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Ion exchange chromatography of monoclonal antibodies: Effect of resin ligand density on dynamic binding capacity

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Abstract

Dynamic binding capacity (DBC) of a monoclonal antibody on agarose based strong cation exchange resins is determined as a function of resin ligand density, apparent pore size of the base matrix, and protein charge. The maximum DBC is found to be unaffected by resin ligand density, apparent pore size, or protein charge within the tested range. The critical conductivity (conductivity at maximum DBC) is seen to vary with ligand density. It is hypothesized that the maximum DBC is determined by the effective size of the proteins and the proximity to which they can approach one another. Once a certain minimum resin ligand density is supplied, additional ligand is not beneficial in terms of resin capacity. Additional ligand can provide flexibility in designing ion exchange resins for a particular application as the critical conductivity could be matched to the feedstock conductivity and it may also affect the selectivity.

Keywords

Ion exchange chromatography; Protein; Monoclonal antibody; Exclusion mechanism; Ligand density; Pore size; Dynamic binding capacity

1. Introduction

Ion exchange chromatography is a common and powerful purification technique in the production of many proteins. In ion exchange chromatography, charged solute molecules are reversibly adsorbed onto oppositely charged porous resin particles. Separation of target molecules from other species present in the mobile phase depends on attraction of these target molecules to the chromatographic resin relative to that of the other species present, as well as the capacity of the resin for the target molecule. Interaction strength and resin capacity have long been known to depend on the ionic charge of the solute molecules as well as buffer ionic strength [1,2]. Recent work has shown that the performance of an ion exchange separation is a complex function of mobile phase conditions, protein properties, and chromatographic support properties [3–13].

Mobile phase conditions affect the maximum binding capacity of the chromatographic support as well as the surface properties of macromolecule solutes, and thus both buffer conductivity and pH are important parameters in determining the behavior of an ion exchange separation [4,10,11,13]. In general, the capacity of a resin for a given protein is expected to decrease with increasing buffer conductivity and with decreasing protein charge

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[14]. Recently published work, however, has identified the existence of two mechanisms in ion exchange chromatography of monoclonal antibodies; exclusion and equilibrium [13]. The exclusion mechanism occurs due to antibodies binding to the outer part of the resin beads and excluding additional antibody from entering the bead by charge repulsion and steric exclusion. An increase in conductivity and pH (decrease in protein net charge) leads to an increase in DBC until a maximum is achieved after which the equilibrium mechanism dominates, where DBC decreases with increasing conductivity and pH. This maximum DBC is a consequence of the trade off between the exclusion and equilibrium mechanisms. The critical conductivity is defined as the conductivity at which the maximum DBC occurs and depends on the buffer pH, and hence the charge of the protein. Under strong binding conditions of low conductivity and high protein net charge, the protein has been shown by confocal microscopy to bind in a narrow region near the surface of the resin while the interior region remains unoccupied. More moderate conditions of slightly increased ionic strength or decreased protein net charge allow increased pore accessibility.

The work presented herein investigates the effect of ligand density, apparent pore size and protein charge on the DBC. To that end, the dynamic binding capacity of a range of strong cation exchange resins for a monoclonal antibody is presented under conditions of varying pH and conductivity. The resins utilized in this study vary by apparent pore size as well as surface ligand density, or total ionic capacity.

2. Methods and materials

2.1. Proteins, chemicals and resins

Eight strong cation exchange resin prototypes based on the base matrices Sepharose™ 6 Fast Flow and Sepharose™ 4 Fast Flow resin chemistry and backbone were obtained from GE Healthcare (Uppsala, Sweden). The distribution coefficients (K_d) of dextran standards were determined for each of the unmodified base matrices with inverse size-exclusion chromatography (ISEC) utilizing refractive index to monitor elution [15–17]. The distribution coefficient corresponding to 110 kDa dextran standard was estimated by interpolation for the two base matrices used for the eight resins. The cation exchange resins were manufactured to have a variety of ligand densities varying from 25 to 240 $\mu\text{mol/mL}$ as shown in Table 1. The resins were packed in columns (Biochem Valve/Omnifit™, Boonton, NJ) with packed bed volumes of 3.4 mL (0.66 cm column diameter, 10 cm bed height). A full-length monoclonal antibody (160 kDa, pI 9.2) from Genentech, Inc. (South San Francisco, CA) was utilized, and is designated Mab1 in this study as well as in a previous related study [13]. Protein net charge versus pH, as calculated from the amino acid sequence, is shown in Fig. 1.

For pH 4 and 5 studies, 15 mM sodium acetate buffer (Fisher Scientific, Hampton, NH) was used. pH adjustment was accomplished by addition of glacial acetic acid (Mallinckrodt, Phillipsburg, NJ). For the pH 6 studies, 14 mM MES (Angus Buffers and Biochemicals, Buffalo Grove, IL) and 10 mM sodium MES (Angus Buffers and Biochemicals) was used. The buffers were pH adjusted with 50% sodium hydroxide (J.T. Baker, Phillipsburg, NJ). Conductivity was adjusted by the addition of 5 M NaCl stock solution. Protein concentration of the load was adjusted to 8 mg/mL (determined by absorption at 280 nm) by ultrafiltration with a 10 kDa composite regenerated cellulose membrane in a Pellicon™ XL cassette (Millipore, Bedford, MA) using a LabScale™ TFF system (Millipore) and then diafiltered into the appropriate load buffer solution. Protein solution conductivity was adjusted by the addition of a stock solution of 5 M NaCl solution to the desired buffer conductivity. A six-column volume (CV) gradient elution was performed with load and elution buffer (load buffer with 1.5 M NaCl).

2.2. Dynamic binding capacity experiments

Dynamic binding capacity was determined for Mab1 at pH 4, 5 and 6 and at conductivities of 1, 5, 10, 15, and 25 mS/cm on the resins listed in Table 1. In some cases, very little change in DBC was observed over the range of conductivity values, and only selected buffer conductivities were tested to conserve time and material. The resins were equilibrated with 5 CV of load buffer, which was sufficient to achieve the target pH and conductivity. Protein was loaded onto the resin to 10% breakthrough ($Q_{B10\%}$) based on absorbance at 280 nm (A_{280}). Unbound protein was washed from the resin with 5 CV of load buffer. Bound protein was eluted using 6 CV gradient with load buffer and elution buffer (load buffer with 1.5 M NaCl), followed by 6 CV of elution buffer. Dynamic binding capacity was calculated according to Eq. (1).

$$Q_{B10\%} = C_p \frac{V_p - V_h}{V_b} \quad (1)$$

where $Q_{B10\%}$ is the dynamic binding capacity (mg/mL), C_p the concentration of protein loaded (mg/mL), V_p the volume of protein solution loaded at 10% breakthrough (mL), V_h the holdup volume of the system (mL), and V_b is the packed bed volume of the resin (mL).

Each condition was run in duplicate, and averages of the capacities are reported. All experiments were conducted at ambient temperature (25–27 °C) and a flow rate of 100 cm/h corresponding to a residence time of 6 min. In all studies, the resins were regenerated and stored using 0.5N NaOH and 0.1N NaOH, respectively.

3. Results

DBC trends were obtained as a function of mobile phase pH or protein net charge, mobile phase conductivity, and resin ligand density for resins based on Sepharose 6 Fast Flow and Sepharose 4 Fast Flow (Figs. 2 and 3). The unfunctionalized base matrix Sepharose 6 Fast Flow has a distribution coefficient (K_d) corresponding to 110 kDa dextran standard of 0.42. With few exceptions such as low resin ligand density, the resins based on this base matrix (Fig. 2) consistently demonstrate an exclusion mechanism, as demonstrated by the discernible optimum in the DBC curves. The Sepharose 4 Fast Flow base matrix by contrast have larger pores ($K_d = 0.60$) and resins based on this base matrix (Fig. 3) demonstrate minimal exclusion only with high resin ligand density and high charge conditions (low pH and low conductivity).

Both ligand density and protein net charge can be seen to have an effect on performance of the resin. The extent to which these system variables affect the critical conductivity, defined as the conductivity at which the maximum DBC occurs, depends on the resin pore size (Fig. 4). For the smaller pore size resins (Fig. 4a), the critical conductivity increases with increasing resin ligand density and protein net charge. The larger pore size resins (Fig. 4b), however, do not demonstrate this same dependence, with a critical conductivity less than 5 mS/cm for all resins tested. The increased differentiation in the pH 4 curve compared to the pH 5 and 6 curves could be due to the increased protein net charge (Fig. 1).

The maximum DBC is shown in Fig. 5 as a function of the protein net charge (mobile phase pH) and the resin ligand density. The maximum DBC value does not vary to a great extent with ligand density for the Sepharose 6 Fast Flow and Sepharose 4 Fast Flow based resins. However, a certain minimum ligand density would be expected to be required for ionic

binding and an indication of a threshold ligand density is seen for the Sepharose 6 Fast Flow based resins (Fig. 5a).

4. Discussion

The current study explores the effect of ligand density, protein net charge and, to a limited extent, apparent pore size on the dynamic binding capacity of monoclonal antibodies. The critical conductivity is seen to be a function of protein net charge (mobile phase pH) and resin ligand density (Fig. 4). The dependence on resin ligand density varies with pH and resin pore size. As noted earlier, an exclusion mechanism is seen in smaller pore size resins, and thus the critical conductivity is dependent on protein net charge (Fig. 4a). This dependence should tend to decrease with increasing pore size as the exclusion effect decreases. This is seen with the larger pore size resins tested where no dependence is seen due to minimal exclusion occurring (Fig. 4b).

The dependence of the critical conductivity on resin ligand density in the smaller pore size resins (Fig. 4a) can be explained in the following manner. Proteins initially entering the resin pore bind to the exterior resin surface. As the resin ligand density is increased, the total surface charge on the resin is increased, resulting in a stronger binding interaction between protein and resin surface. Increasing the mobile phase conductivity decreases this interaction which could result in the initially bound protein being displaced to the interior of the resin by incoming proteins. Increasing the mobile phase conductivity would also decrease the electrostatic repulsion encountered by incoming proteins with bound proteins at the resin's exterior surface, resulting in the incoming proteins bypassing the bound proteins and accessing the resin interior. A higher mobile phase conductivity is thus required to achieve a condition under which the proteins may access the resin interior either due to decreased protein-protein electrostatic repulsion or protein resin surface interaction. This dependency is not present in the more open, larger pore resins because the proteins are not sterically or electrostatically hindered by bound proteins, allowing them to freely diffuse into the resin interior where they may interact with the surface charge groups of the resin. The dependency of the critical conductivity on protein net charge or pH has been previously explained [13].

The effect of resin pore size can be seen in comparing the capacity trends of the two sets of resins tested in this study (Figs. 2 and 3). In general, the Sepharose 6 Fast Flow based resins show a greater sensitivity to mobile phase pH and conductivity, with critical conductivity values spanning the entire test range. These resins also tend to exhibit a more dominant exclusion effect than the larger pore resins based on the Sepharose 4 Fast Flow backbone. Clearly, the steric and electrostatic interactions responsible for the exclusion mechanism will be more significant in smaller pores, as the adsorbed molecules will occlude a larger portion of the pore cross-section. For the larger pore resins (Fig. 3), we see exclusion only at the highest protein charge (pH 4) and ligand density condition studied. This indicates that the adsorbed molecules in general are not occluding a significant portion of the pore cross section, thus allowing free access to the resin interior.

Previous studies have shown that the conductivity at which the maximum DBC occurs as a function of protein net charge condenses into a single trend for SP Sepharose Fast Flow and SP Sepharose XL resins with three different antibodies. This functionality may actually represent more general ion exchange phenomena related to media and protein surface potentials and their influence over the balance of attraction between media and free protein and repulsion between adsorbed and free protein [13]. It is seen in this work, that this can be further extended to all DBC and not just limited to maximum DBC. As shown in Figs. 6 and 7 all the DBC data for a given resin collapse into one trend if graphed as a function of a

normalization parameter, N , calculated as a ratio of the mobile phase conductivity and protein net charge according to Eq. (2):

$$N = \frac{\sigma}{Z_p} \quad (2)$$

where σ is the mobile phase conductivity (mS/cm) and Z_p is the net charge of the protein molecule and is a function of mobile phase pH (Fig. 1). The expansion from mapping just the critical conductivity to the entire DBC versus conductivity and the applicability of the normalization both in presence and absence of exclusion indicates that this could refer to more fundamental ion exchange phenomena. The single trend (Figs. 6 and 7) and the normalization parameter (Eq. (2)) could be utilized to predict capacities at a given pH and conductivity, both in scenarios where exclusion phenomena are present and where more traditional ion exchange behavior dominates. Even more important is the possibility to design screening experiments based on the normalization parameter in both types of scenarios. The relevant range of variations in feed conductivity is dependent on the protein net charge and should therefore be shifted when different pH levels are tested. The design of experiments evaluation is also facilitated by expressing the variations in feed conductivity according to the normalization parameter [18].

It was expected that there would exist some optimum combination of resin ligand density and pore size at which the DBC would be maximized. Surprisingly, however, the magnitude of the maximum DBC is relatively insensitive to the resin ligand density or pore size within the range tested (Fig. 5). The maximum DBC appears to be a function of solution conductivity, protein net charge and the ligand. Increasing solution conductivity decreases the binding strength of the protein to the ligand and also reduces the exclusion effect. Hence, proteins will only pack into a space to a certain density, as electrostatic effects repel adjacent molecules. Once that packing density has been achieved, the repulsive forces will dominate and additional ligand will not be beneficial.

In the Sepharose Fast Flow resins, the charged ligand is limited to the surface of the agarose and may leave the central region of the pore unutilized. In the case of the SP Sepharose XL resin, however, dextran is used as a surface extender creating a lattice that distributes the ligands throughout the three-dimensional space of the resin interior. The distribution of ligand throughout the porous space of the resin allows the protein molecules to more fully utilize the available space within the resin. This leads to a higher total protein capacity with equivalent resin ligand density [19].

5. Conclusions

The effect of resin ligand density and apparent pore size on the dynamic binding capacity of a monoclonal antibody has been investigated. It was shown that the resin pore size is a factor in the occurrence of exclusion, with exclusion being largely absent in the larger pore size Sepharose 4 Fast Flow based resins, but appearing consistently in the smaller pore size Sepharose 6 Fast Flow based resins. The resin ligand density had very little effect on the maximum dynamic binding capacity, however, it affected the critical conductivity. These results suggest that increasing ligand density above a certain minimum value (55 $\mu\text{mol/mL}$ in this case) will not improve resin binding capacity, even if the pore size is simultaneously decreased. In order to increase resin capacity, it appears that it is necessary to increase the efficiency with which the pore space is utilized. This can be accomplished through more efficient distribution of the ligand within the pore space, as is done in the SP Sepharose XL resin and more recently the CaptoTM S. The resin ligand density was shown to affect the

critical conductivity; this can be leveraged in resin optimization by tuning the ligand density to achieve critical conductivities that would suit potential applications.

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Fig. 1. Mab1 net charge as a function of pH. Protein net charge is calculated based on amino acid sequence.

Fig. 2. SP Sepharose 6 Fast Flow based resin dynamic binding capacity (mg/mL resin) as a function of mobile phase conductivity (mS/cm), and resin ligand density (◆, 25 $\mu\text{mol/mL}$; ■, 60 $\mu\text{mol/mL}$; ▲, 110 $\mu\text{mol/mL}$; ●, 190 $\mu\text{mol/mL}$; ○, 240 $\mu\text{mol/mL}$) at mobile phase pH 6.0 (a), mobile phase pH 5.0 (b), and mobile phase pH 4.0 (c).

Fig. 3. SP Sepharose 4 Fast Flow based resin dynamic binding capacity (mg/mL resin) as a function of mobile phase conductivity (mS/cm), and resin ligand density (◆, 55 $\mu\text{mol/mL}$; ■, 130 $\mu\text{mol/mL}$; ▲, 240 $\mu\text{mol/mL}$) at mobile phase pH 6.0 (a), mobile phase pH 5.0 (b), and mobile phase pH 4.0 (c).

Fig. 4.

The effect of mobile phase pH (◆, pH 4; ■, pH 5; ▲, pH 6) and resin ligand density on critical conductivity for SP Sepharose 6 Fast Flow based resins (a) and SP Sepharose 4 Fast Flow based resins (b). The conductivity at which the maximum dynamic binding capacity occurs, i.e. the critical conductivity, is seen to be affected by protein net charge (mobile phase pH) as well as the resin ligand density.

Fig. 5. The effect of mobile phase pH (◆, pH 4; ■, pH 5; ▲, pH 6) and resin ligand density on the magnitude of the maximum dynamic binding capacity for SP Sepharose 6 Fast Flow based resins (a) and SP Sepharose 4 Fast Flow-based resins (b).

Fig. 6. Dynamic binding capacity as a function of the normalization parameter for SP Sepharose 6 Fast Flow resins (◆, pH 6; ■, pH 5; ▲, pH 4) (a) 25 $\mu\text{mol/mL}$, (b) 60 $\mu\text{mol/mL}$, (c) 110 $\mu\text{mol/mL}$, (d) 190 $\mu\text{mol/mL}$ and (e) 240 $\mu\text{mol/mL}$.

Fig. 7. Dynamic binding capacity as a function of the normalization parameter for SP Sepharose 4 Fast Flow based resins (◆, pH 6; ■, pH 5; ▲, pH 4) (a) 55 $\mu\text{mol/mL}$, (b) 130 $\mu\text{mol/mL}$ and (c) 240 $\mu\text{mol/mL}$.

Table 1

Resin ligand densities tested.

Backbone	Sepharose 4 Fast Flow	Sepharose 6 Fast Flow
Ligand	Sulfopropyl	Sulfopropyl
K_d 110 kDa dextran	0.60	0.42
Ligand density ($\mu\text{mol/mL}$)	55, 130, 240	25, 60, 110, 190, 240

Ligand densities are expressed as μmol ligand per mL resin.