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# Insights in understanding aggregate formation and dissociation in cation exchange chromatography for a structurally unstable Fc-fusion protein

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ABSTRACT

Cation-exchange chromatography (CEX) of a structurally unstable Fc-fusion protein exhibited multi-peak elution profile upon a salt-step elution due to protein aggregation during intra-column buffer transition where low pH and high salt coexisted. The protein exhibited a single-peak elution behavior during a pH-step elution; nevertheless, the levels of soluble aggregates (i.e. high molecular weight species, HMW) in the CEX eluate were still found up to 12-fold higher than that for the load material. The amount of the aggregates formed upon the pH-step elution was dependent on column loading with maximum HMW achieved at intermediate loading levels, supporting the hypothesis that the aggregation was the result of both the conformational changes of the bound protein and the solution concentration of the aggregation-susceptible proteins during elution. Factors such as high load pH, short protein/resin contact time, hydrophilic resin surface, and weak ionizable ligand were effective, to some extent, to reduce aggregate formation by improving the structural integrity of the bound protein. An orthogonal technique, differential scanning fluorimetry (DSF) using Sypro Orange dye confirmed that the bound protein exposed more hydrophobic area than the native molecule in free solution, especially in the pH 4–5 range. The Sypro Orange dye study of resin surface property also demonstrated that the poly[styrene-divinylbenzene]-based Poros XS with polyhydroxyl surface coating is more hydrophobic compared to the agarose-based CM Sepharose FF and SP Sepharose FF. The hydrophobic property of Poros XS contributed to stronger interactions with the partially unfolded bound protein and consequently to the higher aggregate levels seen in Poros XS eluate. This work also investigates the aggregation reversibility in CEX eluate where up to 66% of the aggregates were observed to dissociate into native monomers over a period of 120 h, and links the aggregate stability to such conditions as resin surface properties and charged ligand type. Experimental data was correlated semi-quantitatively with theoretical protein charge and hydrophobicity calculations using homology modeling within the BIOVIA Discovery Studio software. Finally, an arginine-sulphopropyl (Arg-SP) agarose resin immobilized with multi-functional ligands was prepared to verify the proposed hypothesis and to eliminate the aggregate formation. The findings of this work provide general insights in understanding aggregate formation and dissociation for structurally unstable proteins in the CEX step.



## 1. Introduction

Monoclonal antibodies (mAbs) and their derivative products (e.g., Fc-fusion proteins) play an important role in treating some of the most challenging human diseases owing to the safety, efficacy and high quality of these types of biologics [1]. The monoclonal antibody market is growing significantly fast, and it is estimated that the combined world-wide sales of monoclonal antibody products will reach approximately $125 billion by 2020 [2]. Therefore, the development of robust commercial chromatography purification processes is of vital importance to patients [3]. Purification processes typically use Protein A chromatography for capture, followed by one or two polishing steps [4]. For those mAbs and Fc-fusion proteins possessing basic isoelectric points (pI), cation-exchange chromatography (CEX) has traditionally been considered a preferred option for polishing step, mainly due to relatively simple column behaviors, efficient removal of product-related impurities (e.g., HMW), and generally minor impact of electrostatic interactions on protein structure [5,6]. In comparison, proteins

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often undergo partial unfolding upon adsorption on surfaces of hydrophobic interaction chromatography (HIC) media [7–9].

In recent years, there has been an increasing number of cases reporting rather unconventional protein binding and elution behaviors in CEX. For example, Voitl et al. [10,11] reported a two-peak elution profile during a linear salt gradient for human serum albumin on Fractogel EMD SE Hicap. Since no HMW increase was observed in the two elution peaks, it was hypothesized that the protein bound to the resin in two different conformations requiring different salt concentrations to fully elute. Gillespie et al. [12] reported a two-peak elution profile for an unstable aglycosylated IgG1 in several CEX resins using a linear salt gradient, and that the late eluting peak contained more aggregates than the early eluting peak. Hydrogen-deuterium exchange and Fourier transform infrared spectroscopy (FTIR) results indicated that the second peak was originated by resin-induced antibody denaturation which could be mitigated by using preferentially excluded solutes, such as arginine [13–15]. The work evaluated many factors related to CEX operation, however, the aggregation mechanisms were not discussed in detail. Guo and Carta [16–18] reported a two-peak CEX elution behavior for a glycosylated IgG2. This effect was prominent for a polymer-functionalized resin Fractogel EMD $SO_3^-$, but virtually absent for a macroporous resin without grafted polymer (e.g. UNOsphere Rapid S), leading to the hypothesis that protein diffusion through the tentacle polymer destabilized protein structure and caused aggregate formation. It was noted that the two-peak elution behavior was seen only when the bound protein was held for extended period of time. Luo et al. [19] reported a two-peak elution behavior of an IgG2 upon salt elution. The work suggested that the IgG2 can form reversible self-association (RSA) at high salt and high protein concentration, and that the RSA species can bind more strongly to the resin than monomer, contributing to the peak splitting. In another study, Luo et al. [20] reported a split peak phenomenon for an IgG4, and linked it to the separation of histidine-protonation-based charge variants. To the best of our knowledge, mechanistic studies on aggregate formation and stability in CEX for Fc-fusion proteins have not yet been reported.

Diversified column behaviors for biologic therapeutics can ultimately be attributed to their complex molecular properties. For example, although mAbs and Fc-fusion proteins share similar fragment crystallizable (Fc) regions, their solution properties can differ significantly due to the highly variable complementarity determining region (CDR) and the glycosylation in the Fc region [21]. It has been reported that some mAbs are prone to denaturation and aggregation at low pH (pH 2–4) and high salt concentration [22]. Buchner et al. [23] reported that immunoglobulin can form an "A-state" under low pH (<3), which is characterized by a high degree of secondary structure with increased hydrophobicity and a tendency towards slow aggregation in high salt. Latypov et al. [24,25] conducted an extensive study for human IgG1 and IgG2 on acid-induced unfolding and aggregation which was primarily determined by the stability of the $CH_2$ domain located in the Fc region. Depending on solution conditions, proteins such as mAbs can partially unfold and form irreversible aggregates or reversible clusters with each other while maintaining their native structure [26,27]. Reversible or irreversible mAb association is often driven by protein–protein attractions due primarily to heterogeneous charge distribution on the mAb surface especially at high protein concentration [28–30], whereas recent report on the formation of dimeric IgG1 mAb microstructure with the presence of electrolytes also suggests important non-electrostatic contributions, such as hydrophobic interactions [26]. Fc-fusion proteins are even more likely to undergo conformational changes compared to complete mAbs due to lack of inter- and intra-molecular domain stability. Fast et al. [31] reported rapid aggregation of an Fc-fusion protein, abatacept (Orencia), when pH was lowered from 7.5 to 6.0 at 40 °C. Conformational changes and aggregate formation were attributed to the instability of CDR (CTLA-4) and $CH_2$ domains which unfold to form a molten globule-like structure that is prone to aggregation.

Furthermore, the solution conditions to which proteins are exposed in the CEX column can be difficult to determine due to complex ionic equilibrium between the mobile and the stationary phases. As a result, situations may arise where the impact of protein solution properties is convoluted with that of the chromatographic processes in understanding complex column phenomena. For example, it was reported that an unexpected pH drop can occur in CEX during salt elution due to competitive equilibrium between buffer salt ions and $H^+/OH^-$ ions [32–34]. When high salt buffer is introduced in the elution step, the cations in the mobile phase displace the $H^+$ ions in the stationary phase. These released $H^+$ ions then enter into the mobile phase and cause the temporary reduction of pH in the eluate. Extra cautions should be taken for unstable proteins in such transient conditions where the local environment of high salt and low pH, in addition to high intra-pore protein concentration upon elution, can cause protein denaturation and aggregation.

This work investigates the CEX behavior of a structurally unstable Fc-fusion protein which lacks disulfide bond between the two single chains and is prone to aggregation and precipitation at low pH and high salt conditions. The elution profile and aggregate formation during CEX is first studied in a condition employing a salt-step elution. The aggregation behavior is then explored in more detail by decoupling the contribution primarily associated with the CEX bind/elute process from the impact of protein solution properties via the use of a pH-step elution. Several factors including loading, pH, contact time, rate of desorption, resin, and ligand type are examined to better understand the underlying mechanism of the aggregation behavior observed in CEX. As adding excipients such as arginine to product-contact solutions (e.g., load, wash, and elution) appeared ineffective to suppress protein aggregation due mainly to poor stability of bound protein, an arginine-sulphopropyl (Arg-SP) functionalized agarose resin was prepared in-house to examine the proposed hypothesis which facilitates the explanation of the aggregate formation and dissociation processes associated with the CEX step.

## 2. Materials and methods

### 2.1. Chemicals, resins and protein

All chemicals were obtained from J.T. Baker (Phillipsburg, NJ, USA) unless otherwise noted. 3-Amino-1-propanesulfonic acid (3APS) and ethanolamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). SP Sepharose Fast Flow (SP SFF) and CM Sepharose Fast Flow (CM SFF) resins were obtained from GE Healthcare Sciences (Uppsala, Sweden). Poros XS resin and *N*-hydroxysuccinimide (NHS)-activated agarose slurry were purchased from Life Technologies (Waltham, MA, USA). UNO sphere Rapid S and gel filtration standard were purchased from Bio-Rad (Philadelphia, PA). The Fc-fusion protein used in this work was expressed in Chinese Hamster Ovary (CHO) cells and produced at Bristol-Myers Squibb, Co. The overall molecular weight of this Fc-fusion protein is 78 kDa. The experimental pI (isoelectric point) is 7.2 determined by Zeta potential measurement. As mentioned earlier, there is no disulfide bond in the hinge region and elsewhere between the two single chains for this Fc-fusion protein. Protein load materials used in this study were obtained by buffer exchanging Protein A purified pool into target solution conditions.

### 2.2. Chromatography instrumentation and methods

All chromatography runs were performed using a GE Healthcare ÄKTA AVANT system installed with Unicorn software version