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Surface induced three-peak elution behavior of a monoclonal antibody during cation exchange chromatography

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ABSTRACT

A monoclonal antibody exhibits a two- or three-peak elution behavior when loaded on the CEX resin POROS XS and eluted with a salt gradient. Two peaks are observed without a hold step while a third more strongly retained peak becomes noticeable with a hold time as low as 10 min. As the hold time is increased further, the first peak gradually disappears, the second peak initially increases and then decreases, and the third peak continuously increases. Dynamic light scattering shows that the third peak contains significant levels of aggregates formed in the column. Circular dichroism, HX-MS analyses of the eluted fraction, in-line fluorescence detection, and bound-state HX-MS analysis indicate that the aggregates derive from an unfolded intermediate that is slowly formed while the protein is bound to the resin. Aggregate formation does not occur on a different CEX resin, Nuvia HR-S, with similar particle size but with a more homogenous structure or when the sodium acetate load buffer is replaced with arginine acetate. The two early eluting peaks observed for POROS XS regardless of hold time are shown to comprise exclusively monomeric species. A set of biophysical measurements as well as mechanistic modeling support the hypothesis that these two peaks form as a result of the presence of weak and strong binding sites on the resin having, respectively, fast and slow binding kinetics.

1. Introduction

Ion exchange chromatography plays an important role in the purification of therapeutic proteins. Cation exchange chromatography (CEX), in particular, is commonly used to remove product related impurities from monoclonal antibodies, such as aggregates and charge variants. Size exclusion chromatography (SEC) can also be used, in principle, as a mild chromatographic modality for aggregate removal, particularly for highly unstable antibodies. However, low selectivity, volume overload limitations, and viscous fingering effects often curb its practical application in large-scale processing. Compared to other retentive chromatographic modalities, such as reverse phase chromatography (RPC) and hydrophobic interaction chromatography (HIC) that often result in protein unfolding and multiple peak elution behaviors [1–4], CEX is generally thought to be milder and less likely to induce protein conformational changes, since it is based primarily on electrostatic interactions. In most cases, the bound protein is eluted from CEX columns in a single peak at a salt concentration that depends on pH and gradient slope [5–7].

Recent studies, however, have shown that multiple peak elution can also occur on CEX columns as a result of different binding conformations of the protein. Voitl et al. [8], for example, described a two-peak elution behavior for human serum albumin on Fractogel SO3 CEX columns and explained the experimental behavior by assuming that the protein was bound in two different conformations with different binding strength and kinetics [9]. In other cases, multiple peak elution in CEX has been shown to occur as a result of protein conformational changes that occur upon binding. In some cases, the latter has also been shown to result in the on-column formation of aggregated species. For example, Gillespie et al. [10] described a two-peak elution behavior for an aglycosylated IgG1 monoclonal antibody (mAb) also on Fractogel SO3 CEX columns with the early eluting peak comprised of monomer and the late-eluting peak containing a high percentage of high molecular weight species that were formed in the column. Analyses by hydrogen-deuterium exchange and Fourier transform infrared spectroscopy (FTIR) showed that the mAb was partially unfolded while bound to the resin forming a destabilized intermediate that aggregated upon elution.

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Although glycosylated mAbs are expected to be more stable than their aglycosylated counterparts [11], recent work has shown that conformational changes upon binding to CEX resins can also occur for these molecules. Luo and co-workers [12,13] showed the occurrence of distinct two-peak elution behaviors for two different glycosylated mAbs. For the first of these mAbs [12], the two-peak elution behavior occurred on both strong and weak CEX resins and was attributed to a resin-induced reversible self-association which resulted in aggregate formation. For the second mAb [13], no aggregates were formed in the column and the two-peak elution behavior was attributed to the slow protonation of a histidine residue, which, in turn, resulted in un-protonated and protonated forms having different binding strength on the CEX surface.

Guo et al. [14,15] also reported a two-peak elution behavior for a glycosylated IgG2 mAb on various CEX resins including Fractogel SO3. In this case, the two-peak elution behavior was accompanied by the on-column formation of more-strongly bound high molecular weight species. Based on solution-phase and on-column hydrogen deuterium exchange mass spectrometry coupled with proteolytic fragmentation (HX-MS) it was found that a portion of the mAb became partially unfolded while bound to the resin resulting in specific areas of the Fc region that became more solvent exposed compared to the native protein. During elution, the native mAb eluted early in the gradient while the destabilized, unfolded bound species eluted late in the gradient, in part refolded to native monomer and in part in the form of aggregates. Further studies indicated that the two-peak elution behavior was dependent on the resin type, composition of the load buffer, and on the inherent stability of the mAb [16]. Aggregate formation was shown to be facilitated by CEX resins containing grafted polymers, such as Fractogel SO3, Eshmuno S, and Nuvia S, and by resins having a bimodal pore size distribution, such as POROS HS, while it was nearly absent for CEX resins with an open-pore structure and no grafted polymers, such as UNOsphere S and Source 30S. In general, weaker binding conditions, including higher pH, higher salt concentration, and replacing sodium with arginine, reduced or eliminate aggregate formation, while different elution conditions did not seem to have a significant effect. Different mAbs also had distinct elution behavior on the same resin under the same operating conditions, and it was shown by circular dichroism (CD) that this effect appears to be correlated with the protein melting process when exposing the protein to increasing temperatures. In all cases, however, the two-peak elution behavior was directly correlated with the length of time that the protein remained bound on the resin indicating that the ensuing two-peak elution behavior was surface catalyzed.

In this work, we explore the behavior of a mAb that exhibits a two-peak elution behavior for short hold times and a three-peak elution behavior for longer hold times on the CEX resin POROS XS. To our knowledge such behavior has not been previously reported. A number of biophysical measurements are used to characterize this system, including off-line and in-line dynamic light scattering (DLS), circular dichroism (CD), in-line fluorescence spectroscopy, and HX-MS. These measurements indicate that the two-peak behavior is associated with heterogeneous binding on the POROS XS resin likely due to existence of weak and strong binding sites. Three elution peaks are observed when the protein held on the stronger binding sites increasingly undergoes conformational changes, which ultimately results in the formation of late eluting aggregated species. The effects of flow rate and that of replacing sodium with arginine as a counter-ion in the buffer are also studied along with the behavior of the same mAb on the open-pore resin Nuvia HR-S. This resin reportedly contains neither grafted polymers nor a significant bimodal pore size distribution [17].

2. Experimental

2.1. Materials

POROS XS was obtained from Applied Biosystems (Life Technologies Corporation, Grand Island, NY, Lot No. XS-040, Date of Manufacture October 2012) and stored per manufacturer instructions in 20% ethanol. According to the manufacturer [18], this resin is based on a crossed-linked poly(styrene-divinylbenzene) backbone with a polyhydroxyl surface coating functionalized with sulfopropyl groups. As shown in previous work, POROS resins have a bimodal pore size distribution, which includes large "through-pores", about 500 nm in diameter, as well as small pores, about 22 nm in diameter [19,20]. The average particle size of the POROS XS resin used in this work is about 50 μm. Nuvia HR-S was obtained from Bio-Rad Laboratories (Hercules, CA, USA) and used for comparison. According to its manufacturer, this resin is based on a hydrophilic polyacrylamide backbone also functionalized with sulfopropyl groups. This resin also has an average particle size of about 50 μm. Basic properties of this resin can be found in ref. [17].

The monoclonal antibody used in this work was provided by Bristol-Myers Squibb (Hopewell, NJ, USA). The pI of the mAb, calculated based on the amino acid sequence, is 7 and the molecular mass is around 150 kDa. Analytical size exclusion chromatography with in-line dynamic light scattering was conducted with a TSKgel 3000SWXL column (Tosoh Bioscience LLC, King of Prussia, PA, USA) in 40 mM sodium acetate at pH 5 and at a flow rate of 0.5 mL/min using the Wyatt DLS detector described below. The SEC results, given in Fig. S1 in the Supplementary material, showed that the fresh mAb sample is essentially only monomer with a hydrodynamic radius of 5.3 ± 0.2 nm. L-Arginine, sodium acetate, acetic acid, sodium chloride, tetra-*n*-butylammonium hydroxide (TBAH), ethylenediaminetetraacetic acid (EDTA), formic acid, trifluoroacetic acid (TFA), guanidine hydrochloride (GdnHCl) were obtained from Fisher Scientific (Houston, TX, USA). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Thermo Scientific (Rockford, IL, USA). Deuterium oxide was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All experiments were conducted at room temperature (22 ± 2 °C) except otherwise noted.

2.2. Methods

2.2.1. Chromatographic experiments

Chromatographic experiments were conducted primarily with a Waters e2695 Alliance HPLC unit (Milford, MA, USA) interfaced with an in-line dynamic light scattering (DLS) instrument, DynaPro NanoStar with a fiber optics connections to a miniDAWN TREOS, obtained from Wyatt Technologies (Santa Barbara, CA, USA). Each resin was packed into 0.5 cm diameter × 5 cm long Tricorn columns from GE Healthcare (Piscataway, NJ, USA) with actual packed bed volumes of 1.0 and 1.1 mL for POROS XS and Nuvia HR-S, respectively. Each column was first equilibrated with 5 column volumes (CV) of load buffer prepared by titrating 40 mM sodium acetate with acetic acid to pH 5. After that the column was loaded with 100 μL of 5 g/L protein and washed with the load buffer for different periods of time from 0 to 240 min at the same flow rate. This time period is referred to as the hold time. For 1000 min hold time, the flow was stopped after loading and restarted after 1000 min. In all cases, after the hold step, the protein was eluted with a 20 CV linear salt gradient from 0 to 1 M NaCl with each buffer containing 40 mM sodium acetate at pH 5. The default load and elution flow rate was 0.5 mL/min but load and elution flow rates of 0.25 and 1 mL/min were also used. UV absorbance was monitored and the hydrodynamic radius of the eluted species was determined by