ELECTRONICALLY REPRINTED FROM DECEMBER 2015
INTERNATIONAL BioPharm
The Science & Business of Biopharmaceuticals

N-Glycan Composition Profiling for Quality Testing of Biotherapeutics

Rebecca Duke and Christopher H. Taron

Advances in glycan analysis are enhancing biologics development and quality control processes.

The pharmaceutical industry is being transformed by the success of protein-based therapeutics (biologics). In 2012, biologics sales in the United States topped $63 billion, up 18.2% from 2011 (1). Additionally, it is estimated that half of new drug approvals in 2015 will be biologics, with this trend forecasted to increase over the next decade (2).

Many classes of biologics are recombinant secretory proteins (e.g., antibodies, fusion proteins, growth factors, cytokines, etc.) that possess post-translational carbohydrate modifications of certain asparagine residues (N-glycans) or serine/threonine residues (O-glycans). The structure and composition of glycans can dramatically affect the stability, bioactivity, and pharmacokinetics of a biologic drug. Most biologics are recombinant N-glycosylated proteins that are derived from non-human protein expression systems such as Chinese hamster ovary (CHO) cells (3). Glycan structure and composition are particularly sensitive to changes in a biologic's manufacturing environment, such as process improvements, scale-up, and method transfers. As such, glycan structure is designated a critical quality attribute (CQA) that must be monitored during manufacturing, and the glycan profile of a finished product is used to assess the consistency of a manufacturing process from batch to batch.

STRUCTURAL ANALYSIS OF N-GLYCANS

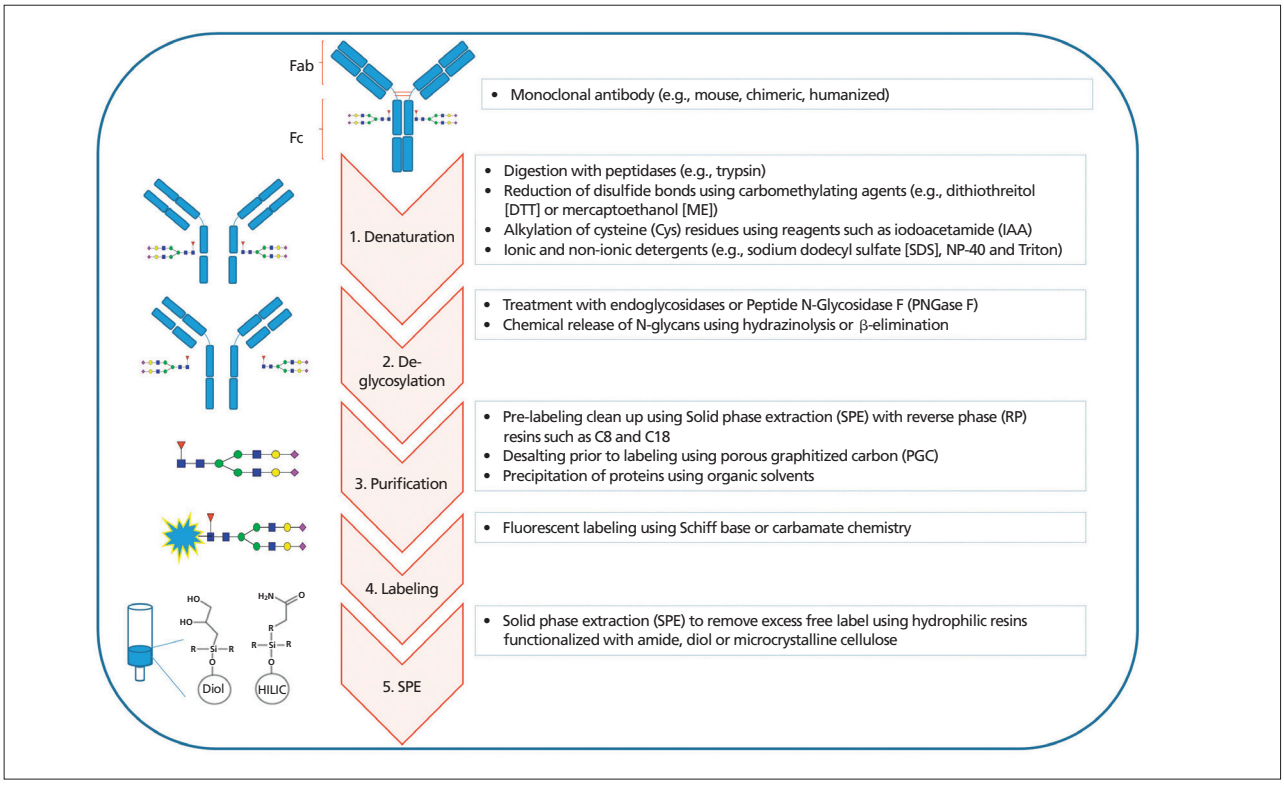
Workflows that enable N-glycan structural analysis are complicated and have historically involved sample-processing methods that are slow, labor intensive, multi-stepped, and dimensional. Conventional preparation of N-glycans derived from monoclonal antibodies (mAbs) and immunoglobulin Gs (IgGs) generally proceeds as outlined in **Figure 1**. Typically, fluorescently labeled N-glycans are produced and analyzed using liquid chromatography (LC) coupled to fluorescent detection (FLR), LC with detection using mass spectrometry (MS), or capillary electrophoresis (CE) with laser-induced fluorescence (LIF). There is currently no single standardized protocol within the pharmaceutical industry for N-glycan analysis and most organizations have developed customized workflows to suit their individual needs (4). This landscape is rapidly changing, however, as industry demands have increased for more standardized workflows that permit faster, more accurate, higher throughput, reproducible, and quantitative structural characterization of N-glycans. Recent advances in sample preparation and analysis of N-glycans that are enabling better quality testing of N-glycans of biologics are reviewed here.

ADVANCES IN N-GLYCAN SAMPLE PREPARATION

The field has seen remarkable recent advances in the range, supply, and quality of reagents, consumables, and analytical instrumentation aimed at improving glycan analysis. An important area of improvement has been in

Rebecca Duke is a postdoctoral researcher, and **Christopher H. Taron** is scientific director, Protein Expression and Modification Division, both at New England Biolabs, Ipswich, MA.

Figure 1: Schematic illustrating the steps of a typical sample processing method for N-glycan analyses of monoclonal antibodies (mAbs) and IgGs. Most N-glycan sample preparation workflows consist of these general steps, but additional technical options could also be employed.



sample preparation methods that have been considerably streamlined from day to hour timescales. New methods are now also higher throughput, and analyses in 96-well formats are feasible using robotic liquid handling platforms. A 384-well sample preparation method was reported for analysis of N-glycans derived from IgG using LC–FLR (5). This study marked a significant step toward bringing the throughput of glycomics closer to the realm of genomics. Additionally, the field has seen the recent emergence of turnkey commercial sample preparation kits that permit glycans to be rapidly liberated from glycoproteins and labeled for analysis.

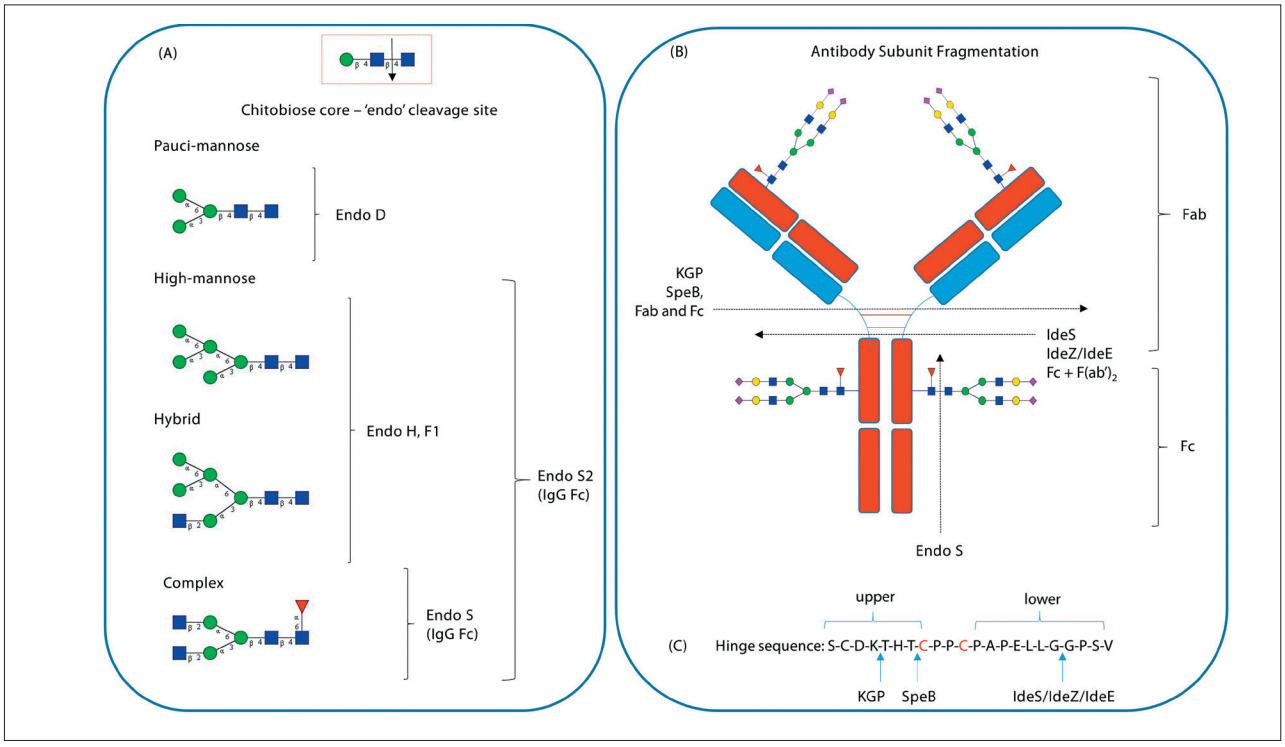
Enzymatic release of N-glycans

A key early step in N-glycan composition profiling involves complete and unbiased removal of N-glycans from a target glycoprotein. N-Glycan release typically involves denaturing a protein with a reducing reagent (e.g., dithiothreitol [DTT]), sodium dodecyl sulfate [SDS], and an alkylating reagent (e.g., iodoacetamide [IAA]), respectively, prior to enzymatic release of glycans with peptide N-glycosidase F (PNGase F). This process has several technical drawbacks: SDS inhibits the function of PNGase F, long PNGase F digestions are typically required (two hours to overnight) and often not all N-glycans are completely released, and the presence of detergent or SDS in a sample is detrimental to downstream analysis using MS.

The field has moved to eliminate these technical complications. A first improvement was the development of RapiGest SF (Waters Corporation), an acid (H+) cleavable MS and PNGase F 'friendly' labile surfactant. RapiGest SF denatures a mAb in approximately three minutes in the absence of DTT, eliminating the need for time consuming clean-up steps prior to MS analysis. RapiGest SF has also been reported to reduce the amount of time needed for PNGase F digestion from overnight to approximately two hours (6); although in this study, N-glycan release was not entirely complete. A second improvement has been the development of Rapid PNGase F (New England Biolabs), a novel MS-compatible composition of the enzyme that permits complete and unbiased release of all N-glycans from mAbs and fusion proteins in 10 minutes (7).

ALL FIGURES ARE COURTESY OF THE AUTHORS

Figure 2: (A) Schematic summarizing the general specificity of selected endoglycosidases. Endo S removes Fc N-glycans from IgG only. (B) Antibody fragmentation enzymes currently available (New England Biolabs, Promega, and Genovis). IdeZ/IdeE (*Steptoccocus equi*) cleaves the upper hinge region of human IgG's but has higher activity towards mouse IgG's than IdeS (*Streptoccocus pyogenes*), particularly mouse IgG2a. GingisKHAN (KGP) cleaves human IgG1 at a single site above the hinge region. SpeB (*Streptoccocus pyogenes*) digests IgG's from many species and subclasses. (C) Hinge sequence showing cleavage specificity. The cysteine residues of disulfide bonds are indicated in red.

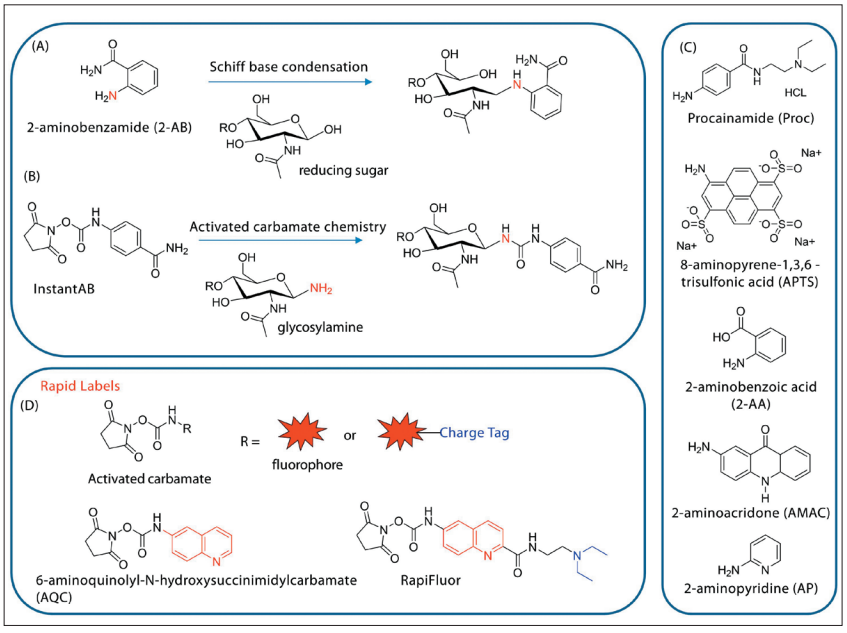


In addition to PNGase F, several other endoglycosidases are now available for application in glycan profiling workflows (8). For example, peptide-N-glycosidase A (PNGase A) can be used for profiling N-glycans from proteins derived from plant or insect cells and that contain a core α(1,3)-linked fucose, a modification that is recalcitrant to PNGase F digestion. Additionally, endoglycosidases (e.g., Endo D, H, F1, S, and S2) cleave within the chitobiose core of N-glycans (**Figure 2A**). Endoglycosidases S and S2 work more efficiently than PNGase F under native conditions and can selectively remove classes of N-glycans from the fragment crystallizable (Fc) region of IgG (i.e., hybrid versus high mannose N-glycans). This method of deglycosylation is often useful for investigation of the role glycosylation plays in the biological activity of a target glycoprotein. Additionally, antibody subunit fragmentation proteases (e.g., single site hinge or upper hinge region cleavage) are commercially available and can be used in combination with endoglycosidases to permit separate analysis of N-glycans derived from the Fc or fragment antigen-binding (Fab) regions of antibodies (**Figure 2B**). These tools are expanding the breadth of applications for which N-glycan profiling can be used.

Fluorescent labeling of glycans

Recent advances in N-glycan labeling chemistries have yielded tremendous improvements in both labeling speed and analytical sensitivity. There are two basic labeling strategies that are typically employed in analytical workflows: Schiff base (SB) condensation and reactive carbamate chemistry (**Figures 3A** and **3B**). Both methods are used after glycans are released from a protein by PNGase F. The PNGase F reaction produces a glycan product with a transient glycosylamine moiety on the *N*-acetylglucosamine (GlcNAc) that was formerly linked to asparagine. This GlcNAc can be converted from a glycosylamine form to a reducing sugar form thereby enabling use of SB condensation with amine functionalized fluorophores (**Figure 3A**). The most frequently used fluorophore is 2-aminobenzamide (2-AB);

Figure 3: (A) 2-Aminobenzamide (2-AB) and (B) InstantAB N-glycan labeling using Schiff Base and reactive carbamate chemistry, respectively. (C) Selection of amine functionalized fluorophores frequently used for Schiff Base labeling. (D) Features of instant labels and the examples AQC and RapiFluor.



however, several other fluorescent compounds are commercially available that are tailored for various downstream applications (e.g., triple charged 8-aminopyrenetrisulfonic acid [APTS] for CE, and protonatable procainamide (Proc) for enhanced ionization in positive mode MS) (**Figure 3C**).

An important advance has been the development of a new generation of 'instant' labels. These compounds are based on the reaction of activated fluorescent carbamates (e.g., InstantAB from Prozyme, 6-aminoquinoyl N-hydroxysuccinimidyl carbamate [AQC], and RapiFluor from Waters) with glycosylamine (**Figure 3D**). Compared to SB condensation that has a reaction time of 2–4 hours at approximately 65 °C, this chemistry takes place at room temperature in just minutes. The RapiFluor label was rationally designed to combine the strong fluorescence emission of AQC and the ionization benefits of a tertiary amine (**Figure 3D**). This label allows a single sample preparation to enable two-dimensional (2-D) glycan analysis using offline LC–FLR and MS or online LC–FLR–MS. It is conceivable that many currently used fluorescent glycan labeling reagents could be synthesized in an 'instant' format by reaction with N-N-disuccinimidyl carbonate. Procainamide is now commercially available as an activated carbamate, referred to as InstantPC (from Prozyme).

Solid-phase extraction (SPE)

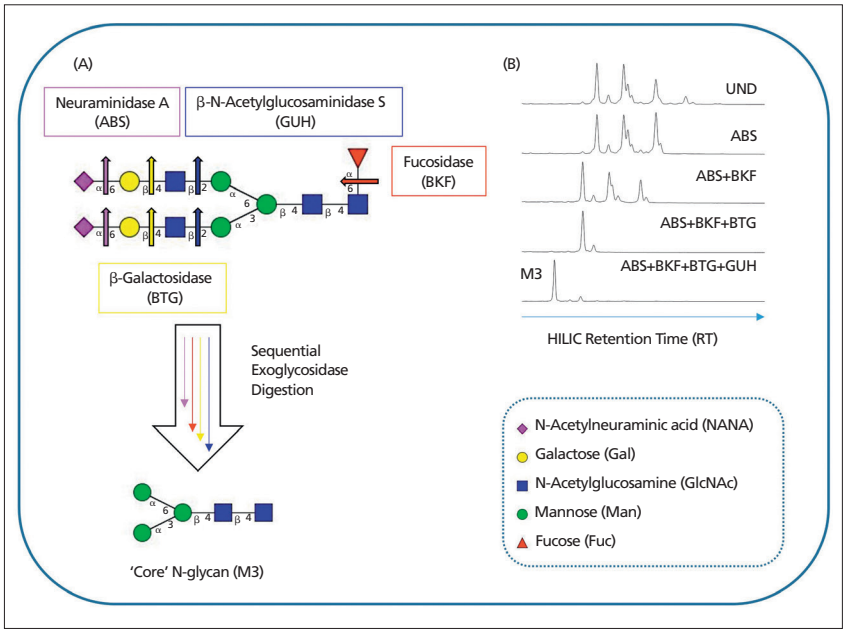
Another important aspect of N-glycan sample preparation is solid-phase extraction (SPE). SPE is commonly used to separate glycans that have been fluorescently derivatized from excess free fluorophore that can obscure glycan peaks during chromatography. Often the mAbs analyzed in biopharmaceutical quality control environments are pure finished products. In this case, development of an effective SPE method can be relatively straightforward, especially when the expected repertoire of glycan structures (e.g., glycan size and charge state) is known. When the sample matrix is more complicated and biological analytes and impurities are present, an effective SPE strategy that does not result in a large decrease in glycan yield must be determined empirically. In fact, such samples may require more rigorous clean up, and pre-labeling SPE may also be required.

Recently, an alternative to the traditional cartridge-based SPE was developed. It involves the extraction of free N-glycans from solution using hydrazide functionalized beads (9). This approach, termed glycoblotting, was used to profile N-glycans from human serum proteins and IgG, cells, organs, and plant tissues in high throughput (HTP) formats. Its success led to the commercialization of high-density hydrazide beads, BlotGlyco (S-BIO). With the emergence of HTP N-glycan profiling, SPE in a 96-well format has become more commonplace with options including HyperSep Diol (Thermo Scientific) and hydrophilic interaction liquid chromatography (HILIC) (Waters and Nest Group).

ADVANCES IN GLYCAN SEPARATIONS AND DATA ANALYSIS

Advances in separation technologies have played a critical role in the progress of glycoanalytics. Several instrument configurations permit separation and analysis of fluorescently labeled glycans. Common approaches involve first separating labeled glycans by LC or CE ahead of their structural analysis. Both technologies have significantly improved in resolution and throughput over the past decade.

Figure 4: (A) Exoglycosiadase enzymes typically used to digest fluorescently labeled N-glycans derived from a mAb produced in Chinese hamster ovary (CHO) cells. Enzymes are Neuraminidase A from *Arthrobacter ureafaciens* (ABS) for removal of α(2-3,6,8,9) linked sialic acid, Bovine Kidney Fucosidase (BKF) for removal of core and outer arm fucose in α(1-2,3,4,6) linkages, β-N-Acetylglucosaminidase S from *Streptoccocus pneumoniae* (GUH) for removal of β(1-2,3,4,6) linked N-acetylglucosamine and Bovine Testes Galactosidase (BTG) for removal of β(1-3,4) linked galactose. (B) hydrophilic interaction liquid chromatography-fluorescent (HILIC–FLR) profiles depicting enzyme array digestion of 2-AB labeled human serum IgG N-glycans.



Ultra-High Performance Liquid Chromatography

A major advance has been the development and commercialization of ultra-high performance liquid chromatography (UHPLC), liquid chromatography systems capable of functioning at high pressures (e.g., 10,000 psi). These systems offer shorter sample run times and greater resolution than older HPLC systems. Additionally, in the past, characterization of complex N-glycan structures typically required multiple offline chromatographic techniques (referred to as 2- and 3-D analyses). Considerable time is now being saved by online coupling of UHPLC systems fitted with HILIC columns to both fluorescence detection (FLR) and tandem mass spectrometry (MS/MS) systems. Such powerful analytical systems are now becoming mainstream and are amenable for use in biopharma for simultaneous relative quantification of N-glycans (FLR) and structure confirmation using accurate mass and fragmentation. Adding to the strength of HILIC–UPLC platforms is the relational database GlycoBase3+ (NIBRT), which contains normalized retention times for glycans expressed as glucose unit (GU) values (10). This resource contains more than 600 GU values and is a powerful tool that can assist in structural assignments.

Capillary electrophoresis

Capillary electrophoresis (CE) is an established analytical technique routinely used in the biopharmaceutical industry for the separation of fluorescently labeled N-glycans. CE utilizes an applied voltage to separate ions based on their electrophoretic mobility. Typical instrumentation consists of a high-voltage power supply; a sub-millimeter, micro, or nanofluidic capillary; and a LIF or MS detector. For N-glycan profiling, CE sample run times are fast, and the technique is ideally suited to multiplexing and running high numbers of samples with low concentration, while maintaining high resolution. Multicapillary CE, as designed for DNA sequencing, is now routinely combined with on-column LIF detection. Direct structural information on the glycan peaks in the electrophoretic profile, however, is not obtained. This is changing with the development of glycan mobility databases and related bioinformatics tools to help assist structure assignments. Currently, characterization is performed using co-migration with commercially available standards and exoglycosidase treatment followed by analysis of migration shifts. In 2014, Agilent and Picometrics described the first online CE–LIF–MS instrumentation for biopharmaceutical applications (11). Also, recent efforts have focused on developing methodology to deliver CE–MS profiles that can be compared to those generated using standard CE–LIF of ATPS labeled glycans, so that unidentified peaks can be more easily assigned (12).

Exoglycosidase sequencing

The structures in each chromatographic peak of a glycan profile must be sequenced to ensure the quality of a biopharmaceutical. This is often done with fragmentation MS. Treatment of samples with exoglycosidases having

highly defined specificities, however, can enhance structural interpretations. Exoglycosidases sequentially remove monosaccharides from the non-reducing end of a glycan, and give information about the type of sugar removed and how it was linked (anomer and linkage) to the glycan (**Figure 4**) (2). The use of exoglycosidase digestion panels to decipher N-glycan structure is an experimentally straightforward approach that was first described in the 1980s (13). For many years, however, the supply and purity of these reagents was inconsistent. An import advance has been the development of recombinant and highly pure versions of the field's preferred exoglycosidases for glycan sequencing applications that are manufactured under ISO9001 and ISO13485 quality standards (New England Biolabs).

FUTURE TRENDS IN GLYCAN ANALYSIS

Technical advances in glycan analysis will enable better definition of the guidelines for assessing the glycan composition of biologics. Currently, there is still a lack of clarification on the level of glycan characterization required for any given biologic, or what deviations in glycosylation are acceptable, generating potential commercial and legal risks for companies (14). Federal agencies such as FDA, European Medicines Agency (EMA), and National Institute for Standards and Technology (NIST), as well as academic organizations like the Minimum Information Required for a Glycomics Experiment (MIRAGE), are improving guidelines and analytical standards. These improved guidelines have aided many companies in devising custom in-house programs to show that they understand, measure, and control the glycosylation of their products. However, more rigorous industry standardization is needed.

Advances in N-glycan analysis will continue to enable important possibilities in biotech and drug manufacturing beyond quality control of biotherapeutics. The ability to more readily assess glycan structure opens a world of opportunities that affect many aspects of drug development. In the next decade, it is likely that faster and simpler glycan analysis will be routinely used on-line to address glycan composition during a manufacturing process. This will allow faster troubleshooting of altered glycosylation and the effect of varying bioprocessing parameters on glycan structure to be better understood. Improved analytics will allow the tailoring (glycoengineering) of glycan composition to create more efficacious biologics or bio-betters to become more commonplace in the drug-discovery process. Glycoengineering may also provide new options for developing intellectual property positions around novel drugs. Finally, increases in the throughput of glycan analysis promises to improve cell line development processes, whereby manufacturing lines that have the best yields and most desired glycan profiles can be identified.

With regards to glycan analysis workflows and instrumentation, the field will continue improving on speed, automation, throughput, and sensitivity. Sample preparation methods will likely continue toward higher throughput platforms (e.g., 384 well) and those capable of profiling highly heterogeneous glycosylation from complex sample matrices. Additionally, use of exoglycosidase arrays as part of sample preparation will likely become more commonplace and will improve the quality of data interpretation. Finally, instrumentation may move closer toward the dream of fully automated sample preparation, analysis, and interpretation of data in a single machine. For example, HPLC chip technology systems have implemented reusable microfluidic chips for the online de-glycosylation of mAbs, followed by enrichment, separation, and ionization of glycans. It is anticipated that future methods may further explore miniaturization and automation of sample preparation and analysis. Such concepts may one day help move glycan analysis from the realm of a specialty skill to that of a routine analysis that can be performed by any technician.

REFERENCES

1. S. Aggarwal, *Nat. Biotechnol.* 32 (1), 32-39 (2014).
2. S. A. Berkowitz, J. R. Engen, J. R. Mazzeo, and G. B. Jones, *Nat. Rev. Drug Discov.* 11(7), 527-540 (2012).
3. O. Hossler, S.F Khattak, and Z.J. Li, *Glycobiology*. 19 (9), 936–949 (2009).
4. K. Mariño, J. Bones, J. J. Kattla, and P. M Rudd, *Nature Chem. Biol.* (6), 713-723 (2010).
5. H. Stöckmann, R. M. Duke, S. M. Martín, and P. M. Rudd, *Anal. Chem.* 87 (16), 8316–8322 (2015).
6. Y. Q Yu, M. Gilar, J. Kaska, and J. C. Gebler, *Rapid Commun. Mass Spectrom*; 19, 2331–2336 (2005).
7. A. Bielik et al., "Novel Formats of PNGase F for N-Glycan Removal Workflows and Applications", DOI: 10.13140/RG.2.1.3263.3442, abstracts of Well Characterized Biotechnology Pharmaceutical (WCBP) Conference (Washington, DC, Jan, 2015).
8. M. Collin and, A. Olsen, *EMBO J.* 20 (15), 3046-3055 (2001).
9. M. Abe; *Polym. J.* 44, 269–277 (2012).
10. M. P. Campbell, L. Royle, C. M. Radcliffe, R. A. Dwek and, P. M. Rudd, *Bioinformatics*. 24, 1214–1216 (2008).
11. L. A. Gennaro and O. Salas-Solano, *Anal. Chem*. 80 (10), 3838-3845 (2008).
12. S. C. Bunz, E. Rapp, and C. Neususs, *Anal. Chem*. 85, 10218-10224 (2013).
13. P. M. Rudd, et al, *Nature*. 388, 205-207 (1997).
14. J. Fournier, *BioPharm International* 28 (10), 32-37 (2015). ◆