 1120 (2019) 29–40.

[18] L. Zhang, S. Luo, B. Zhang, Glycan analysis of therapeutic glycoproteins, mAbs 8 (2016) 205–215.

[19] A. Shajahan, C. Heiss, M. Ishihara, P. Azadi, Glycomic and glycoproteomic analysis of glycoproteins-a tutorial, Anal. Bioanal. Chem. 409 (2017) 4483–4505.

[20] F. Tousi, W. Hancock, M. Hincapie, Technologies and strategies for glycoproteomics and glycomics and their application to clinical biomarker research, Analytical Methods 3 (2011) 20–32.

[21] R. Rocklin, C. Pohl, Determination of carbohydrates by anion-exchange chromatography with pulsed amperometric detection, J. Liq. Chromatogr. 6 (1983) 1577–1590.

[22] J.S. Rohrer, L. Basumallick, D. Hurum, High-performance anion-exchange chromatography with pulsed amperometric detection for carbohydrate analysis of glycoproteins, Biochemistry (Mosc.) 78 (2013) 697–709.

[23] J.R. Barr, K.R. Anumula, M.B. Vettese, P.B. Taylor, S.A. Carr, Structural classification of carbohydrates in glycoproteins by mass spectrometry and high-performance anion-exchange chromatography, Anal. Biochem. 192 (1991) 181–192.

[24] M.R. Hardy, R.R. Townsend, Y.C. Lee, Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection, Anal. Biochem. 170 (1988) 54–62.

[25] W. Morelle, J.C. Michalski, Analysis of protein glycosylation by mass spectrometry, Nat. Protoc. 2 (2007) 1585–1602.

[26] D. Reusch, M. Haberger, D. Falck, B. Peter, B. Maier, J. Gassner, M. Hook, K. Wagner, L. Bonnington, P. Bulau, M. Wuhrer, Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-Part 2: mass spectrometric methods, mAbs 7 (2015) 732–742.

[27] N. Leymarie, J. Zaia, Effective use of mass spectrometry for glycan and glycopeptide structural analysis, Anal. Chem. 84 (2012) 3040–3048.

[28] H. Geyer, S. Schmitt, M. Wuhrer, R. Geyer, Structural analysis of glycoconjugates by on-target enzymatic digestion and MALDI-TOF-MS, Anal. Chem. 71 (1999) 476–482.

[29] S.J. North, H.H. Huang, S. Sundaram, J. Jang-Lee, A.T. Etienne, A. Trollope, S. Chalabi, A. Dell, P. Stanley, S.M. Haslam, Glycomics profiling of Chinese hamster ovary cell glycosylation mutants reveals N-glycans of a novel size and complexity, J. Biol. Chem. 285 (2010) 5759–5775.

[30] A.W. Barb, J.H. Prestegard, NMR analysis demonstrates immunoglobulin G N-glycans are accessible and dynamic, Nat. Chem. Biol. 7 (2011) 147–153.

[31] M. Lundborg, G. Widmalm, Structural analysis of glycans by NMR chemical shift prediction, Anal. Chem. 83 (2011) 1514–1517.

[32] M. Lundborg, G. Widmalm, NMR chemical shift prediction of glycans: application of the computer program CASPER in structural analysis, Methods Mol. Biol. 1273 (2015) 29–40.

[33] M.D. Battistel, H.F. Azurmendi, B. Yu, D.I. Freedberg, NMR of glycans: shedding new light on old problems, Prog. Nucl. Magn. Reson. Spectrosc. 79 (2014) 48–68.

[34] Z. Zhang, N.M. Khan, K.M. Nunez, E.K. Chess, C.M. Szabo, Complete monosaccharide analysis by high-performance anion-exchange chromatography with pulsed amperometric detection, Anal. Chem. 84 (2012) 4104–4110.

[35] T.R. Cataldi, D. Nardiello, Determination of free proline and monosaccharides in wine samples by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), J. Agric. Food Chem. 51 (2003) 3737–3742.

[36] L.D. Salvador, T. Suganuma, K. Kitahara, H. Tanoue, M. Ichiki, Monosaccharide composition of sweetpotato fiber and cell wall polysaccharides from sweetpotato, cassava, and potato analyzed by the high-performance anion exchange chromatography with pulsed amperometric detection method, J. Agric. Food Chem. 48 (2000) 3448–3454.

[37] M. Melmer, T. Stangler, M. Schiefermeier, W. Brunner, H. Toll, A. Rupprechter, W. Lindner, A. Premstaller, HILIC analysis of fluorescence-labeled N-glycans from recombinant biopharmaceuticals, Anal. Bioanal. Chem. 398 (2010) 905–914.

[38] L.R. Ruhaak, G. Zauner, C. Huhn, C. Bruggink, A.M. Deelder, M. Wuhrer, Glycan labeling strategies and their use in identification and quantification, Anal. Bioanal. Chem. 397 (2010) 3457–3481.

[39] Z. Szabo, A. Guttman, J. Bones, B.L. Karger, Rapid high-resolution characterization of functionally important monoclonal antibody N-glycans by capillary electrophoresis, Anal. Chem. 83 (2011) 5329–5336.

[40] V. Agrawal, I. Slivac, S. Perret, L. Bisson, G. St-Laurent, Y. Murad, J. Zhang, Y. Durocher, Stable expression of chimeric heavy chain antibodies in CHO cells, Methods Mol. Biol. 911 (2012) 287–303.

[41] N. Pristovšek, H.G. Hansen, D. Sergeeva, N. Borth, G.M. Lee, M.R. Andersen, H. F. Kildegaard, Using titer and titer normalized to confluence are complementary strategies for obtaining Chinese hamster ovary cell lines with high volumetric productivity of etanercept, Biotechnol. J. 13 (2018), e1700216.

[42] W. Strober, Trypan blue exclusion test of cell viability, Curr Protoc Immunol, Appendix 3 (2001). Appendix 3B.

[43] S. Hober, K. Nord, M. Linhult, Protein A chromatography for antibody purification, J Chromatogr B Analyt Technol Biomed Life Sci 848 (2007) 40–47.

[44] A.C. Grodzki, E. Berenstein, Antibody purification: affinity chromatography - protein A and protein G Sepharose, Methods Mol. Biol. 588 (2010) 33–41.

[45] Y. Maynard, M.G. Scott, M.H. Nahm, J.H. Ladenson, Turbidimetric assay of IgG with use of single monoclonal antibodies, Clin. Chem. 32 (1986) 752–757.

[46] P.M. McCue, Evaluation of a turbidimetric immunoassay for measurement of plasma IgG concentration in foals, Am. J. Vet. Res. 68 (2007) 1005–1009.

[47] S. Zhao, I. Walsh, J. Abrahams, L. Royle, T. Nguyen-Khuong, D. Spencer, D. Fernandes, N. Packer, P. Rudd, M. Campbell, GlycoStore: a database of retention properties for glycan analysis, Bioinformatics 34 (2018) 3231–3232.

[48] J. Zhang, X. Liu, A. Bell, R. To, T. Baral, A. Azizi, J. Li, B. Cass, Y. Durocher, Transient expression and purification of chimeric heavy chain antibodies, Protein Expr. Purif. 65 (2009) 77–82.

[49] A. Bell, Z. Wang, M. Arbabi-Ghahroudi, T. Chang, Y. Durocher, U. Trojahn, J. Baardsnes, M. Jaramillo, S. Li, T. Baral, M. O'Connor-McCourt, R. MacKenzie, J. Zhang, Differential tumor-targeting abilities of three single-domain antibody formats, Canc. Lett. 289 (2010) 81–90.

[50] G. Santos, F. Shepherd, M. Tsao, A. Abbas, S. Galli, P. Howley, EGFR mutations and lung cancer, Annu. Rev. Pathol. 6 (6) (2011) 49–69.

[51] R.P. Kozak, C.B. Tortosa, D.L. Fernandes, D.I. Spencer, Comparison of procainamide and 2-aminobenzamide labeling for profiling and identification of glycans by liquid chromatography with fluorescence detection coupled to electrospray ionization-mass spectrometry, Anal. Biochem. 486 (2015) 38–40.

[52] T. Keser, T. Pavić, G. Lauc, O. Gornik, Comparison of 2-aminobenzamide, procainamide and*Rapi*Fluor-MS as derivatizing agents for high-throughput HILIC-UPLC-FLR-MS N-glycan analysis, Front Chem 6 (2018) 324.

[53] T. Pierpont, C. Limper, K. Richards, Past, present, and future of rituximab-the world's first oncology monoclonal antibody therapy, Frontiers in Oncology 8 (2018).

[54] M. Smith, Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance, Oncogene 22 (2003) 7359–7368.

[55] M. Bakovic, M. Selman, M. Hoffmann, I. Rudan, H. Campbell, A. Deelder, G. Lauc, M. Wuhrer, High-throughput IgG fc N-glycosylation profiling by mass spectrometry of glycopeptides, J. Proteome Res. 12 (2013) 821–831.

[56] U. Aich, D.C. Hurum, L. Basumallick, S. Rao, C. Pohl, J.S. Rohrer, S. Kandzia, Evaluation of desialylation during 2-amino benzamide labeling of asparagine-linked oligosaccharides, Anal. Biochem. 458 (2014) 27–36.

[57] R. O'Flaherty, A.M. Harbison, P.J. Hanley, C.H. Taron, E. Fadda, P.M. Rudd, Aminoquinoline fluorescent labels obstruct efficient removal of N-glycan core α(1-

6) fucose by bovine kidney α-l-Fucosidase (BKF), J. Proteome Res. 16 (2017) 4237–4243.
[58] S. Vainauskas, C. Kirk, L. Petralia, E. Guthrie, E. McLeod, A. Bielik, A. Luebbers, J. Foster, C. Hokke, P. Rudd, X. Shi, C. Taron, A novel broad specificity fucosidase capable of core alpha 1-6 fucose release from N-glycans labeled with urea-linked fluorescent dyes, Sci. Rep. 8 (2018).
[59] H. Chew, S. Miyamoto, H. An, D. Rocke, C. Lebrilla, Serum glycan analysis in metastatic breast cancer, J. Clin. Oncol. 25 (2007).
[60] R. Saldova, A. Shehni, V. Haakensen, I. Steinfeld, M. Hilliard, I. Kifer, A. Helland, Z. Yakhini, A. Borresen-Dale, P. Rudd, Association of N-glycosylation with breast carcinoma and systemic features using high-resolution quantitative UPLC, J. Proteome Res. 13 (2014) 2314–2327.
[61] L. Veillon, S. Zhou, Y. Mechref, Quantitative glycomics: a combined analytical and bioinformatics approach, Methods Enzymol. 585 (2017) 431–477.
[62] V.S. Tayi, M. Butler, Isolation and quantification of N-glycans from immunoglobulin G antibodies for quantitative glycosylation analysis, J Biol Methods 2 (2) (2015), https://doi.org/10.14440/jbm.2015.52 e19.
[63] J.D. Karkas, E. Chargaff, Studies ON the stability OF simple derivatives OF sialic acid, J. Biol. Chem. 239 (1964) 949–957.
[64] A. Varki, Essentials of Glycobiology, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2008.
[65] M. Morpurgo, E.A. Bayer, M. Wilchek, N-hydroxysuccinimide carbonates and carbamates are useful reactive reagents for coupling ligands to lysines on proteins, J. Biochem. Biophys. Methods 38 (1999) 17–28.
[66] Z. Segu, T. Stone, C. Berdugo, A. Roberts, E. Doud, Y. Li, A rapid method for relative quantification of *N* -glycans from a therapeutic monoclonal antibody during trastuzumab biosimilar development, mAbs 12 (2020) 1750794.
[67] S. Yang, H. Zhang, Solid-phase glycan isolation for glycomics analysis, Proteonomics Clin. Appl. 6 (2012) 596–608.
[68] Q. Zhang, H. Li, X. Feng, B.F. Liu, X. Liu, Purification of derivatized oligosaccharides by solid phase extraction for glycomic analysis, PloS One 9 (2014), e94232.
[69] P. Kang, Y. Mechref, I. Klouckova, M.V. Novotny, Solid-phase permethylation of glycans for mass spectrometric analysis, Rapid Commun. Mass Spectrom. 19 (2005) 3421–3428.
[70] J. Delaney, P. Vouros, Liquid chromatography ion trap mass spectrometric analysis of oligosaccharides using permethylated derivatives, Rapid Commun. Mass Spectrom. 15 (2001) 325–334.
[71] C. Wang, Y. Lu, J. Han, W. Jin, L. Li, Y. Zhang, X. Song, L. Huang, Z. Wang, Simultaneous release and labeling of O- and N-glycans allowing for rapid glycomic analysis by online LC-UV-ESI-MS/MS, J. Proteome Res. 17 (2018) 2345–2357.
[72] G. Zauner, C.A. Koeleman, A.M. Deelder, M. Wuhrer, Mass spectrometric O-glycan analysis after combined O-glycan release by beta-elimination and 1-phenyl-3-methyl-5-pyrazolone labeling, Biochim. Biophys. Acta 1820 (2012) 1420–1428.
[73] S.J. Yang, H. Zhang, Glycan analysis by reversible reaction to hydrazide beads and mass spectrometry, Anal. Chem. 84 (2012) 2232–2238.
[74] L. Veillon, S. Zhou, Y. Mechref, A. Shukla, Quantitative glycomics: a combined analytical and bioinformatics approach, Proteomics in Biology, Pt a 585 (2017) 431–477.
[75] D.R. Wing, B. Garner, V. Hunnam, G. Reinkensmeier, U. Andersson, D.J. Harvey, R. A. Dwek, F.M. Platt, T.D. Butters, High-performance liquid chromatography analysis of ganglioside carbohydrates at the picomole level after ceramide glycanase digestion and fluorescent labeling with 2-aminobenzamide, Anal. Biochem. 298 (2001) 207–217.
[76] K. Mariño, J.A. Lane, J.L. Abrahams, W.B. Struwe, D.J. Harvey, M. Marotta, R. M. Hickey, P.M. Rudd, Method for milk oligosaccharide profiling by 2-aminobenzamide labeling and hydrophilic interaction chromatography, Glycobiology 21 (2011) 1317–1330.
[77] N. Ahmed, P.J. Thornalley, Chromatographic assay of glycation adducts in human serum albumin glycated in vitro by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate and intrinsic fluorescence, Biochem. J. 364 (2002) 15–24.
[78] R. Ullmer, A. Plematl, A. Rizzi, Derivatization by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate for enhancing the ionization yield of small peptides and glycopeptides in matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 1469–1479.
[79] Y. Wu, Q. Sha, J. Du, C. Wang, L. Zhang, B.F. Liu, Y. Lin, X. Liu, Determination of N-glycans by high performance liquid chromatography using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as the glycosylamine labeling reagent, J. Chromatogr. A 1535 (2018) 114–122.
[80] X. Wang, X. Chen, L. Chen, B. Wang, C. Peng, C. He, M. Tang, F. Zhang, J. Hu, R. Li, X. Zhao, Y. Wei, Optimizing high-performance liquid chromatography method for quantification of glucosamine using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization in rat plasma: application to a pharmacokinetic study, Biomed. Chromatogr. 22 (2008) 1265–1271.
[81] M. Kimzey, Z. Szabo, V. Sharma, A. Gynes, S. Tep, A. Taylor, A. Jones, J. Hyche, T. Haxo, S. Vlasenko, Development of an instant glycan labeling dye for high throughput analysis by mass spectrometry, Glycobiology 25 (2015), 1295-1295.
[82] T. Keser, T. Pavic, G. Lauc, O. Gornik, Comparison of 2-aminobenzamide, procainamide and RapiFluor-MS as derivatizing agents for high-throughput HILIC-UPLC-FLR-MS N-glycan analysis, Front Chem 6 (2018) 324.