Cell Culture Optimization Using an Agilent Bio-Monolith Protein A Column and LC/MS

Application Note

Biopharmaceuticals and Biosimilars

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Abstract

This application note describes how the Agilent Bio-Monolith Protein A column was applied to the optimization of Chinese hamster ovary cell-culture conditions to produce a recombinant monoclonal antibody with desired structural characteristics, focusing on glycosylation. The workflow demonstrates the versatility and wide applicability of the column in biopharmaceutical and biosimilar development.

Agilent Technologies

Introduction

Monoclonal antibodies (mAbs) represent the fastest growing class of therapeutics. Currently, more than 30 monoclonal antibodies are marketed and several have acquired major importance in the recent past [1]. The knowledge that the top-selling mAbs are, or will become, open to the market in the coming years has resulted in an explosion of biosimilar activities. The first two monoclonal antibody biosimilars were approved in 2013, and both contained the same active substance, infliximab [2].

An earlier Agilent application note [3] described the use of the Agilent Bio-Monolith Protein A column for the selection of trastuzumab-producing Chinese hamster ovary (CHO) clones in the context of a herceptin biomarker development program. Herceptin, which is being used in the treatment of HER2-positive breast cancer [4], will be open to the European market in 2014 and the US market in 2018. The Bio-Monolith Protein A successfully guided the clone selection process based on mAb titer and structural characteristics. It was demonstrated that the herceptin originators and clone-derived trastuzumab biosimilar displayed the same amino acid sequence. In addition, the same type of complex N-glycans were observed on the heavy chain of the mAbs. However, in comparison to the Herceptin originators, the glycosylation profile differed quantitatively, with an overrepresentation of G0F species. Since glycosylation is a critical quality attribute [1], this under-galactosylation does not make the product similar enough to be considered by regulatory authorities as a Herceptin biosimilar.

This application note describes the use of the Agilent Bio-Monolith Protein A column in guiding cell-culture optimization to bring the glycosylation profile of the biosimilar to within the originator specifications. To do this, the CHO cell culture was tuned by feeding uridine, galactose, and manganese chloride at different concentrations [5]. These are the substrates and activator of the galactosyltransferase responsible for donating galactose residues to G0F and G1F acceptors. Trastuzumab was harvested from the culture medium using the Protein A column. The mAb titer was determined making use of a calibration curve generated with the Herceptin originator, and structural characteristics were revealed by mass spectrometry and compared to the originator molecule.

Experimental

Materials

Acetonitrile, water, and isopropanol were obtained from Biosolve (Valkenswaard, The Netherlands). Citric acid, formic acid, NaH_2PO_4, Na_2HPO_4, and *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). Trastuzumab biosimilar CHO cell-culture supernatants were obtained from a local biotechnology company.

Sample preparation

Herceptin stock solution present at 21 mg/mL was diluted in mobile phase A for construction of the calibration curves. Cell supernatants were diluted 1:1 in 50 mM Na_2HPO_4. Supernatants were centrifuged at 5.000 g for 5 minutes prior to injection. Collected fractions were reduced at room temperature for 1 hour by adding 10 mM TCEP.

Instrumentation

Bio-Monolith Protein A measurements were performed on:

- Agilent 1100 Series Quaternary Pump (G1311A)
- Agilent 1100 Series Autosampler (G1313A)
- Agilent 1100 Series Diode Array Detector (G1315A)
- Agilent 1200 Infinity Series Analytical-scale Fraction Collector (G1364C)

LC/MS measurements were performed on:

Agilent 1290 Infinity Binary LC equipped with:

- Agilent 1290 Infinity Binary Pump (G4220B)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF with Agilent Jet Stream LC/MS (G6540A)

Software

- Agilent Technologies OpenLAB CDS ChemStation revision C01.05 (35)
- Agilent Technologies MassHunter for instrument control (B05.01)
- Agilent Technologies MassHunter for data analysis (B06.00)
- Agilent Technologies BioConfirm software for MassHunter (B06.00)

Conditions, Bio-Monolith Protein A column

Column:	Agilent Bio-Monolith Protein A (p/n 5069-3639)
Mobile phase:	A) 50 mM phosphate, pH 7.4 B) 100 mM citric acid, pH 2.8
Gradient:	Time (min) % B 0 to 0.5 0 (binding) 0.6 to 1.7 100 (elution) 1.8 to 3.5 0 (regeneration)
Flow rate:	1 mL/min
Injection volume:	50 µL
Detection:	UV at 280 nm
Fraction collection:	Time-based

Conditions, LC/MS

Cartridge:	Online desalting cartridge, 2.1 × 10 mm
Mobile phase:	A, 0.1% formic acid in water (v:v); B, 0.1% formic acid in acetonitrile (v:v)
Flow rate:	400 µL/min
Injection volume:	Variable (corresponding to a protein amount of 1 µg)
Needle wash solvent:	60% acetonitrile, 35% water, 5% isopropanol
Autosampler temperature:	7 °C
Gradient:	Time (min) % B 0 5 0.5 5 2 80.0 3 80.0 3.10 5 5 5
Q-TOF source:	Agilent Jet Stream, positive ionization mode
Drying gas temperature:	300 °C
Drying gas flow rate:	8 L/min
Nebulizer pressure:	35 psig
Sheath gas temperature:	350 °C
Sheath gas flow rate:	11 L/min
Nozzle voltage:	1,000 V
Capillary voltage:	3,500 V
Fragmentor voltage:	200 V
Q-TOF detection:	Mass range 3,200 amu
Data acquisition range:	500 to 3,200 m/z
High-resolution mode (4 GHz)	
Data acquisition rate:	1 spectrum per s
Profile acquisition	
Diverter valve:	Time (min) Flow to 0 waste 1 MS 3.5 waste

Results and Discussion

Figure 1 shows an overlay of the Protein A chromatograms of the supernatant of a selected trastuzumab-producing clone (clone 9) grown at different concentrations of galactose, uridine, and manganese chloride (denoted as 0x, 4x, 8x, 16x, 24x). The unbound material eluted in the flow-through while the mAb was retained and released after lowering the pH. The mAb fractions were collected from the Protein A column and measured by high-resolution mass spectrometry following disulfide bond reduction, giving rise to the light and heavy chains. This strategy allowed verification of the amino acid sequence and revealed the glycosylation pattern. To reduce the mAb directly in the collection vial containing acidic buffer, TCEP was chosen due to its reducing capacities over a broad pH range. Reduced fractions were delivered to the MS system following online desalting. Figures 2 and 3 show the deconvoluted heavy chain spectra of four different Herceptin-originator production batches and the trastuzumab biosimilar obtained by growing the CHO clone at different galactose, uridine, and manganese chloride concentrations.

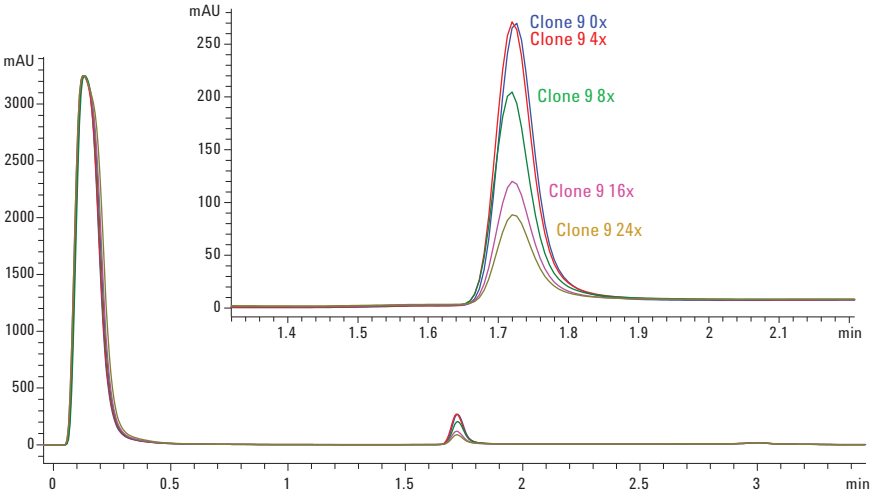

Figure 1. Overlaid UV 280 nm Agilent Bio-Monolith Protein A chromatograms of a trastuzumab-producing CHO clone grown under different cell culture conditions, with increasing concentrations of galactose, uridine, and manganese chloride.

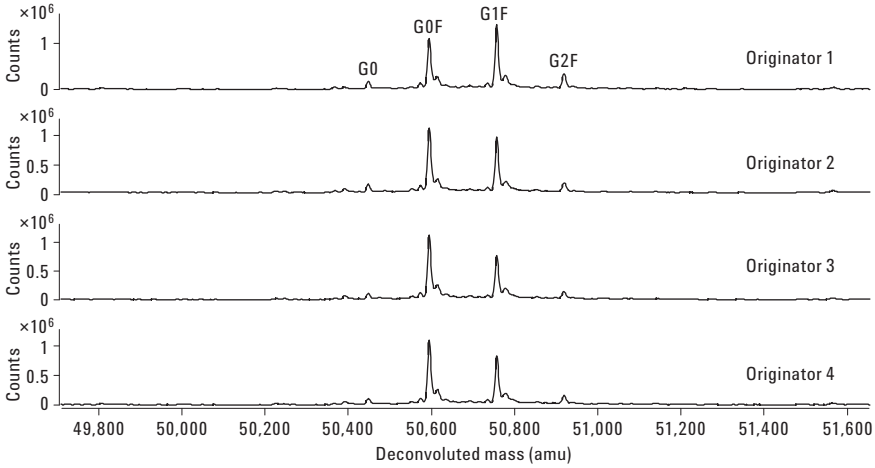

Figure 2. Deconvoluted heavy chain spectra of four different production batches of the Herceptin originator. The abbreviations G0, G0F, G1, G2F refer to the N-glycans attached to the mAb backbone.

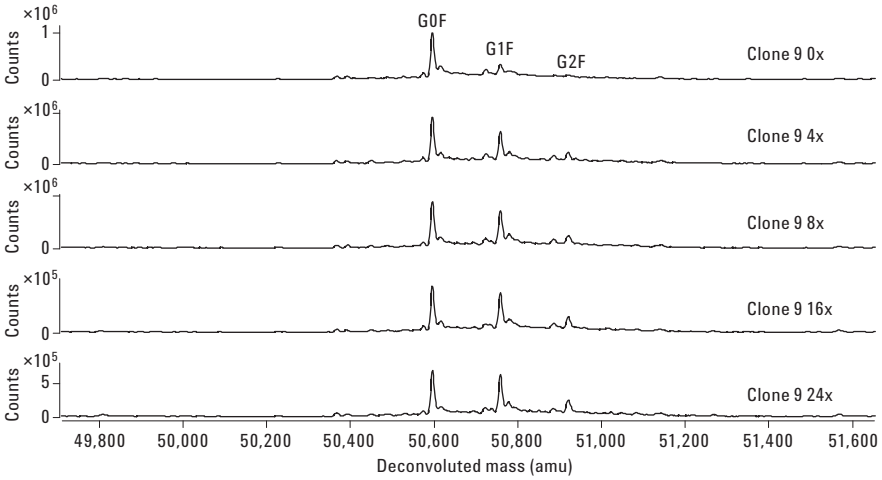

Figure 3. Deconvoluted heavy chain spectra of trastuzumab biosimilar obtained by growing the CHO clone at different galactose, uridine, and manganese chloride concentrations.

Table 1 displays the relative intensity of the main glycoforms in the different samples, as well as the average relative intensity and standard deviation observed in the different Herceptin production batches, thereby illustrating lot-to-lot variation. It was observed that the ratio G1F:G0F increased with increasing concentration of galactose, uridine, and manganese chloride. From these results, it was concluded that conditions could be found that matched the glycosylation of the biosimilar within the originator specification.

Table 1. Relative intensity of the main glycoforms in four Herceptin-originator production batches and in the trastuzumab biosimilar obtained by growing the CHO clone at different galactose, uridine, and manganese chloride concentrations.

Glycoform	Originator 1	Originator 2	Originator 3	Originator 4	Originator avg/std dev
% Man 5	1.6	1.6	1.3	1.1	1.4 ± 0.2
% G0F-GlcNAc	1.5	2.7	3.3	2.4	2.5 ± 0.7
% G0	5.7	5.9	5.0	4.9	5.4 ± 0.5
% G0F	**35.2**	**44.8**	**50.5**	**48.2**	**44.7 ± 6.7**
% G1F	**45.2**	**38.4**	**34.0**	**36.8**	**38.6 ± 4.8**
% G2F	10.7	6.6	5.9	6.7	7.5 ± 2.2

Glycoform	Clone 9 0x	Clone 9 4x	Clone 9 8x	Clone 9 16x	Clone 9 24x
% Man 5	4.9	3.9	3.3	3.7	4.1
% G0F-GlcNAc	4.7	2.7	3.1	2.4	2.6
% G0	2.4	3.3	2.6	2.3	2.2
% G0F	**64.1**	**47.8**	**44.4**	**41.8**	**40.8**
% G1F	**19.0**	**31.8**	**35.8**	**36.7**	**36.9**
% G2F	5.0	10.5	10.8	13.0	13.3

Next to the structural characteristics, the second important criterion in selecting the optimal growth media conditions is based on the mAb titer. From the chromatograms shown in Figure 1, a distinction can already be made between low- and high-mAb-producing conditions. Absolute mAb concentrations were determined by linking the peak areas to an external calibration curve. The calibration curve constructed by diluting a Herceptin originator is shown in Figure 4. Good linearity was obtained between 0.02 and 2 mg/mL, which is the typical mAb titer range in CHO cells.

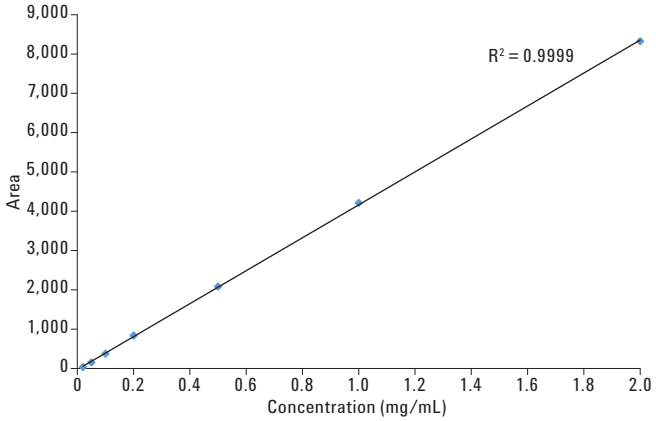

Figure 4. Herceptin Agilent Bio-Monolith Protein A calibration curve, 0.02 to 2 mg/mL.

Obtained mAb titers are reported in Table 2. A drop in antibody concentration was noticed with increasing galactose, uridine, and manganese chloride concentrations.

From these findings, clear decisions could be made for further biosimilar development, that is, a compromise was reached between mAb titer and structural characteristics at intermediate galactose, uridine, and manganese-chloride feeding concentrations (4x and 8x).

Table 2. Absolute mAb concentrations determined in the trastuzumab CHO clone grown at different concentrations of galactose, uridine, and manganese chloride.

CHO Clone 9	Concentration (mg/mL)
0x	0.458
4x	0.470
8x	0.350
16x	0.202
24x	0.142

Conclusions

The Agilent Bio-Monolith Protein A column was successfully applied in the optimization of trastuzumab-producing CHO cell-culture conditions. The glycosylation profile of a trastuzumab biosimilar in development was brought within the originator specifications by tuning the cell culture conditions by supplementing the growth medium with galactose, uridine, and manganese chloride.

References

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