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Unfolding and aggregation of monoclonal antibodies on cation exchange columns: Effects of resin type, load buffer, and protein stability

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ABSTRACT

The chromatographic behavior of a monoclonal antibody (mAb) that exhibits a pronounced two-peak elution behavior is studied for a range of strong cation exchange resins and with varying load buffer pH and composition. Six stationary phases are considered, including two tentacle-type resins (Fractogel EMD SO3-(M) and Eshmuno S), a resin with grafted polymeric surface extenders (Nuvia S), a resin with a bimodal pore size distribution (POROS HS 50), and two macroporous resins without polymer grafts (Source 30S and UNOsphere Rapid S). The two-peak elution behavior is very pronounced for the tentacle and polymer-grafted resins and for POROS HS 50, but is essentially absent for the two macroporous resins. The extent of this behavior decreases as the buffer pH and concentration increase and, consequently, mAb binding becomes weaker. Replacing sodium with arginine as the buffer counterion, which is expected to decrease the mAb binding strength, nearly completely eliminates the two-peak behavior, while replacing sodium with tetra-n-butylammonium hydroxide, which is expected to increase the mAb binding strength, dramatically exacerbate the effect. As shown by hydrogen–deuterium exchange mass spectrometry (HX-MS), the two-peak elution behavior is related to conformational changes that occur when the mAb binds. These changes result in increased solvent exposure of specific peptides in the Fc-region for either the Fractogel or the Nuvia resin. No significant conformational changes were seen by HX-MS when the mAb was bound to the UNOsphere resin or on the Fractogel resin when arginine was used in lieu of sodium as the load buffer counterion. Experiments with two additional mAbs on the Fractogel resin show that the two-peak elution behavior is dependent on the particular antibody. Circular dichroism suggests that the propensity of different mAbs to either precipitate directly or to form stabilizing intermolecular structures upon exposure to thermal stress can be related to their chromatographic behaviors.

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1. Introduction

Unfolding and aggregation of monoclonal antibodies (mAbs) and other proteins during downstream process is an important concern since protein aggregates can affect the activity, half-life, and immunogenicity of proteins [1]. Since the pI of many mAbs is relatively high, cation exchange chromatography (CEX) is often used as a tool for mAb purification both to capture the antibody and to remove impurities including aggregates and charge variants [2,3]. Despite the proven effectiveness of CEX, new approaches to improve performance, streamline method development, and characterize impurities separated with these processes continue to be sought as demonstrated by many recent publications in this area (e.g. [4–7]). In general, unlike other techniques such as hydrophobic interaction chromatography (HIC) where protein binding is often associated with significant protein conformational changes and unfolding [8–11], CEX is thought of as a mild processing step unlikely to affect the protein structure. CEX depends largely on electrostatic interactions between the charged chromatographic media and the protein charged residues most of which are located on the outer protein surface [12]. As a result, binding without conformational change or unfolding is thought to be possible [13]. A few different authors, however, have recently reported multiple peak elution behavior for various proteins on CEX resins, which have been attributed to conformational changes, and on-column aggregation. Voitl et al. [14], for example, reported a

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two-peak elution behavior when pure human serum albumin was injected on columns packed with the strong cation exchange resins Fractogel EMD SO3- and Fractogel EMD SE Hicap and eluted with a salt gradient. They hypothesized that these two peaks were the results of different binding conformations. Gillespie et al. [15] also reported a two-peak elution behavior when a purified aglycosylated IgG1 antibody was loaded on a Fractogel EMD SO3 column and eluted with a salt gradient. Size exclusion chromatography indicated that the late eluting peak contained both mAb monomeric species as well as aggregates that were apparently formed in the column. Hydrogen–deuterium exchange and Fourier transform infrared spectroscopy (FTIR) suggest that conformational changes occurred during the binding step and were identified as the driving force for on-column aggregate formation. In a more recent study, Luo et al. [16] reported a two-peak elution behavior of an IgG2 antibody upon salt gradient elution in a few different CEX columns. They hypothesized that the late-eluting species were caused by reversible self-association (RSA) of the mAb that occurred at the high NaCl and high protein concentrations encountered during the elution step. Using lower NaCl concentrations or using stabilizing additives were suggested as a means of suppressing RSA.

In our previous work [17,18], we have also reported a two-peak elution behavior for a glycosylated IgG2 on a Fractogel SO3 (M) column. In our study, we found that the percentage of the protein that eluted late was higher when the initial protein binding strength was greater, which occurred either at a lower pH or at a lower salt concentration, when the hold time between load and elution steps was longer, when the protein load was smaller, and when the load flow rate was higher. The late eluting peak was found to be a mixture of monomers and higher molecular mass species that appeared to be fairly stable over long periods of time. The CEX resin structure was also found to affect this behavior. For example, while the "tentacle type" Fractogel SO3 resin gave a strong two-peak elution behavior, only one peak eluted from a macroporous CEX resin without tentacles (UNOsphere Rapid S) for otherwise identical conditions. Confocal laser scanning microscopy (CLSM) observations of protein binding in individual resin particles revealed that protein binding in the Fractogel and UNOsphere resins occurred by different mechanisms. While the bound protein remained immobilized in an area near the outer edge of the UNOsphere particles during the hold step after partial loading of the resin, the protein appeared to retain diffusional mobility after binding on the Fractogel resin, which resulted in a redistribution of the protein throughout particle prior to elution. This redistribution resulted in a low local bound protein concentration, which, in turn, was shown to facilitate protein conformational changes. Hydrogen–deuterium exchange mass spectrometry (HX-MS) coupled with proteolytic fragmentation was used to determine the conformation changes involved in this process. The results showed that a strongly bound unfolded intermediate, characterized by greater solvent exposure of residues in the Fc region of the mAb, was formed gradually as a function of time following binding to the Fractogel resin. During elution, the native protein, which was shown to bind more weakly, eluted at low salt concentration while the destabilized unfolded intermediate eluted only at high salt concentrations, in part refolding to the native protein conformation and in part forming aggregates with unfolding pattern similar to that of the bound intermediate.

This work has four primary objectives. The first is to extend our studies to a broader range of resin structures including CEX resins that contain different types of surface extenders and different pores sizes and pore size distributions. The second objective is to determine how load and elution conditions affect conformational changes of the bound protein and the ensuing two-peak elution behavior. While our previous data were limited to a few different load salt concentrations, which affected the protein binding strength, the type of counterion used in the load buffer can also have significant effects in this regard. Perez-Almodovar et al. [19], for example, showed that different protein binding strengths resulted when using acetate buffers prepared with tetra-n-butylammonium (TBAH), arginine, or calcium instead of sodium. By studying the counterion effect we can separate the effects of ionic strength from those that result from specific ion exchange interactions. Moreover, arginine has been suggested as a mean of suppressing protein aggregation and improving recovery in different types of chromatography, including affinity chromatography [20], HIC [21], and multimodal chromatography [22]. Gillespie et al. also reported that arginine reduced the percentage of protein eluting late and hypothesized that arginine prevented protein unfolding by decreasing protein binding strength in the load/wash step and by facilitating refolding and inhibiting aggregation during the elution step. Luo et al. also observed that arginine reduced the percentage of the late eluting peak, but, unlike Gillespie et al., they hypothesized that arginine worked only by inhibiting reversible self-association (RSA) induced by high NaCl concentrations. Arakawa at el. [23] and Das at al. [24] provide mechanistic explanations of the arginine effects on preventing aggregate formation in concentrated mAb solutions, but it is unclear whether these mechanisms are at play in the resin-induced aggregation. Thus, establishing the effects of arginine on the structure of the bound protein and chromatographic behavior can help establish the dominant mechanism.

The third objective is to determine the effects of resin type and load conditions on conformational changes using, as shown in our prior work, HX-MS coupled with proteolytic fragmentation. As previously discussed, this method is based on the dependence of the exchange of deuterium for amide hydrogen on the degree of their solvent exposure. Since buried amides exchange very slowly or not at all while solvent exposed amides exchange quickly, determining the degree of deuterium exchange provides a measure of unfolding. When coupled with proteolytic fragmentation and HPLC separation of the ensuing peptides, the method also provides information about which regions of the protein structure are affected [25–36].

The fourth and final objective is to extend our previous work to other mAbs and to determine whether a correlation exists between the intrinsic stability of the protein and the surface-induced conformational changes that lead to the two-peak elution behavior and on-column aggregate formation. For this purpose, CEX gradient elution experiments are conducted with two additional mAbs. Circular dichroism (CD) is used to determine their stability both by measuring their melting temperature and by analyzing the patterns of change of the CD spectra as a function of temperature [37–41].

2. Experimental

2.1. Materials

The resins used in this work were obtained as follows. Fractogel EMD SO3-(M) and Eshmuno S were obtained from EMD Millipore (Darmstad, Germany). According to the manufacturer, both of these two resins have a polyacrylate backbone grafted with charged polymers or "tentacles" to facilitate binding with proteins. Nuvia S and UNOsphere Rapid S were obtained from Bio-Rad Laboratories (Hercules, CA). Both these resins are based on an acrylamide polymeric backbone but while Nuvia S is grafted with polymeric surface extenders, UNOsphere Rapid S does not contain grafted polymers. POROS HS 50 and Source 30 S were obtained from Applied Biosystems (Life Technologies Corporation, Grand

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