Title: Recent Advances in Biologic Therapeutic N-Glycan Preparation Techniques and Analytical Methods for Facilitating Biomanufacturing Automation

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Abstract
Post translational modification such as N-Glycosylation on biologics during production of monoclonal antibody (mAb) based therapeutics is a critical quality attribute that dictates safety and efficacy. Variability is introduced in the cell culture process which influences, the glycosylation pattern which is known to be highly heterogenous and must be tightly controlled during the manufacturing process. Techniques have been developed for glycan screening through the use of new denaturation techniques; deglycosylation, fluorescent labeling, and analysis coupled to state-of-the-art tools consisting of multi attribute methods and multi attribute chromatography. In this review, we delve into advances within sample preparation techniques that allow for rapid and robust sample preparation as well as how these techniques are being used for innovative at-line high-throughput screening and PAT focused systems. Finally, we foresee how these advances will influence current manufacturing practices and enable bioprocess automation. The future state of biomanufacturing looks to decrease process costs while increasing process understanding and quality for novel biologic candidates and biosimilars.

Keywords
Automation, glycosylation, analysis, antibody drug(s), monoclonal antibody(s), process analytical technology (PAT), HPLC (High Performance/Pressure Liquid Chromatography), Liquid Chromatography-Mass spectrometry (LC-MS), Capillary Electrophoresis,

Abbreviations
mAb, monoclonal antibody; CHO, Chinese hamster ovarian; NS0, nonsecreting murine myeloma; HEK, Human embryonic kidney; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement dependent cytotoxicity; PAT, process analytical technology; CQA, critical quality attribute; LS, Lauroylsarcosine; DTT, Dithiothreitol; RFMS, RapiFluor-MS; IPC, InstantPC; V-tag, Velocity tag; MAM, multi-attribute method; MAST, modular automated sampling technology

1. Introduction to Carbohydrates and Their Importance in Biopharmaceuticals

Prior to 2014, less than 5 antibody therapeutics were approved by the FDA per year whereas, after 2014 seven or more antibody therapeutics have been approved each year with the maximum of 17 approvals in 2017, 2019, and 2020[1].The FDA has released critical guidance to implement process analytical technology (PAT)[2] within a manufacturing process to monitor and control the process and impact on quality attributes in real-time rather than traditionally at the end of the manufacturing process which enables continuous manufacturing[3,4]. However, due to the complexity of the molecular machinery and cell culture conditions during the post translational modification process, mAbs are found to change immensely during the cell culture process, leading to varying glycoforms, a protein or mAb that differs only with respect to type of number of attached glycans, being found in the final drug substance. Because of this variability, the glycosylation process must be tightly controlled during the manufacturing process. Importantly, the glycan profile is widely regarded as a key critical quality attribute (CQA)[5,6,7]; as process parameters such as media, feed, and feeding strategy[5,8] play a key role in the glycosylation process. Physical attributes within the bioreactor such as , pH[5,8] , shear stress[5], dissolved oxygen, and osmolarity, can also have a large impact on the final N-linked glycan profile. As the bioreactor conditions immensely impact the glycosylation profile it is paramount to monitor and control the profile throughout the production process especially during process development.

In short, following protein biosynthesis, post translational modification occurs where the protein at one or more amino acids is biochemically modified. N-Linked glycosylation is a common post translational modification (PTM) that occurs during biologic production, such as with monoclonal antibody (mAb) based therapeutics expressed in eukaryotic hybridoma cell lines, like Chinese hamster ovary (CHO), nonsecreting murine myeloma (NS0) and Human embryonic kidney (HEK) cells. N-linked glycans covalently bonded at the Asn297 residue of the CH2 domain of the heavy chain of monoclonal antibodies[9] play an important role in pharmacokinetics, efficacy, and safety[10]. Due to the location of the N-linked glycans, they are vital for Fc effector function including antibody-dependent cellular cytotoxicity (ADCC)[11], complement dependent cytotoxicity (CDC)[11], and antibody-dependent cellular phagocytosis, which ultimately impacts molecular recognition, adhesion, cell signaling[12,13] and consequently impacting the binding affinity of the antibody to their Fcγ receptor.

In general, the most prevalent strategies for glycan analysis of mAb profiles are depicted in Figure 1. The most common practices (in order of least to most sensitivity) are intact mAb analysis, glycopeptide analysis, and free glycan analysis. The sample preparation for free glycan analysis is traditionally lengthy: purified mAb samples are prepared at a desired concentration usually between 0.2-2 ug/uL and N-linked glycans are enzymatically released using Peptide: N-glycosidase F (PNGase F). Released glycans are fluorescently labeled using chemicals such as 2-aminobenzamide (2AB), 2-aminobenzoic acid (2AA), procainamide, or 8- aminopyrene-1,3,6-trisulfonate (APTS). Then cleaned from excess label and salt via solid phase extraction (SPE). The analysis is conducted using high performance liquid chromatography (HPLC) typically via hydrophilic interaction chromatography (HILIC) and detected by fluorescence (FLR) detection and or mass spectroscopy (MS). This results in an analysis that is difficult to conduct and leads to large analytical lead times due to complex, laborious sample preparation, complex chromatographic profiles and/or need of expertise with mass spectrometry interpretation.

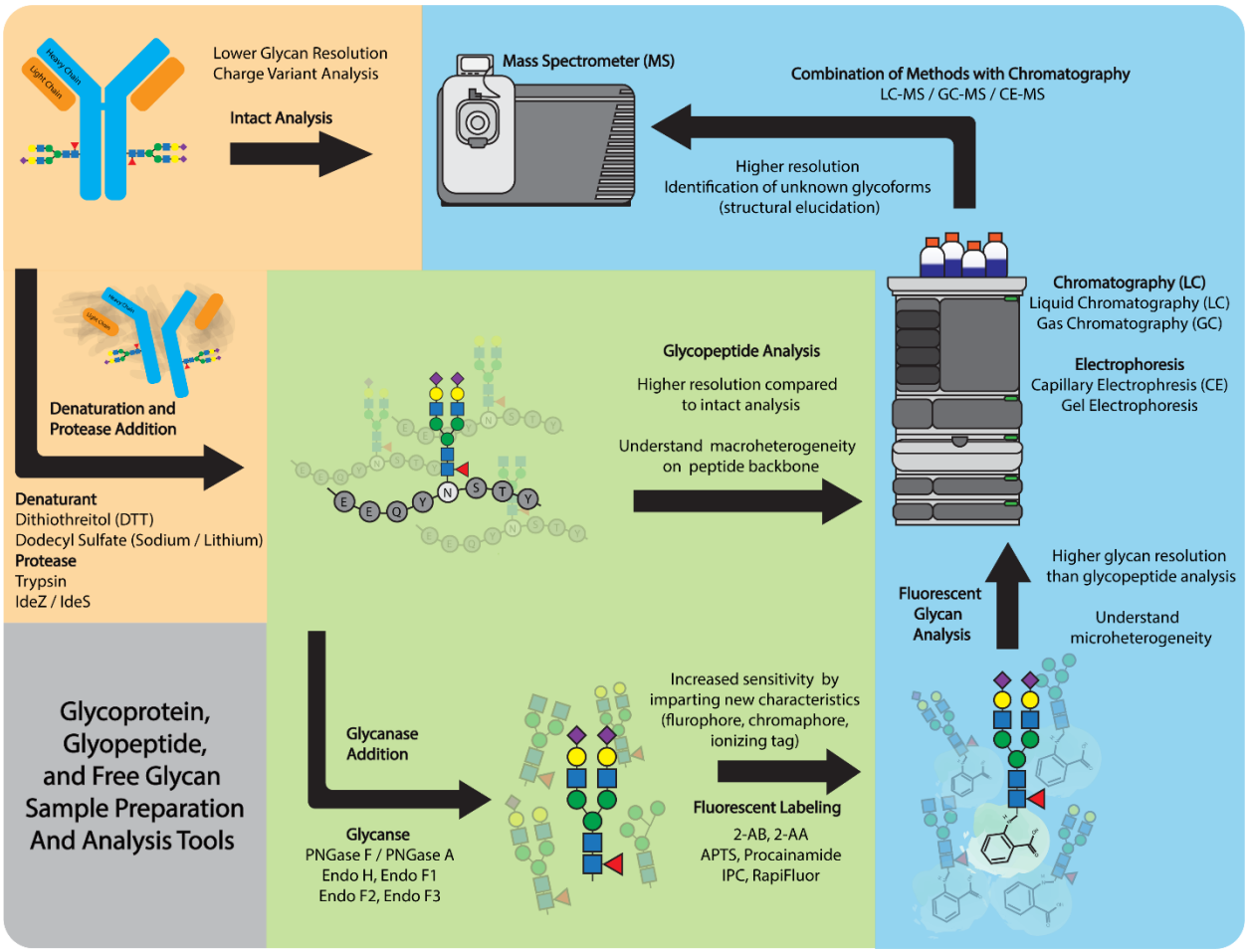

Figure 1: Common strategies for glycan analysis of mAbs

In this review, we will discuss recent advances in samples preparation specifically in denaturation, enzymatic, derivatization methods, and automation as well as advances in characterization of released and intact N-glycans using chromatography, capillary electrophoresis, and mass spectrometry techniques with a focus on rapid, automated approaches to support analysis of glycosylation profiles of biopharmaceuticals which can be summarized in Table 1. Advances include improved deglycosylation methods using surfactants for denaturation and rapid enzymatic deglycosylation. In addition, rapid, and sensitive labelling reagents to enable capillary electrophoretic, chromatography, and mass spectrometry with a focus on automated, high throughput approaches using pipetting robots, and liquid handling systems as well as novel process analytical technology (PAT) approaches.

Table 1: Recent advances in sample preparation and analytical method to support rapid N-Glycan analysis

Summary of advances in denaturation methods and enzymatic release methods of N-Glycans

Denaturation/Release Method	Incubation Time	Advances	Literature References
Lauroyl sarcosine (LS) + Dithiothreitol (DTT) +PNGaseF	0.5% LS wt/wt + 20 mM DTT 3 mins @ 50C deglycosylation	Surfactant and reducing agent to denature and reduce tertiary and secondary structures of the difficult to denature mAbs. Over an order of magnitude more effective than using a surfactant alone. A one pot reaction with surfactant, reducing agent, and enzyme result in a rapid deglycosylation method.	14,15
Sodium Deoxylcholate (SDC) + DTT + PNGaseF	2% SDC wt/wt + 4mM DTT 3 mins @ 50C deglycoslyation		
*Rapi*Gest™	N/A	Contains a novel acid-labile surfactant as the main ingredient. Increases speed and recovery of enzymatic digestions of proteins. Advantages for sample preparations that can initially benefit from the presence of a surfactant and then ultimate removal of surfactant to enable various analyses.	15–17
Gly-X Denaturant + PNGaseF	3 mins	Composed of tertiary or quaternary ammonium cations as main ingredient to effectively and efficiently denature proteins. Gly-X Denaturant plus PNGaseF can be used in a one-pot reaction for rapid deglycosylation.	18

Resin-bound trypsin (Thermo Scientific SMART Digest Kit)	60 mins	Thermally stable resin-bound protease allows high heat digestions to occur. High heat denatures the protein without deactivating the proteases. Allows difficult to digest proteins to be rapidly digested without the use of typical reduction and alkylation steps. Quick cleanup via a solid phase extraction or centrifugal filter procedure.	[19,20]
Endoglycosidases (EndoH, EndoS, EndoS2)	60 mins	Endoglycosidases may be used when deglycosylation of specific glycans are necessary, such as high mannose and hybrid glycans (EndoH), complex glycans (EndoS). EndoS2 is specific to all these groups.	[21]
IdeS/IdeZ	30 mins	Highly specific and reproducible cleavage at a single site below the hinge region between two Glycine residues. Often used for mass spec analysis.	[22–24]

Summary of derivatization techniques of released N-glycans

Label	Labeling Time	Advances	Literature References
Aminoxy tandem mass tag (aminoxyTMT)	22 hrs	Rapid label, sample multiplexing, minimal post labeling cleanup, and reduced spectral complexity. Limited to mass spectrometry analyses.	[25,26,27]
RapiFluor-MS (or RFMS)	5 mins	Rapid label that can be detected by fluorescence, mass spectrometry, and electrospray ionization positive ion mode compatible. High sensitivity ability to detect and characterize low abundance glycan species.	[28,25,29]
InstantPC (modified procaine and dye solvent)	3 mins	Rapid label that can be detected by fluorescence, mass spectrometry. High sensitivity ability to detect and characterize low abundance glycan species.	[30,31]
Velocity tag (V-tag)	15 mins	Rapid label that can be detected by fluorescence, mass spectrometry, and MALDI- MS negative ion mode compatible,	[32]

		high sensitivity ability to detect and characterize low abundance glycan species.	

Summary of advances in methods to analyze N-linked glycans

Technology	Separation/Detection	Advances	Literature References
Capillary Electrophoresis – Mass Spectrometry (CE-MS)	Charge based separation, mass detection	Multi-attribute method with potential for PAT with automation. Analysis of cell culture supernatant (no proA purification), upon heavy label or enzymatic treatment and sample reduction glycosylation profile and titer information obtained.	[33]
Capillary Nanogel Electrophoresis	Lectin nanogel, glycan affinity	Using a lectin nanogel zone in CE, detection of specific glycans based on lectin specificity.	[34,35]
ZipChip CE-MS	Charge based separation, mass detection	Reduced mass analysis that requires no purification of cell culture components. Multi-attribute method for glycosylation and titer analysis. With automation, future PAT to monitor both titer and glycosylation product quality in real-time	[33,26]
Raman Spectroscopy	Light scattering	Real-time monitoring in situ. Future PAT tool to monitor nutrients in cell culture and predict glycosylation variants based on models from Raman spectroscopy data.	[36–38]
LC-MS, LC	LC-MS	Increased capabilities for protein characterization based off mass-to-charge ratio (M/Z) especially for unknown glycan validation. Automated aseptic sampling from bioreactor with modular purification and preparation to enable a peptide-based MAM.	[39]

HILIC separation	Superior glycoform separation with MS compatible buffers. This can be adapted for fully automated online tool called N-GLYcanyzer that finishes with a HILIC separation for N-glycan analysis	40

Summary of advances in automation to prepare and analyze N-glycans

Platform	Advances	Literature References
Coated 96 well plates	Protein G and Protein A coated plates can be used to process cell culture supernatants from protein respectively.	41
Coated Tips	C4 coated tips can separate proteins of interest, followed by tryptic digestion where C18 tips are used to separate glycosylated fragments from non-glycosylated fragment followed by further digestion and separation of free glycans from non-glycosylated fragments using C18 tips.	42–44
Liquid Handling Systems	Systems like, Thermo Fisher Scientific Versette and Hamiliton Star in conjunction with 96 well plates and/or specialized tips have been shown to fully automate preparation for N-Glycan analysis.	39,41,42
Flow Automation (FIA)	Fully customizable flow system for glycan analysis, integrated for LC and/or MS based analyses such as N-GLYcanyzer for real-time measurements and MAM tools rapid characterization.	39,40

2. Advances in mAb Denaturation and Deglycosylation Methods

Most commonly, N-glycans are deglycosylated from the mAb using PNGase F, a glycosidase that cleaves the oligosaccharides between asparagine and the innermost N-acetylglucosamine residue[21] of glycoproteins (which is conserved as an aspartic acid residue) which allows analysis of the entirety of the oligosaccharides. The most common alternative is the use of endoglycosidases including EndoH, EndoS, EndoS2. These enzymes have advantages and limitations due to specificity of certain groups of glycans. EndoH is highly specific to high mannose and hybrid glycans, EndoS to complex glycans, and EndoS2 specific to all of these groups[21]. Advantages being specificity towards specific glycans and is more cost effective compared to PNGase F. Whereas, the limitations lie within digestion time and while advantageous for quantification of specific glycans, endoglycosidases do not allow for complete non-bias glycan analysis Using these traditional enzymes digestions (glycosidases and endoglycosidases) can take up to an hour increasing the preparation time required on an already laborious sample preparation to analyze N-linked glycans. For semi-quantitative studies, in conjunction with PNGase F for complete digestion, glycan trimming chemicals can be used to validate unknown glycans when high resolution mass spectrometry is not available. Thus, advances in denaturation methods and enzymatic release of glycans have been studied.

Commonly, a surfactant is added to solution to aid in solubilizing and denaturing large molecules like mAbs before the digestion occurs. Sodium dodecylsulfate (SDS) is a common anionic surfactant used however impacts sensitivity of mass spectrometry and is difficult to buffer exchange due to formation of emulsions.

To overcome this, *Rapi*Gest™ (Waters, Milford, Mass.) is a destructible anionic surfactant that can be selectively broken up at low pH conditions[16]. Specifically, *Rapi*Gest™ is composed of a novel acid-labile surfactant, sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxyl]-1-propanesulfonate, as the main ingredient which has been shown to increase speed and recovery of enzymatic digestions of proteins[15,17] This allows for sample preparations that can initially benefit from the presence of a surfactant and ultimate removal of surfactant to allow for various analyses. This is ultimately coupled with rapid PNGase F (New England BioLabs Inc.) and *Rapi*Fluor (Waters, Milford Mass.).. Alternatively, Agilent Technologies Inc. (Santa Clara, CA) who acquired ProZyme a leading provider of glycan reagents and kits in 2018, patented the use of tertiary or quaternary ammonium cations (patent # 11,111,268) for protein denaturation to effectively and efficiently denature proteins[18] and subsequently deglycosylate with PNGase F Ultra. Lastly, in 2016, New England Biolabs Inc. (Ipswich, MA) patented (# 9,964,548) deglycosylation reagents and methods for efficiently and completely deglycosylating mAbs. They found that including a carboxylated surfactant, lauroylsarcosine (LS) or sodium deoxylcholate (SDC), along with Dithiothreitol (DTT) and PNGase F, which is typically used for deglycosylation of mAbs, that a substantially deglycosylated antibody could be achieved in a 3 minute reaction at 50° C[14]. To demonstrate their reagents and protocols were superior to traditional use of PNGase F and DTT they compared their method to various conditions including high concentrations of PNGase F and DTT, extended incubation with nominal levels of PNGase F, and against another commercially available surfactant, *Rapi*Gest™ compared to LS. High concentrations of PNGase F and DTT are used to achieve a more rapid reaction however, this often results in partial deglycosylation and bias towards to more abundant glycoforms. Using high concentrations of PNGase F and DTT often lead to scalability and cost issues. When comparing their methods to an extended 16-hour incubation with PNGase F, the extended incubation did not result in substantial deglycosylation of the antibody heavy chain. New England BioLabs Inc. also compared their deglycosylation protocol using LS, DTT, and PNGase F to achieve a 3 min digestion to another commercially available reagent, *Rapi*Gest™ which is added prior to PNGase F digestion. This also resulted in partially deglycosylated antibody when analyzed via ESI-MS-TOF[14]. . With the use of *Rapi*Gest™, quaternary ammonium cations, or carboxylate detergents in solution it increases solubility of hydrophobic proteins and glycoproteins which were previously resistant to proteolysis from inaccessible cleavage sites. This reduces miscleavages and increases efficiency of the denaturation and release step.

In addition, with the introduction of ThermoFisher Scientific (Waltham, MA) SMART™ Digest kit to the market, advances in site specific glycosylation (glycopeptide) analysis compared to typical trypsin digestions (reduction, alkylation, digestion) have improved in terms of efficiencies and reproducibility. The SMART Digest kit consists of thermally stable, resin-bound proteases. Thermal stability of the proteases allows for high heat digestions to occur that denature the protein without deactivating the proteases[45]. This allows for difficult to digest proteins to be digested without the use of typical reduction and alkylation steps.[19] Additionally, the resin-bound proteases allow for quick clean up via a solid phase extraction or centrifugal filter procedure. Overall, trypsin digestion occurs within one hour reducing the time it takes to conduct a typically laborious trypsin digestion[45]. With this kit improved efficiencies, reproducibility, and compatibility for future automation are the main advantages to improve this technique.

3. Advances in labeling/derivatization techniques of released Glycans

Since glycans lack a chromophore, traditional ultraviolet–visible (UV) spectroscopy analyses are not applicable however there are quite a few strategies utilized to derivatize glycans such that they can be analyzed. Schiff base and reductive amination are the most common strategy to attach glycans with a label like 2AB, 2AA, and APTS[27].Similarly, to the labels just mentioned, procainamide uses the same reductive amination mechanism to bind with the reducing end of the glycan as the others however exhibits increased fluorescence and superior ionization performance compared to 2-AB which is advantageous for mass spectrometry analyses[25,29,46].

Conventional labeling strategies with 2AA and 2AB are often employed due to high molar labeling efficiencies independent of quantity of glycans and independent of glycan structures with limited levels of desialylation. Both labeling strategies result in an accurate representation of glycan profiles and allows for relative quantification of individual glycans with 2AB being compatible for chromatographic separations and

2AA with electrophoretic seperations[47]. The labeling reaction occurs in a two-step reductive amination manner where glycans with a free reductive sugar undergo a Schiff base formation and the carbonyl carbon of the reducing sugar is nucleophilically attacked by the dye. Upon addition of sodium cyanoborohydride or 2-picoline borane, a non-toxic alternative with equivalent performance[48], the resulting imine group is reduced, and a stable label is formed. However, the drawback to these techniques is sample preparation time becomes a bottleneck for analysis as 2AB and 2AA sample preparation methods take upwards to 40 hours.

New derivatization techniques have been studied to increase sensitivity and drive sample preparation efficiencies. A novel derivatization technique including an aminoxy tandem mass tag (aminoxyTMT) has a carbonyl reactive aminoxy group that allows for protein carbonylation of the amino acid side chains. This allows for multiplexed characterization and quantitative comparison of complex glycans of biological samples for mass spectrometry analyses. AminoxyTMT exhibits superior labeling efficiency and reduced mass spectral complexity while supporting a simple labeling procedure[27,49]. This tag reduces labeling times by half compared to traditional methods with poor mass spectrometry sensitivity however sample preparation time is still a bottleneck for sample analysis albeit quicker. The Rapi*Fluor*-MS (Waters, Milford MA) label uses a rapid tagging functional group, N-hydroxysuccinimide (NHS) carbamate, which allows for even quicker labeling efficiencies to occur in 5 minutes. When coupled with Rapid PNGase F (New England BioLabs, Ipswich MA) enzymatic digestion sample preparation times are decreased to twenty minutes. This being a major advantage compared to the other labels when sample preparation time is a paramount. The *Rapi*Fluor-MS label also allows for fluorescence detection due to the quinoline fluorophore and enhanced positive mode electrospray ionization due to the basic tertiary amine[50]. Additionally, increased sensitivity of fluorescence and mass spectrometry detection is observed with a novel labeling reagent composed of an activated form of procaine called Instant PC (IPC) (Agilent Technologies, Inc., Santa Clara CA). This reagent labels glycosylamines digested with PNGase F by forming a stable urea linkage with the N-glycan and rapidly within 5 minutes, making it comparable in sample preparation time with the Waters method. Their IPC reagent is the main ingredient used for labeling in Agilent Technologies Inc. GlykoPrep® Rapid N-Glycan Preparation with InstantPC Kit[31]. Lastly, the Velocity Tag (V-tag) by Ludger Ltd (Oxfordshire UK) is a novel fluorophore containing an amine reactive succinimidyl ester that reacts with the N-terminus amine moiety connected by a simple alkyl group to the fluorescent group containing a sulfate group that improves sensitivity in negative ion mode using MALDI-MS[51] and can be used for chromatographic separations. With advances in the aminoxyTMT, *Rapi*Fluor-MS, IPC, and V-tag labels, upon protease digestion labeling can occur in less than an hour with all labels exhibiting superior sensitivity for chromatographic and mass spectrometry analyses.

4. Advances in N-linked Glycan Analysis Methods

Due to the complexity of the biopharmaceutical process it is advantageous and encouraged to monitor in real time the product quality of the mAb. However, due to the cell culture and the heterogeneity of the mAb product this is often difficult. One strategy is sampling by day and analyzing the samples offline however employing real-time PAT for monitoring is more favorable. Unfortunately, there are not many tools specific enough to analyze the product quality of the product rather than analyzing the cell culture impurities. Advances in both in-process and real-time monitoring have been made. There is an emergence using electrophoretic techniques as compared to traditional chromatographic techniques and an increased focus on multi-attribute method (MAM) and multiattribute chromatography (MAC) technology. In the area of real-time monitoring there is a focus on PAT tools such as Raman spectroscopy[37,38], CE-MS[52], sequential injection analysis with chromatography and increased efforts to develop automated[53,54] and/or high throughput technologies to reduce time and labor-intensive offline analyses.

Capillary electrophoresis (CE) has become a pervasive technique in the analysis of N-glycans. There are several advantages to this technique including: femtoliter to nanoliter range injection volumes; milliliters worth of running buffers; laser induced fluorescence (LIF) detection limit is within femtomolar range; and the use of high electric fields results in fast separation times and high efficiency[26]. Yamada et al. employed electrophoresis paired with mass spec analysis to develop an orthogonal method to SDS-PAGE to separate the mAb glycan heterogeneities, recovered them using an SDC-containing buffer as the extraction solvent and subsequently performed intact mass analysis to demonstrate a direct, rapid, and precise analysis of *N*-

glycan variants[55].This method allows for a rapid and precise alternative to a traditionally laborious and time-consuming SDS-PAGE separation for *N*-glycan analysis.

Recently, another approach using capillary electrophoresis has emerged specifically, with the use of lectin nanogels incorporated in the capillary as a zone. Lectins are known to have great potential for use in analytical methods to detect and identify glycans due to their specificity for various glycoforms such as fucosylated, galactosylated, sialyated, and bisected N-acetylglucosamines and can be used as a method to identify glycosylation profile[34]. Lu and Holland used four lectins with specificity to the various common human IgG N-glycans. This included *aurantia* lectin, *Erythrina cristagalli* lectin (ECL), *Sambucus nigra* lectin, and *Phaseolus vulgaris Erythroagglutinin* (PHA-E) lectics which have specificity for and are used to identify fucose, galactose, α2–6-linked sialic acid, and bisected *N*-acetylglucosamine, respectively[35]. This approach has great promise and specificity for the glycans of interest yet misses the mark for total N-glycan analysis required for mAb product quality.

Both Raman spectroscopy and capillary electrophoresis technologies have been investigated for their utility as a PAT to monitor the cell culture nutrients and extrapolate glycosylation profile and titer of cell culture samples. With the use of calibration curves from cell culture nutrients and models developed from offline analyses, Raman spectroscopy has shown promise to predict levels of glucose and lactate in cell culture in real-time which ultimately impacts the final glycosylation profile.[37] With this information models can be developed to predict the final glycosylation profile using the information gained from Raman spectroscopy regarding cell culture nutrients and offline glycan profile analysis[37,38]. Raman spectroscopy and other techniques have displayed utility in monitoring process parameters that can be used to predict quality attributes however has yet to realize correlative and quantitative results in the glycosylation profile thus increasing focus on methods that are able to monitor glycosylation profile in real time have taken precedent. Wang, Feng, Sosic, and Zang developed a fast ZipChip CE-MS reduced mass analysis that required no purification of the cell culture which is necessary for real-time analyses. The supernatant was desalted and buffer exchanged. Heavy label mAb control and reducing reagent, DTT, added to the sample to be analyzed for titer while for glycosylation analysis there was an enzymatic treatment and addition of DTT to the sample prior to analysis. Both these sample preparation procedures are rapid with potential for automation. Upon analysis via microfluidic ZipChip CE-MS sample results are turned around within 1.5 hours[33]. With model-based prediction and other mathematical modeling more correlated techniques for Raman spectroscopy is to be expected to emerge in the future.

5. High-Throughput and Automated Approaches

Automation of sample preparation has the greatest potential to reduce laborious sample preparations whether it be rapid or complex sample preparations. Stockmann et al. developed an automated and high throughput glycan profiling method. They developed a rapid, protein G coated 96-well filter plate to isolate the cell culture supernatants then the filtrate moved to a protein A coated 96 well plate to isolate the purified monoclonal antibody followed by a hydrazide-mediated glycan clean up. After clean-up, a fluorescent labeling mix (30:70) of (350 mM 2-aminobenzamide, 1 M sodium cyanoborohydride in acetic acid/dimethyl sulfoxide) was dispensed into each well, and the plate was incubated at 70°C. After incubation, a solid phase extraction is performed, and samples concentrated. All of this is done using robotics and in an automated fashion[53].

While, Stockmann et al. developed an automated, high throughput end to end sample preparation process, Shajahan et al. developed a rapid labeling and clean-up permethylation method that is compatible with ESI-MS and MALDI-MS analyses. Using an automated pipet robot, released glycans plated in a 96-well plate format can be permethylated in less than an hour significantly reducing the amount of time that the labeling step traditionally takes. The samples are then cleaned up using solid phase extraction with C18 coated tips[54].

Liquid handling systems like the ThermoFisher Scientific Versette and Hamilton Star were used to develop automated sample preparation processes able to prepare samples for intact and released N-Glycan analyses. Chen et al developed a sample preparation scheme that leveraged the affinity of three different

tip-based procedures: C4 digestion, C18/MAX enrichment, and C18 desalting. The C4 tips bind the proteins of interest and are followed by a protease digestion. Then C18/MAX-Tips are used to bind the tryptic digested proteins and separate them from non-glycopeptides as those remain bound to the MAX resin. Following release and elution of the glycopeptides from the tips an aliquot of the intact glycan samples can be further digested in PNGase to aid in released glycan analyses and C18 tips used to separate the deglycosylated peptides and glycans[42]. Upon centrifugation to remove cell debris, Lanter et al. incorporated the Hamilton Star system to create an automated intact mass analysis and reduced mass analysis sample preparation workflow using samples directly from the bioreactor that had undergone centrifugation and plated directly into a 96 well plate ready for intact LC-MS glycan analysis and a reduction step prior to reduced mass analysis for analysis of light and heavy chains[39].

With this integration of automation for sampling and sample preparation, Liu et al. developed a fully integrated online platform for real time monitoring of multiple product quality attributes including glycosylation. This fully integrated method automates sample preparation under aseptic conditions with modular automated sampling technology (MAST), ProSIA which enables multi-sample preparation programming, and sample analysis via UHPLC and mass spectrometry[56]. After direct aseptic sample, particulates and cellular debris are removed from the sample using a hollow fiber filtering system. The cell free sample is further proA purified before sample analysis. Using a Python to control these steps the sample can then be further processed for peptide-based MAM method where the sample preparation occurs in a designed fluidic network of valves, tubing coils, and reaction chambers to allow for automated enzymatic digestion preceding sample analysis[56]. This technology enables end to end sample lifecycle from sampling from a bioreactor, purification, sample preparation, and sample analysis. This advancement realizes the FDA's call to shift the testing and monitoring of a process at the end of it's process to a focus on testing and monitoring the process while in-process.

With proof of concept demonstrated automation of sample prep would allow this to become a future PAT to monitor both titer and glycosylation product quality in real-time[52]. In fact, Liu et al. developed a fully integrated online platform for real time monitoring of multiple product quality attributes including glycosylation. Additionally, Gyorgypal and Chundawat developed a fully automated online tool called the N-GLYcanyzer which also allows for automated and integrated online sample preparation and analysis within 3 hours of sampling from a bioreactor and concludes with a HILIC separation which will allow for real-time glycosylation control[40].

In addition to possible PATs described above, like the ZipChip CE-MS method that can monitor both titer and glycosylation profile[52] and Liu et al. fully integrated online platform[56] the importance of multi-attribute methods to support process development have increased in prevalence. An intact mass analysis method was developed for open access use to provide rapid, comparable results regarding light chain and heavy chain identification, light chain glycation, and non-glycosylated heavy chain (NGHC) compared to current LC-MS/MS peptide based MAM method for comparison of light chain and heavy chain identification and light chain glycation and compared to the current CGE method for NGHC comparison[39]. With this method analytical analysis can be performed with little analyst interaction and drastically reduces the number of analytical methods necessary to be conducted to gain similar product quality information. With implementation of automated sampling this could further reduce the need to analyst interaction and become another PAT tool.

6. Conclusion and Future directions

Recent advances in sample preparation techniques particularly in denaturation methods using novel surfactants, novel enzymatic treatments, and novel labels that allow for rapid labeling with improved fluorescence and mass spectrometry sensitivity have enabled rapid and sensitive N-glycan analyses. Additionally, focus on advances in methods used to analyze N-glycans include capillary electrophoresis, Raman spectroscopy, and multi-attribute methods including chromatography and/or mass spectrometry modalities. Improvements in monitoring culture nutrients may be used in the future to create predictive models for glycosylation profiles[37,38,57]. Most notably, multi attribute methods that incorporate bioreactor sampling, sample preparation, and various sample analyses have the most promise and utility in the field of N-glycan analysis biopharmaceuticals. Lastly, advances in automation to sample preparation using liquid

handling systems and pipet robots have reduced laborious sample preparations. With the increase focus on rapid, automated sample preparations and latest advances in analytical methods the biopharmaceutical industry is closer to realizing a completely online and real-time PAT that can be used in the adoption from fed-batch to continuous biomanufacturing in the coming future.

With diminishing returns in advances in sample preparation by getting close to instant labeling efficiencies the next bottleneck the next steps are ensuring a sensitive and robust PAT system that can enable continuous manufacturing and allowing for more in depth models and artificial intelligence/neural networks. This will be enabled through quicker insights in glycosylation flux pathways and how the glycosylation profile is changing on a transient level. Once we have a transient understanding, a control model can be built to aid in the control of CQA. This will allow for more safe and efficacious for drugs and biosimilars to be brought to market.

Conflict of Interest
The authors declare no competing financial interest.

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