Biotechnol Lett (2015) 37:1371–1377
DOI 10.1007/s10529-015-1815-3

ORIGINAL RESEARCH PAPER

# Monitoring sialylation levels of Fc-fusion protein using size-exclusion chromatography as a process analytical technology tool

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Received: 10 December 2014 / Accepted: 12 March 2015 / Published online: 18 March 2015


**Abstract**
*Objective* To develop a rapid process analytical technology (PAT) tool that can measure sialic acid content of an Fc-fusion protein from cell culture samples.
*Results* A statistical significant correlation between the sialic acid content and size-exclusion chromatography (SEC)–HPLC retention time of an Fc-fusion protein was observed when analyzing the titer of the samples. Using linear fitting analysis, the data fit the model well with $R^2 = 0.985$. Based on the SDS-PAGE and oligosaccharide analysis, we speculate that the amounts of the glycans could expand the structure of the Fc-fusion protein. This was manifested by the SEC–HPLC method in which proteins were separated based on its molecular size. In order to development a robust PAT method, an internal standard was used to improve the precision of the method by reducing systematic errors. We found the change of SEC retention time (*delta t*) and sialic acid content were highly correlated ($R^2 = 0.992$). This method was further validated by a 1500 l production process.
*Conclusion* SEC–HPLC is a promising PAT tool to monitor the sialic acid content of Fc-fusion protein during biomanufacturing or medium optimization processes.

**Keywords** Cell culture · Critical quality attributes · Critical quality parameters · Fc-fusion protein · Glycosylation · Process analytical technology · SEC–HPLC · Sialic acid

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## Introduction

Many regulatory authorities encourage implementation of the quality by design (QbD) and process analytical technology (PAT) for the process development of new drugs (Rathore and Winkle 2009). The term PAT was introduced as an initiative to bring an improved understanding of pharmaceutical manufacturing processes to increase the quality of the products (Glassey et al. 2011). PAT plays an important role in evaluating critical quality attributes (CQAs), as well as monitoring the critical process parameters (CPPs) that affect the CQAs (Paris et al. 2006; Rathore et al. 2010). The PAT principles have been widely adopted in the chemical and pharmaceutical industry. However, challenges remain in the development PAT for biomanufacturing processes, because of the complexity of biopharmaceuticals and their production systems (Kuribayashi et al. 2012).

Approximately 70 % of therapeutic proteins are glycoproteins (Sethuraman and Stadheim 2006). The sialic acid content of glycoprotein is commonly considered as a CQA due to its pivotal role in plasma half-life of protein therapeutics (Jedrzejewski et al. 2013; Rathore et al. 2010). In production of therapeutic glycoproteins, many parameters can affect the sialic acid content of recombinant glycoproteins (Hossler et al. 2009; Ngantung et al. 2006). Process monitoring and optimization, therefore, are required to ensure consistent and optimal protein sialylation. These tasks can be accomplished if a rapid PAT method that can measure sialic acid content of cell culture samples is available. During the past few decades, many colorimetric, chromatographic, enzymatic and fluorescence methods have been developed for quantifying the concentration of sialic acid (Markely et al. 2010). These methods, though powerful, still require chromatographic purification and titer detection prior to sialic acid content analysis, which are the major bottlenecks for using them as PAT methods.

In our current study, a rapid process development analytic method using SEC–HPLC was proposed. This method can be successfully applied as a PAT tool for the analysis of the sialic acid content and glycosylation of Fc-fusion protein during the cell culture process.

## Materials and methods

### Cell line and cell culture

The stable cell line used in this study was a gift from Hisun Biopharmaceutical Co., Ltd. (China). The cell line was derived from a CHO-K1 cell line, and was transfected with a proprietary recombinant vector containing both the glutamine synthetase (GS) and Fc-fusion protein genes. The cells have been tested for mycoplasms. The Mycoplasma-free cells were grown in shake-flasks at 37 °C with shaking at 120 rpm.

### Fc-fusion protein purification

Product quality assays were performed on samples purified by Protein A chromatography using Mab Select SuRe resin (GE Healthcare, UK). The column was equilibrated in a Tris/NaCl chloride buffer at pH 7.4. Bioreactor supernatant samples were loaded directly onto the column, washed with Tris/NaCl buffer at pH 7.4, eluted in citrate buffer at pH 3.6, and neutralized using a Tris/HCl buffer (pH 8).

### Total sialic acid content assay

Total sialic acid content was measured using the resorcinol method (Svennerholm 1957). In brief, purified samples was mixed with resorcinol reagent, the mixture was then heated in a water bath at 100 °C for 30 min. An extraction solution (*N*-butyl acetate/1-butanol, 4:1 v/v) was then added after the mixture had cooled to room temperature. The mixture was shaken vigorously and kept still for 10 min to allow the separation of organic layer from the aqueous layer. The absorption of the organic layer was read at 580 nm. A blank organic solution was used as the control. The sialic acid content of the glycoprotein is determined against a calibration curve created with the standard sialic acid analyzed under the same condition.

### Size-exclusion chromatography (SEC)

Fc-fusion protein titer was analyzed by HPLC using a size-exclusion analytical column (TSKgel G3000SWXL; 7.8 × 300 mm, Tosoh Bioscience LLC, Japan) at 25 °C. Protein (20 μg) was loaded onto the column and eluted isocratically with 20 mM sodium phosphate/200 mM NaCl, pH 7.4, at 0.5 ml/min 30 min. The eluate was nominated at 280 nm. For the modified SEC method, a mixture of equal volume of 4 mg albumin/ml (Hisun Pharmaceutical, China) and sample was loaded onto the column.

### SDS-PAGE

SDS-PAGE was performed using a vertical Bio-Rad Mini-Protean 3 gel electrophoresis system (Bio-Rad, USA) under non-reducing conditions using Mini-Protean TGX gels (Bio-Rad). 5 μl protein sample (2 mg protein/ml) was added to each well after incubating (95 °C, 5 min) with 5 μl of sample buffer (Tris/HCl, glycerol, SDS, Bromophenol Blue). A broad range molecular weight marker was used (Bio-Rad). The gels were stained with Coomassie Blue.

### Protein glycosylation analysis

The *N*-linked glycans of Fc-fusion protein were analyzed by an NP-HPLC method (Royle et al. 2008). In short, *N*-linked glycans were released from purified samples by overnight incubation with peptide-*N*-glycosidase F (Prozyme, USA). The released glycans were recovered and labeled with the fluorophore 2-aminobenzamide (2-AB; Prozyme). A cleaning cartridge (Prozyme) was used to remove excess 2-AB. Glycans were analyzed on a TSKgel Amide-80 (150 × 4.6 mm) column (Tosoh Bioscience, Japan) equilibrated in acetonitrile and eluted with a gradient of 125 mM ammonium formate, pH 4.4 on an HPLC with a multiple-fluorescence detector (excitation at 330 nm, emission at 420 nm). The amount of each structure such as A1, A1F, A2 etc., was expressed as the percentage of total peak area.

## Results and discussion

### Correlation of SEC–HPLC retention time and sialic acid content of Fc-fusion protein

The impact of two media components, the concentration of uridine and galactose (Sigma, USA), on the sialylation of the Fc-fusion protein was studied. A 2-factor, 3 level full factorial design with two additional replicates on the center point (Table 1) was conducted using the Design Expert 8 software (State-Ease Inc., USA). The sialic acid content is shown in Fig. 1. Interestingly, deviations of the retention times of samples were observed when the titers of the protein were measured using the SEC–HPLC method. The retention time showed a statistical significant correlation ($R^2 = 0.985$) to the sialic acid content of the Fc-fusion protein measured by the traditional method (Fig. 2). Figure 2 shows that the sialic acid content and SEC–HPLC retention time of the Fc-fusion protein were negatively correlated. This result indicated that the SEC–HPLC method can potentially used as a PAT to precisely analyse the sialic acid content of an Fc-fusion protein during the titer evaluation of the protein. Using the SEC–HPLC retention time as an indicator for sialic acid content of an Fc-fusion protein has a significant advantage of convenience, since traditional sialic acid content measurement requires sample purification and titer measurement that could take up to 12 h.

In order to gain insight into the correlation between the decline of sialic acid content and the increase of SEC–HPLC retention time, a series of studies were conducted.

**Table 1** Design of experiments for the study of protein sialylation

| Run | Uridine (mM) | Galactose (mM) |
|---|---|---|
| 1 | 1 | 10 |
| 2 | 1 | 15 |
| 3 | 1 | 20 |
| 4 | 5 | 10 |
| 5 | 5 | 15 |
| 6 | 5 | 15 |
| 7 | 5 | 15 |
| 8 | 5 | 20 |
| 9 | 9 | 10 |
| 10 | 9 | 15 |
| 11 | 9 | 20 |

### The potential relationship between sialic acid content and SEC retention time

Size-exclusion chromatography separates molecules according to their molecular size or weight based on the theory that large molecules elute earlier than smaller molecules due to its incapability to penetrate

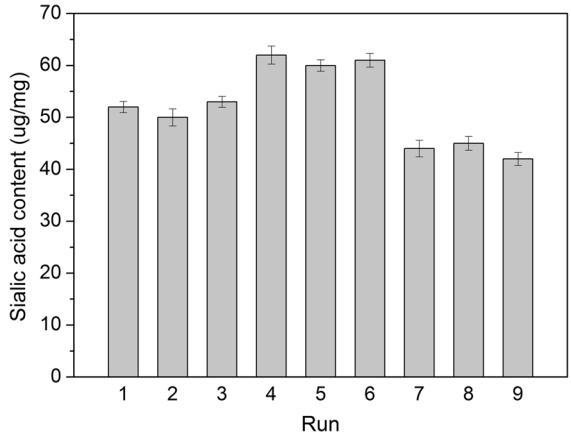



**Fig. 1** Sialic acid content of DOE samples. Samples were taken at the end of the cultures and analyzed using resorcine method after protein purification. Values of each parameter are reported as average ± standard deviation (n = 3)

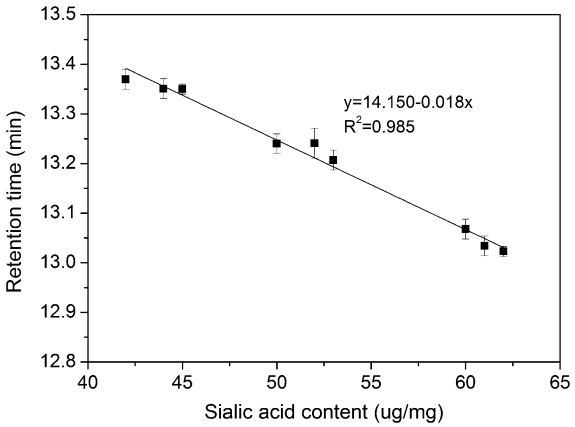



**Fig. 2** Correlations between SEC retention time and sialic acid content. Where, $x$ is the sialic acid content and $y$ is the SEC–HPLC retention time. The equation was obtained using linear fit analysis. Values of each parameter are reported as average ± standard deviation (n = 3)

the pores of the packing material (Hong et al. 2012). Therefore, we examined the molecular size of samples with different levels of sialic acid content using SDS-PAGE.

We chose the samples of runs 3, 6 and 9, since these samples showed the most significantly alternation in the sialic acid content (Fig. 1). Figure 3 shows that there was no significant difference of the molecular size in the three samples. One possible reason is that the change of molecular size is not detectable by SDS-PAGE. This result indicated that the amount of glycans on a protein must be sufficient to be detected by the PAT method. In our study, the Fc-fusion protein consists of 934 amino acids with an apparent molecular weight of 140 kDa. The protein is highly glycosylated containing six *N*-glycans and 26 *O*-glycans, a number that is higher than antibodies or other recombinant proteins. Therefore, it was reasonable to imagine that the amount of the glycans could expand the spatial structure of the Fc-fusion protein (Petrescu et al. 2004).

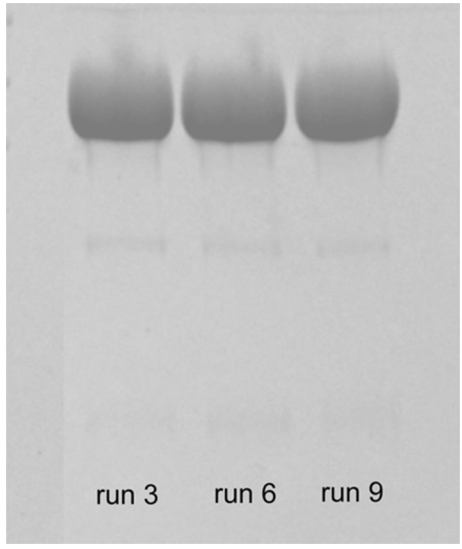



**Fig. 3** SDS-PAGE analysis of Fc-fusion protein secreted at different run

Due to the different sialic acid contents of runs 3, 6 and 9, it was reasonable to hypothesize that the glycan profiles were significantly different (Butler 2006). To evaluate this possibility, we examined the glycans of these samples (Table 2). Oligosaccharide analysis showed that the total sialylated glycans dropped from 41.4 to 28.8 %, when the retention time increased from 13.04 to 13.21 min; and further decreased to 19 % where the retention time increased to 13.43 min (Table 3). The decrease of galactosylated glycans with run 3 and run 9 was 13.1 and 24.6 % respectively, compared to run 6. Therefore, both the galactosylation levels and sialylation levels were decreased with the increasing of the SEC retention time. Hence, the PAT assay used in this work was also a useful predictor of the glycosylation of the Fc-fusion protein.

## Modeling of SEC–HPLC retention time and sialic acid content of Fc-fusion protein

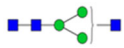
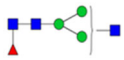
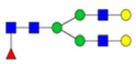
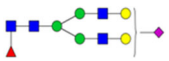
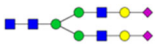
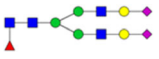
Systematic errors of retention time may occur when using different HPLC systems, running at different time, changing HPLC columns or decreased column performance, etc. In order to eliminate these systematic errors during analysis, albumin was used as an internal standard during the retention time measurement. Data from HPLC chromatograph was analyzed using the follow equation:

$$\Delta t = t_2 - t_1 \quad (1)$$

where $\Delta t$ is the delta time, $t_2$ is the retention time of albumin, while $t_1$ is the retention time of Fc-fusion protein.

Using this method, we analyzed 100 samples from medium optimization experiments. All data shown in Fig. 4 was utilized to create a linear equation using Origin 8 software (OriginLab, USA) with $R^2 = 0.992$.

**Table 2** The glycosylations of *N*-linked glycans under different culture conditions

| Structure[a] | Proposed structure[b] | Percentage of glycans (%) | | |
|---|---|---|---|---|
| | | Run 3 | Run 6 | Run 9 |
|  | A1 | 1 ± 0.1 | 0.4 ± 0.1 | 1.2 ± 0.2 |
|  | A1F | 0.7 ± 0.2 | 0.3 ± 0.1 | 0.8 ± 0.1 |
|  | A2 | 7.0 ± 0.1 | 3.2 ± 0.3 | 11.6 ± 0.7 |
|  | A2F | 24.1 ± 2.1 | 16.7 ± 1.3 | 30 ± 1 |
|  | Man5 | 3.3 ± 0.4 | 2.5 ± 0.2 | 3.8 ± 0.4 |
|  | A2G1 | 4.2 ± 0.1 | 2.5 ± 0.3 | 4.9 ± 0.2 |
|  | A2G1F | 12.2 ± 1.3 | 12.8 ± 0.2 | 12.2 ± 0.6 |
|  | A2G2 | 6.6 ± 0.3 | 6.7 ± 0.2 | 5.7 ± 0.3 |
|  | A2G2F | 11 ± 1.1 | 12.5 ± 0.6 | 9.5 ± 0.3 |
|  | A2G2S1 | 8.1 ± 0.7 | 13 ± 0.3 | 5 ± 0.4 |
|  | A2G2S1F | 14.7 ± 0.6 | 19 ± 2 | 9.1 ± 1.2 |
|  | A2G2S2 | 1.7 ± 0.2 | 3.2 ± 0.2 | 1.4 ± 0.1 |
|  | A2G2S2F | 4.3 ± 0.3 | 6.2 ± 0.2 | 3.5 ± 0.2 |

Data represent means of three experiments with the standard deviations

[a] Assignment of symbols: blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose; red triangle, fucose. Glycan structures are drawn according to the symbol nomenclature defined by the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml)

[b] *A* antennary, *G* galactose, *S* sialic acid, *F* fucose, *Man* mannose

**Table 3** The relative percentage of *N*-linked glycans under different culture conditions

| Run | Total galactosylated glycans (%) | Total sialylated glycans (%) | Fucosylated (%) |
|---|---|---|---|
| 3 | 62.8 ± 1.4 | 28.8 ± 1.7 | 67.7 ± 1.1 |
| 6 | 75.9 ± 1.6 | 41.4 ± 1.2 | 68.2 ± 1.4 |
| 9 | 51.3 ± 1.7 | 19.0 ± 1.4 | 66.0 ± 1 |

The proportion of glycans was estimated using the relative peak area from Table 2

## Verification of the mathematical model

In order to test the applicability of our PAT method, an additional experiment was performed using SEC–HPLC as a PAT tool to monitor the sialic acid content of the Fc-fusion protein during a fed-batch culture process on an 1500 l industrialized production scale. Figure 5 shows the comparison between the predicted sialic acid content calculated by the model and the sialic acid content measured by the resorcine method during the Fc-fusion protein production. There was no significant difference between the results obtained by our model and those obtained by the resorcine method. This result shows that it is feasible to determine the sialic acid content based on the SEC retention time as determined by the SEC–HPLC analysis. In summary, implementation of a PAT based control scheme for a protein production process is feasible.

The traditional methods for quantifying the concentration of sialic acid, though powerful, still require chromatographic purification and titer detection prior to sialic acid content analysis, which are major bottlenecks used as PAT methods. Some high-throughput methods (HTMs) for sialic acid assay have

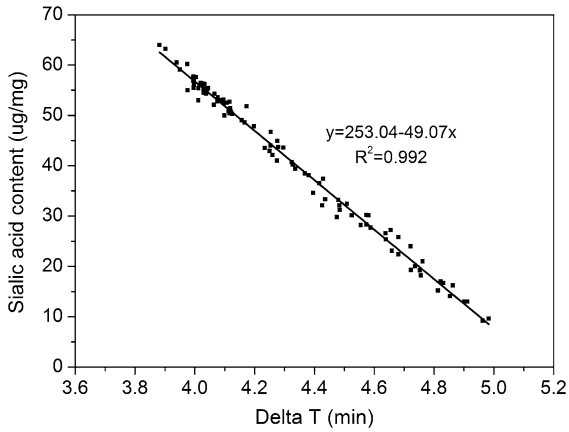



**Fig. 4** Mathematical model of sialic acid content and SEC delta time. The equation was obtained using linear fitting analysis. Where, *x* is the delta time, which was calculated by the SEC retention time of Fc-fusion protein and albumin. *y* is the sialic acid content of different samples, which was analysed using the resorcine method

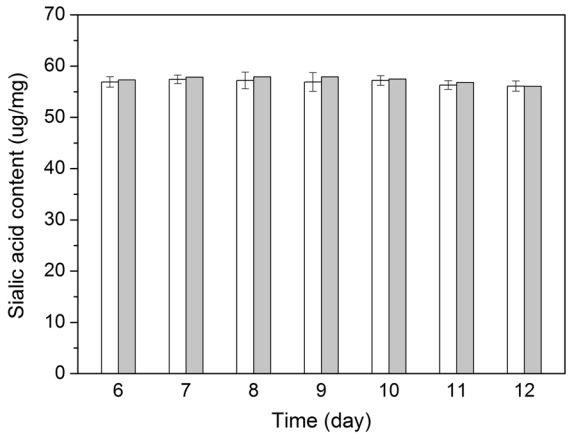



**Fig. 5** Comparison between the predicted (*shaded blocks*) and actual (*square blocks*) sialic acid content of the Fc-fusion protein. Samples were taken daily from the bioreactor and analyzed by the mathematical model and resorcine method respectively. The *error bars* showed the standard deviations of three repeats

been developed (Markely et al. 2010; Park et al. 2010). These methods can rapidly analyze the sialylation of recombinant proteins in many samples in parallel without protein purification. However, only the overall sialylation of all the proteins in a culture sample could be measured. Compared to these methods, there are still two limitations about this PAT method. First, the protein used for this method must contain a large amount of glycans. Proteins with insufficient glycans will not be detectable for different levels of sialylation. Second, the PAT method takes 30 min to analyze a sample, which was slower than those HTMs (15 min). However, the PAT method can be used to specifically measure the sialylation of the product (μg sialic acid per mg product) instead of overall sialylation. In addition, the PAT method provides a direct method for quantifying sialic acid, without introducing any organic solution or fluorescence agent, thereby reducing the assay complexity, cost, and time.

## Conclusion

An HPLC system has been developed that quickly evaluates the sialic acid content of an Fc-fusion protein. This method not only can be used as a PAT tool to monitor the sialic acid content and glycosylation of an Fc-fusion protein during the manufacturing process, it can also be used as a high-throughput technology for analyzing the titer and sialic content of an Fc-fusion protein simultaneously during process development. Further studies need to investigate the universality of this technology to other proteins with different degrees of glycosylation. Nevertheless, our study has shown the feasibility and significance of using SEC–HPLC as a PAT for sialic acid determination of an Fc-fusion proteins in the cultivation process.

**Acknowledgments** This work was supported by the National Natural Science Foundation of China (Nos. 21106045, 21206040, 21406066), the National High Technology Research and Development Program of China (863 Program) (No. 2012AA02A303), the National Science and Technology Major Project (No. 2013ZX10004003-003-003), the Fundamental Research Funds for the Central Universities (WF1214035).

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