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Heterogeneous glycoform separation by process chromatography: I Monomer purification and characterization

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ABSTRACT

Fc fusion proteins with high and low sialylation were purified and separated by preparative ion-exchange and hydrophobic interaction chromatography. Heterogeneity in sialylation and glycosylation led to variation in surface charge and hydrophobicity, and resulted in multiple distinct glycoform populations in response to various purification conditions. Monomer with high sialic acid content has higher surface charge and adsorbs stronger to ion-exchange resin, while the less sialylated monomer interacts more favorably with hydrophobic resin. Extensive biophysical characterization was carried out for purified monomers at different level of sialylation. In general, different monomeric glycoforms have different surface charge and hydrophobicity, different thermal stability, and different aggregation propensity. The surface charge corresponds well with sialic acid content, as evidenced by electrophoresis, N-link domain analysis, and zeta potential results. The sialylation also contributes to minor modification of protein size, molecular mass and tertiary structure. Notably, fluorescence emission spectra and thermal transition became less distinguishable when the monomers containing low and high sialic acid were prepared in high ionic strength solution. Such finding reiterates the fact that the electrostatic forces, which are largely dependent on sialic acid content of protein, plays a dominant role in many intra- and inter-molecular interactions. Overall, the characterization data agreed well with separation behaviors and provided valuable insight to control of glycoform profile in purification process.

1. Introduction

Protein glycosylation is a crucial post-translational modification. It serves a major role in stability, efficacy, immunogenicity, pharmacodynamics, and pharmacokinetics of biotherapeutics [1–3]. For example, the sialylation level is related to glycoprotein's circulatory lifetime and thus a high number of sialic acid is a preferred product quality attribute [4–6]. Therefore, monitoring sialylation and glycosylation has become increasingly important during manufacture. The potential impact of glycosylation on safety and efficacy of therapeutic proteins and increasing regulatory scrutiny have prompted more stringent process control. Previous studies have reported progress in understanding of glycosylation control through cellular engineering, cell line selection, cell culture media optimization, and bioreactor operation improvement [7–9]. However, relatively less known is to what extent heterogeneous glycoform populations can be resolved by the platform purification process.

Most reported separation methods for glycoforms are at analytical scale, such as ion-change or hydrophobic interaction high performance chromatography [10–12], capillary electrophoresis [13], or affinity extraction [14]. Preparative scale purification to isolate a particular glycoform has not been fully established. Presumably, absolute resolution among individual glycoforms is difficult to achieve since a broad glycoform distribution often exists owing to a combination of intracellular protein packaging and extracellular enzymatic modification [2,15]. Affinity chromatography using lectin or boronate probably provides the best resolution capability because the binding mechanisms explore subtle difference in carbohydrate structure [16,17]. However, their implementation at industrial scale has not yet been demonstrated. Lack of process robustness and commercial experiences often limit those techniques in a cGMP manufacture application. For Fc fusion

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protein or monoclonal antibody, the downstream platform has long been proven to accomplish acceptable process yield and product quality. Further understanding the capability within platform process to isolate glycoform population would provide a practical opportunity to control this critical quality attribute.

The glycosylation heterogeneity resides in both the glycosylation sites in the protein backbone as well as the individual glycan components [2,18]. This is often referred to as macro- and micro heterogeneity, respectively. In either configuration, the variation among sugar groups and sialic acids in glycan can lead to variation in surface charge and surface hydrophobicity. They are governing mechanisms for common polishing chromatography steps. In a typical downstream platform, antibody product is first captured by a Protein-A affinity chromatography where most host cell impurities are removed. One or two polishing chromatography may follow to achieve additional purification or to control product variants. The extensively studied product variant is the high molecular species (HMW) due to safety and immunogenicity concerns. But for product variants originated from different glycosylation patterns, partial separation can be achieved by employing ion-exchange principle [10,19]. Conventionally, glycoform separation based on surface charge differentiation is often the first choice due to abundant experience in analytical work. However, the yield and selectivity from a large scale process column typically deviates from a high performance analytical column. Moreover, how the ion-exchange chromatography (IEX) can align with other platform unit operations to achieve overall purification and enrichment goals is yet to be thoroughly investigated. For example, the knowledge of glycoform resolution in preparative hydrophobic interaction chromatography (HIC) is much inadequate. To what extend the glycosylation can modify protein surface hydrophobicity is less known. Therefore, defining general separation behaviors of different glycoforms is the key step to build a rational purification scheme.

To address many of the above questions, this work explores purification behavior of a heavily glycosylated Fc fusion protein (of which oligosaccharides account for approximately 15% of the protein mass) by both ion-exchange and hydrophobic interaction chromatography. This protein was produced from CHO cell culture with a broad distribution of glycoforms in both monomer and HMW species. The downstream objective is to isolate highly sialylated monomers from less sialylated monomers, as well as to clear HMW species and other host cell impurities. Host cell impurity removal was mostly accomplished by the Protein-A capture step. The separation between monomer and HMW species was accomplished by subsequent polishing steps, and will be discussed extensively elsewhere [20]. The separation between different monomers (monomeric glycoforms) was also accomplished by the polishing column steps. An integrated approach is employed in this study to understand both the monomer separation and its structural characterization: first, the separation profile of low and high sialylated monomer populations were compared in both column and batch mode; next, the two populations were purified respectively by multiple column steps, and each population was characterized by an array of biochemical and biophysical techniques. The characterization include glycan structure, size, secondary and tertiary structure, surface charge and hydrophobicity, conformational stability, and aggregation propensity. Although much of characterization work for other glycoproteins are well documented, the past effort was largely focused on glycosylation pattern itself [2,21,22]. The impacted by glycosylation heterogeneity on process relevant characteristics such as bulk surface properties (i.e. charge and hydrophobicity) has not been well studied. This work is intended to better understand glycoform separation mechanisms by more in-depth molecular structure characterization.

2. Materials and methods

2.1. Cell culture production

The Fc-fusion protein used in this work was expressed in Chinese Hamster Ovary (CHO cells) and produced in protein-free proprietary growth and feed media. The protein is heavily glycosylated and is typically composed of a broad distribution of glycoforms with significant variation in oligosaccharides and sialic acids. Bioreactor conditions such as pH, dissolved oxygen, temperature shift were maintained to accomplish desired titer and sialylation. The harvest was clarified for further purification.

2.2. Chromatography purification

MabSelect Protein-A (GE Healthcare, Uppsala, Sweden), POROS 50HQ (Life Technologies, MA, USA), and Phenyl Sepharose 6 Fast Flow High Sub (GE Healthcare) were packed in 1.0–5.0 cm i.d. columns with bed height between 20 and 30 cm. Pilot scale columns were also used in selected cases. Lab scale chromatography experiments were carried out on AKTA AVANT station (GE healthcare). The clarified harvest was first loaded to a Protein-A column for initial purification, before processed by ion-exchange and/or hydrophobic interaction chromatography for further polishing. Sodium acetate or sodium chloride concentration gradient up to 1 M in 25 mM phosphate buffer, pH 7.0 was used during wash and elution of IEX step. High concentration of sodium sulfate was used to strip the IEX column. In HIC, the column was equilibrated by sodium sulfate in phosphate or HEPES buffer at pH 7.0, and the feed was adjusted to designated sodium sulfate or sodium chloride concentration at pH 7.0 before loaded to column. A chase using the same equilibration buffer is employed at the end of loading. The column was stripped by water after the flowthrough and chase.

Batch adsorption experiments were carried out using purified monomer materials at different levels of sialylation. The IEX isotherm was carried out using Poros 50 HQ resin in 10 mM Phosphate buffer, 120 mM NaCl, pH 7.0. The HIC isotherm was done using phenyl FF HS resin in 25 mM HEPES buffer, 850 mM NaCl, pH 7.0. The high and low SA monomers were generated by a Protein-A purification to remove impurities, an IEX fractionation of different sialylated species by designated salt gradient, and a HIC polishing to remove HMW. Various IEX fractionation methods were used to obtain different sialic acid content in collected monomer populations. Those monomer populations were also subjected to various biophysical characterizations.

IEX and HIC equilibrium batch adsorption experiments were performed using pre-equilibrated resin slurry with a designated protein load and specific protein concentration. Both resin slurry and protein solution were prepared with the same buffer and mixed in microcentrifuge tubes. The adsorption experiments were conducted overnight on a rotator (Glas-Col, Terre Haute, IN, USA) for continuous gentle mixing. The tubes were spun down using a benchtop centrifuge (Eppendorf AG, Germany) for 10 min at 3000 rpm, after which the final protein concentration in supernatant was measured by UV absorbance using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The adsorbed protein amount was calculated by mass balance. All experiments were performed at room temperature.

2.3. Monomer purity and quality

Host cell proteins were measured using the Cygnus commercial kit (Cat # F550, Cygnus Technologies, Southport, NC), while residual Protein-A was quantified using Repligen Protein-A kit (Cat # 9000-1, Repligen Corporation, USA). Both ELISA assays were performed according to manufacturer's protocol. Absorbance measurement