Analytical Biochemistry 442 (2013) 10–18

Contents lists available at ScienceDirect

Analytical Biochemistry

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

An automated robotic platform for rapid profiling oligosaccharide analysis of monoclonal antibodies directly from cell culture

Margaret Doherty [a,*], Jonathan Bones [a], Niaobh McLoughlin [a], Jayne E. Telford [a], Bryan Harmon [b], Michael R. DeFelippis [b], Pauline M. Rudd [a]

[a] National Institute of Bioprocessing Research and Training (NIBRT), Mount Merrion, Blackrock, Dublin 4, Ireland
[b] Bioproduct Research and Development, Lilly Research Laboratories, Eli Lilly, Indianapolis, IN 46285, USA

ARTICLE INFO

Article history:
Received 23 May 2013
Received in revised form 26 June 2013
Accepted 1 July 2013
Available online 16 July 2013

Keywords:
Monoclonal antibodies
Oligosaccharides
Automation
Glycomics
Ultra-performance liquid chromatography
Cell culture media

ABSTRACT

Oligosaccharides attached to Asn297 in each of the C_H2 domains of monoclonal antibodies play an important role in antibody effector functions by modulating the affinity of interaction with Fc receptors displayed on cells of the innate immune system. Rapid, detailed, and quantitative *N*-glycan analysis is required at all stages of bioprocess development to ensure the safety and efficacy of the therapeutic. The high sample numbers generated during quality by design (QbD) and process analytical technology (PAT) create a demand for high-performance, high-throughput analytical technologies for comprehensive oligosaccharide analysis. We have developed an automated 96-well plate-based sample preparation platform for high-throughput *N*-glycan analysis using a liquid handling robotic system. Complete process automation includes monoclonal antibody (mAb) purification directly from bioreactor media, glycan release, fluorescent labeling, purification, and subsequent ultra-performance liquid chromatography (UPLC) analysis. The entire sample preparation and commencement of analysis is achieved within a 5-h timeframe. The automated sample preparation platform can easily be interfaced with other downstream analytical technologies, including mass spectrometry (MS) and capillary electrophoresis (CE), for rapid characterization of oligosaccharides present on therapeutic antibodies.

Monoclonal antibodies (mAbs)[1] represent a large proportion of approved and in-pipeline biotherapeutics for use in oncology, treatment of autoimmune diseases, and the prevention of xenograft rejection [1]. The majority of approved therapeutic mAbs are of the immunoglobulin G1 (IgG1) isotype. IgG1 is composed of four polypeptide chains, two heavy and two lights chains, covalently linked by disulfide bonds [2]. A single highly conserved N-linked glycosylation site occurs at Asn297 in each C_H2 domain in the Fc region; however, glycosylation may also occur in the antigen binding region of the IgG molecule [3]. The C_H2 glycosylation can play an important role in the monoclonal antibody's effector functions by modulating the affinity of interaction with Fc receptors or C1q complement displayed on cells of the innate immune system [4,5]. However, the distributions of oligosaccharide structures present on mAbs can vary significantly depending on the bioprocess parameters used, including the cell line used for production [6], the amount of dissolved oxygen [7,8], the nutrients available [9], and the manufacturing mode (i.e., batch-fed or perfusion-based bioreactors) [10,11]. Regulatory authorities expect thorough characterization of the glycosylation profile and understanding of its relationship to the bioprocessing parameters. In certain cases, routine testing may be required to demonstrate that glycan structures are maintained within specific ranges in order to confirm batch-to-batch consistency and ensure product safety and efficacy [12].

The rapidly expanding mAb market has led to an increased demand for the development and implementation of high-throughput analytical technologies for the characterization of the glycosylation at all stages of bioprocess development and production. This demand is set to increase further, especially with the emergence of biosimilar products [13–15]. Current limitations in glycosylation analysis include the hands-on time required for sample preparation and analysis. There has been a committed effort to address this shortfall, and an integrated microfluidic chip for glycan profiling of mAbs with combined sample preparation and sample analysis time has been developed with a timeframe of just 10 min [16]. Another strategy proposed by Ruhaak and coworkers

* Corresponding author. Fax: +353 1 215 8116.
E-mail address: margaret.doherty@nibrt.ie (M. Doherty).

[1] *Abbreviations used:* mAb, monoclonal antibody; IgG, immunoglobulin G; CGE–LIF, capillary gel electrophoresis with laser-induced fluorescence detection; PNGaseF, peptide *N*-glycosidase F; APTS, 8-aminopyrene-1,3,6-trisulfonic acid; ESI, electrospray ionization; HILIC, hydrophobic interaction liquid chromatography; SPE, solid phase extraction; UPLC, ultra-performance liquid chromatography; HTS, high-throughput screening; CE, capillary electrophoresis; MS, mass spectrometry; HPLC, high-performance liquid chromatography; 2-AB, 2-aminobenzamide; PBS, phosphate-buffered saline solution; GU, glucose unit.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.07.005

performs *N*-glycan analysis by capillary gel electrophoresis with laser-induced fluorescence detection (CGE–LIF) employing a multicapillary format using a DNA sequencer following *N*-glycan release by peptide *N*-glycosidase F (PNGaseF) [17]. Optimization of the derivatization reaction was accomplished by varying the concentrations of the APTS (8-aminopyrene-1,3,6-trisulfonic acid) labeling reagent as well as the type and amount of reducing agent. Attention also has been focused on increasing the speed of the derivatization reaction, and citric acid has proved to be a more powerful catalyst than the conventionally used acetic acid [18]. More recently, an automated method was developed based on glycopeptide analysis consisting of two steps. First IgG was purified out of the supernatant, followed by a tryptic digest. The glycopeptides were subsequently purified from the tryptic digest and analyzed [19].

Although viable methods for particular applications, these methods also have several drawbacks; the preparation of the APTS-labeled *N*-glycans prior to multiplexed CGE–LIF requires two overnight incubations, and there is also the requirement for synthetic standards for each glycan analyzed [17]. Using the mAb chip, the method requires optimization for each sample analyzed, and the potential for cross-contamination could be a concern because the same enzyme is used repeatedly and could have major implications when performing analysis in biopharmaceutical processes. In positive mode electrospray ionization (ESI), this microfluidic chip method uses the glucosylamine intermediate glycan to achieve improved sensitivity [16]. However, these intermediates are relatively unstable even under mild acidic conditions. Furthermore, both anomeric forms of the free reducing end glycan are present. To obtain accurate quantitation, it is preferable to use a single glycan standard rather than a potentially unstable glucosylamine intermediate. In addition, glycans may exist as both protonated and sodiated adducts using positive mode ESI, further complicating the characterization process.

Previously, Royle and coworkers [20] described a 96-well plate analytical platform that included sample immobilization, enzymatic *N*-glycan release, fluorescent labeling, and quantitative hydrophobic interaction liquid chromatography (HILIC)–fluorescence-based profiling. In the current article, we describe further development, modification, and automation of the entire procedure using a robotic platform that is capable of sample analysis directly from a bioreactor. mAbs were purified and captured from cell culture media on protein A resin in a 96-well plate format. After washing, the glycans were removed enzymatically via *N*-glycanase while the mAbs were still immobilized on the protein A. The released glycans were fluorescently labeled, and excess underivatized material was removed via solid phase extraction (SPE) using a synthetic polyamide stationary phase packed in 96-well plates. Furthermore, method transfer to ultra-performance liquid chromatography (UPLC) using a 1.7-μm HILIC phase allowed for a 12-fold reduction in analysis time, from 180 min to just 15 min. Using the automated platform, the time taken to achieve this overall process is approximately 5 h as opposed to a number of days for classical analysis. The automated platform is directly suited to the biopharmaceutical industry, where high-throughput methods are required for development activities such as high-throughput screening (HTS) of clones for cell line selection and design of experiment (DOE) studies supporting quality by design (QbD) approaches to understanding the impact of process parameters on product quality. A particular advantage of this automated method is its amenability to multiplexing with other analytical devices such as direct coupling to UPLC, capillary electrophoresis (CE), and mass spectrometry (MS). This high-throughput strategy bridges the interface among bioprocessing operations, sample preparation, and analysis, allowing for rapid data generation for the processes under optimization.

Materials and methods

Chemicals

Reagent water used throughout this study was obtained from a MilliQ Gradient A10 Elix system (Millipore, Bedford, MA, USA) and was 18.2 MΩ or greater with a total organic carbon (TOC) content less than 5 parts per billion (ppb). All solvents used were high-performance liquid chromatography (HPLC) gradient grade and received from Sigma–Aldrich (Dublin, Ireland). Chemicals were of the highest possible grade and were obtained from Aldrich. Protein A plates were purchased from Pierce (Rockford, IL, USA). Clarified Chinese hamster ovary (CHO) cell culture supernatant was provided by Eli Lilly (Indianapolis, IN, USA). PNGaseF was purchased from Prozyme (Hayward, CA, USA). 2-Aminobenzamide (2-AB) *N*-glycan labeling was performed using the 2-AB LudgerTag labeling kit (Ludger, Oxfordshire, UK).

Liquid handling parameter programming

All methods and scripts were programmed using Vector, the Hamilton MicroLab STAR software (Reno, NV, USA). A graphical user interface facilitated the setup of both the glycan release and sample preparation method and the deck layout of the platform. All reagents were loaded into the appropriate reservoirs, and interactive functionality prompted the user to conduct visual checks on the platform before progressing with the liquid handling. All errors were detected by the automatic error response device, allowing the user to respond appropriately. Liquid handling steps were performed employing the 8-channel head, whereas sample transfer was accomplished by operating the 96-channel head. Disposable tips were used at all times. Test methods were run in simulation mode before initiating the actual run to maximize productivity and minimize any potential errors.

Automated glycan preparation on a robotic platform

Protein A plates (Pierce) were equilibrated with 200 μl of phosphate-buffered saline solution (PBS) and vacuumed to waste. Briefly, 200 μl of clarified bioreactor cell culture supernatants was applied to the protein A plate and incubated at room temperature for 10 min to enable IgG1 capture. The plate was washed with 200 μl of PBS twice to remove unbound contaminants and vacuumed to waste. *N*-Glycans were released from the IgG while immobilized to the protein A resin with the addition of 50 μl of 0.25 U/ml PNGaseF (Prozyme, San Leandro, CA, USA) in 20 mM $NaHCO_3$ (pH 7.2). The samples were moved to the integrated 37 °C incubation via the iSWAP robotic plate handler using the CORE gripper tools and incubated for 60 min. Glycans were eluted with 5 × 100 μl of water and concentrated in a centrifugal evaporator (Thermo, Basingstoke, Hampshire, UK). The eluted glycans were labeled with 2-AB using the LudgerTag 2-AB kit.

Glycan labeling and cleanup

Labeling was performed via reductive amination using the LudgerTag 2-AB labeling reagent kit prepared according to the manufacturer's instructions. In all instances, 5 μl of labeling reagent was added to each dried glycan sample in a deep well plate on the robotic platform, and the plate was subsequently transferred to the integrated incubator and incubated at 65 °C for 2 h. All samples were prepared in triplicate for each experiment. Postlabeling sample cleanup was accomplished by conditioning a 96-well SPE plate packed with 25 mg of the synthetic polyamide polymer DPA-6S material (Sigma–Aldrich, Poole, Dorset, UK) with