The effect of pH on antibody retention in multimodal cation exchange chromatographic systems

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Highlights (3-5 points, 85 characters)

- Evaluated mAb retention, domain contributions, and surface properties from pH 5-7
- One mAb showed a pH-dependent spectrum of domain contributions
- pH can tune the relative importance of Fab vs. Fc binding sites for some mAbs
- The titration of Histidine residues plays an important role in this pH range

Abstract

The present paper builds upon previous work on mAb domain contributions to multimodal (MM) chromatography by examining how pH can impact mAb surface properties and retention in these systems. Linear salt gradient experiments were carried out between pH 5-7 for several mAbs with different pI and surface hydrophobicities in four different MM CEX resins at two ligand densities. mAb retention showed an inverse, non-linear correlation with pH. Changing pH affected the elution order, creating unique windows of selectivity in each of the MM CEX resins. One mAb showed a pH-dependent spectrum of domain contributions, demonstrating that pH can be used to tune the relative importance of the $(Fab)_2$ and Fc domains for some mAbs in MM systems. Positive, negative, and hydrophobic patches were calculated between pH 5-7 for the mAbs. Visualizing these patches on the protein surface demonstrated that each mAb showed a unique distribution of surface charge and hydrophobicity that changed with pH. The sum of patch areas was tracked across this pH range to quantitatively understand how pH impacted these important surface properties. The quantitative analysis then was narrowed to consider only patches in the CDR loops, which were hypothesized to be an important interaction site for some mAbs in these systems. Interestingly, differences in the titration of CDR loop patches for each mAb were shown to be a result of Histidine titrations and patches in this region were qualitatively correlated with experimental trends including the observed elution order reversals. These results indicate that pH potentially can be employed as a lever for the strategic design of multimodal steps to create flow through, bind and elute, or weak partitioning operations with important implications for the design of integrated and/or continuous downstream purification processes. Furthermore, the ability to tune domain contributions in MM separations using pH

creates intriguing possibilities for current downstream challenges such as the removal of product-related impurities, as well as the purification of bispecific mAbs.

Keywords: multimodal chromatography; antibodies; pH; surface properties; domain contributions

1. Introduction

A growing body of work has helped elucidate the complex mechanisms that underpin the unique selectivities observed for mAbs in multimodal chromatography [1]. This improved understanding has led to the increased evaluation of multimodal resins as capture steps [2-4] or as polishing steps for the removal of problematic HCPs [3] and aggregates [5-8]. Multimodal steps are also valuable for the purification of non-traditional mAb therapeutics including bispecific antibodies [9, 10], minibodies [11], and other formats [12].

Extensive work has been carried out to evaluate protein retention at constant pH in salt gradients for multimodal anion (MM AEX) [13, 14] and cation exchange (MM CEX) [15-19] systems. The effect of pH in multimodal systems has also been studied, although to a lesser extent. For hydrophobic charge induction chromatography (HCIC), a step change in pH induces a change in the ligand charge state resulting in protein elution [20]. The magnitude of this pH change and the mechanism of interaction in HCIC have been studied extensively [6, 21-25]. A recent paper compared model protein retention in linear salt gradient experiments at pH 5 and 6 for homologous MM CEX ligands and showed that retention was generally stronger at pH 5 [15]. Much of the additional work to investigate pH in weak MM CEX and MM AEX systems utilized pH gradients. On Capto MMC, pH gradients provided unique selectivities compared to salt gradients for model proteins [26]. Dual salt and pH gradients were employed for the separation of mAb charge variants, fragments, and aggregates on Capto MMC ImpRes as well as Eshmuno HCX [27]. Further, pH gradients were employed in conjunction with constant pH salt gradients on MM CEX, MM AEX, and HCIC resins as a screening tool for the synthesis of integrated downstream processes [28].

Several thermodynamic models have been developed to describe the pH dependence of protein retention in multimodal systems. Lee *et al.* adapted Yamamoto's LGE model [29] to describe protein retention in dual salt and pH gradients [27]. The same group also published a model to describe retention in multimodal systems that built on Mollerup's thermodynamic framework [30] and Nfor's multimodal isotherm [31] by expressing protein characteristic charge as a function of pH [9]. The authors demonstrated that this model can be used to predict protein retention in pH gradients at constant ionic strength or in salt gradients at constant pH [9]. While these thermodynamic models have been demonstrated to accurately predict protein behavior, a deep understanding of how pH affects interaction mechanisms in multimodal systems is still lacking.

Work with model proteins demonstrated that protein retention in pH gradients is distinct compared to that in salt gradients due to shifting contributions from charge and hydrophobicity [15]. pH has been shown to change the curve width of U-shaped distribution coefficient plots for a bispecific mAb in multimodal systems, further illustrating the impact of pH on both hydrophobic and electrostatic interactions [9]. Furthermore, for multimodal ligands with weak charge groups, these shifting interactions result not only from titrating the protein surface, but also from changes affected on the resin surface as a result of titrating the ligands [15].

The present investigation builds on this existing body of work to advance the understanding of how pH impacts mAb retention in multimodal systems. The objectives of the present work were twofold: 1. To evaluate mAb interactions in MM CEX systems as a function of pH; and 2. To connect mAb behavior at the various pH conditions to changing protein and ligand surface properties. To this end, linear salt gradient experiments were carried out between pH 5-7 for several mAbs in different MM CEX resins at two ligand densities. Based on the interesting

119 elution trends observed with the intact antibodies, domain contributions were then evaluated for
120 one mAb between pH 5-6 to provide further insight into the mechanism of mAb interactions.
121 Positive, negative, and hydrophobic property patches were calculated between pH 5-7 and
122 visualized on the antibody surface to understand the patch distribution and learn how this distri-
123 bution changed with pH. The sum of patch areas was tracked across this pH range to
124 quantitatively understand how pH impacted these important surface properties. A quantitative
125 analysis of surface properties focused on patches in the CDR loops. Differences in the titration of
126 CDR loop patches were shown to be a result of Histidine titrations and were qualitatively corre-
127 lated with experimental trends such as elution order reversals.
128

129 ## 2. Materials and methods

130 ### 2.1 Materials

131 Three IgG1 antibodies with pI ranging from 7.6 to 8.3 were produced and purified at Merck
132 and Co., Inc. (Kenilworth, NJ, USA). Buffer salts and centrifugal spin filters were purchased
133 from Sigma-Aldrich (St. Louis, MO). Capto MMC and Capto MMC ImpRes resins were pur-
134 chased from GE Healthcare (Uppsala, Sweden). Supplies for the mAb enzymatic digests,
135 including papain and pepsin immobilized on agarose resin beads, Pierce centrifuge columns, and
136 Zeba spin desalting columns were purchased from Thermo Fisher Scientific (Waltham, MA).
137 The Nuvia cPrime, Prototype 4, and Prototype 5 high and low ligand density resins were sup-
138 plied by Bio-Rad Laboratories (Hercules, CA). The prototype ligands are a subset of a ligand
139 library previously developed and evaluated by our group using both experimental and *in silico*
140 techniques [15, 18, 32]. Table 1 summarizes the ligand densities and particle size for each resin
141 evaluated in this work. For Capto MMC, high ligand density was within 70-90 μmol/mL and low

ligand density was 25-39 μmol/mL. Capto MMC ImpRes had a smaller particle size than Capto MMC in addition to a decreased ligand density. For Nuvia cPrime and the two prototypes, high ligand density resins were within 95-126 μmol/mL while low ligand density resins were within 70-75 μmol/mL.

2.2 Methods

2.2.1 Chromatography experiments

Glass chromatography columns (5x50 mm) were packed with MM CEX media in 20% ethanol, 400 mM NaCl packing buffer for a total column volume of 0.8-1.2 mL. An acetone pulse was used to verify that column asymmetry was within 0.90-1.10. Using an Äkta Explorer with Unicorn 5.31 software, linear salt gradient experiments were conducted from 0-1.5M NaCl over 60 CV (column volumes; pH 5 and 5.5) or from 0-1M NaCl over 40 CV (pH 6-7). The gradient slope was kept constant for all experiments although the total salt concentration changed. The flow rate was normalized to 1 CV/minute for a constant residence time through each column. The equilibration buffer compositions were as follows: 20 mM Acetate (pH 5 and 5.5), 20 mM Tris, Acetate (pH 6), and 20 mM Histidine (pH 6.5 and 7). High salt buffers were prepared by the addition of 1M NaCl (pH 6-7) or 1.5M NaCl (pH 5-5.5) to the equilibration buffers. The antibodies were buffer exchanged into equilibration buffer and adjusted to a concentration between 3-5 mg/mL. Samples were equilibrated overnight prior to chromatography experiments. For each column experiment, 200 μL of sample was injected. Retention was determined using moment analysis and reported as the average elution salt concentration of duplicate salt gradient experiments.

2.2.2 Enzymatic digestions of antibody samples

Papain and pepsin were used to cleave antibodies into Fab, Fc, and $(Fab)_2$ samples according to the procedure described by the enzyme manufacturer (ThermoFisher) and also described previously [19].

2.2.3 Surface property patch calculations

mAb homology models were provided by our industrial collaborators at Merck and Co., Inc. (Kenilworth, NJ, USA). To calculate positive/negative charge and hydrophobic surface patches, the mAb structures were energy minimized and titrated using the Molecular Operating Environment (MOE) 2018.01 software from Chemical Computing Group (Montreal, Canada). The "Protein Surfaces and Maps" tool was used to calculate surface patches using the parameters listed in Table 2. Charged patches were identified as regions of the protein surface where an absolute electrostatic potential of at least 50 kcal/mol/C existed for an area of 40 $Å^2$ or greater. Hydrophobic patches were identified as regions for which a hydrophobic potential of at least 0.09 kcal/mol existed over 50 $Å^2$ or larger. For each mAb, patches were computed between pH 5-7 at a 0.25 pH unit resolution. All calculations were performed using the Amber10:EHT force field.

3. Results and discussion

In addition to salt, solution pH is an important although less studied determinant of mAb interactions in multimodal systems. As indicated in the introduction, a growing body of work has recently been published on the use of pH gradients in multimodal systems [9, 15, 26-28]. In practice, step changes in pH are employed more commonly than gradient operations. The current work builds on the existing pH investigations through a detailed characterization of mAb retention in MM CEX systems as a function of pH. This work investigates how mAb retention and

surface properties change as a function of pH and lays the foundation for a deeper understanding of how pH affects the interaction mechanism in multimodal systems.

3.1 mAb retention as a function of pH

In this work, mAb behavior was evaluated in four MM CEX resin systems. The resins represented a mix of commercial (Capto MMC and Nuvia cPrime) and prototype (Prototypes 4 and 5) structures that have previously shown unique selectivities for a wide variety of proteins including model proteins [16], Fab variants [17, 18], as well as mAbs [19]. The ligand structures for each of the resins are shown in Figure 1. As can be seen in the figure, each ligand contained a carboxylic acid charge group and an aromatic hydrophobic group, although these groups differed in their structural arrangements. The two prototype ligands are structural analogs of Nuvia cPrime that were engineered to have increased aromatic (Prototype 4) or aliphatic (Prototype 5) hydrophobic character.

Linear salt gradient experiments were performed at 0.5 unit pH increments within the range of pH 5-7 from 0-1.5M NaCl (pH 5 and 5.5) or 0-1M NaCl (pH 6, 6.5, and 7). As outlined in the methods section, the gradient length was adjusted such that the slope remained constant between the different pH conditions. The three mAbs employed in this work ranged in pI from 7.6 to 8.3 and had a range of hydrophobicities. The mAbs shared a common Fc domain but differed in the Fab domains and were each directed against different antigens. The multimodal behavior of these mAbs at a single pH (pH 6) previously was studied in depth [19].

Figure 2 plots mAb retention in linear salt gradients as a function of pH from pH 5-7 on the four different resins. The mAb retention showed an inverse, non-linear dependence on pH. Stronger retention at lower pH was expected since the mAbs became more positively charged below the pI and was in line with trends previously reported in the literature [16, 33]. As pH

decreases, the sulfonate groups on the ligands become protonated and the ligands become more hydrophobic as the charges are neutralized, likely also contributing to the increased retention at lower pH.

mAb retention on Capto MMC is shown in Figure 2A. As can be seen in the figure, the elution order did not follow the order of the mAb pI values. This trend was in line with previous work that demonstrated that charge distribution, especially in relation to hydrophobicity, is important in multimodal systems [34-36]. mAb B eluted first between pH 5-7, despite having an intermediate pI. The elution order between mAb A and C changed as a function of pH. At pH <6, mAb A was more retained than mAb C despite having a lower pI, while mAb C became more retained at pH >6. The transition between mAb A or mAb C eluting first (indicated in the figure by the arrow) led to unique selectivity windows between these mAbs. Selectivity between mAbs A and C increased as the pH diverged from pH 6. These results open up some intriguing possibilities for creating flow through, bind/elute, or weak partitioning separations for these mAbs. For example, the operation of Capto MMC between pH 6-7 could enable mAb A to flow through while mAb C bound, and vice versa at pH 5-6. On the other hand, no selectivity reversals were observed for mAb B in the Capto system as mAb B eluted before mAbs A and C for the entire pH range examined.

Figures 2B and 2C show mAb retention on Nuvia cPrime and Prototype 4, respectively. Nuvia cPrime and Prototype 4 showed similar trends to those observed with Capto MMC. mAb B eluted earlier than mAbs A and C and a selectivity reversal was observed for mAbs A and C, albeit at slightly higher pH (6.25 and 6.5 for Prototype 4 and Nuvia cPrime, respectively). The ability to shift the pH of the transition between mAbs A and C with different ligands could provide increased flexibility for the design of integrated and/or continuous downstream purifica-

tion processes. Within the downstream mAb platform, subtle pH changes could also allow for purification of mAb products from challenging product related impurities and pH optimization should be an important component of process development.

Figure 2D shows mAb retention on Prototype 5, the ligand with increased aliphatic nature. In comparison to the other MM CEX systems, Prototype 5 showed very different behavior, particularly with respect to mAb B. Whereas mAb B eluted first on the other resins, mAb B showed similar behavior to mAb A at pH 5 and 5.5 and was more strongly retained than mAb C at pH 5.5 on Prototype 5. As reported previously, mAb B was more retained than mAbs A or C on a HIC column [19]. Here, it is hypothesized that Prototype 5 showed different selectivity trends due to a stronger hydrophobic character resulting from the aliphatic linker proximal to the aromatic ring. This unique elution order on Prototype 5 could enable the design of purification steps not possible in the other multimodal resins. The different trends on Prototype 5 also raised the question of the minimum set of ligands required to "cover the spectrum" of possible separations for a given set of proteins. Ongoing work in our lab is investigating this "orthogonality" between multimodal ligands in detail.

In addition to studying the contributions from the different ligand chemistries, mAb retention was evaluated on resins with different ligand densities. Varying stationary phase ligand density has the potential to affect interactions between adjacent ligands. Our group recently demonstrated that Capto MMC and Nuvia cPrime exhibit very different clustering behavior at the resin surface [37] At a microscopic scale, ligand clustering patterns could impact the nature of important protein surface patches or even selectivity. As discussed in the methods section, each ligand was evaluated at a high and low different ligand density (Table 1) to determine if this parameter caused changes in retention behavior. Varying ligand density caused only subtle

changes in selectivity, most noticeably at pH 5.5 approaching the ligand pKa values (values for the commercial resins given in [38]. Generally, decreasing ligand density resulted in decreased retention and this difference was inversely correlated with pH. Slightly larger differences were observed on Capto MMC as compared to Nuvia cPrime or the prototype resins (Supplemental Figures S1 and S2). Previous work from our group had demonstrated that a critical ligand density exists at which protein retention is significantly affected [16]. In the current work, it is likely that the ligand densities were above this critical ligand density value and future work will examine the behavior for mAb domains at lower ligand densities in more detail.

3.2 Effect of pH on domain contributions

As discussed above, mAb B showed significantly different behavior on Prototype 5 as compared to the other resins. Figure 3 highlights this unique behavior, focusing on the retention of mAb B in the various resin systems. mAb B was more strongly retained on Prototype 5 than the other multimodal resins between pH 5-6, with the exception of pH 5 where mAb B did not elute at 1.5M NaCl on either of the prototypes. Recent work from our lab demonstrated that mAb B exhibited unique domain contributions at pH 6 on Prototype 5 as compared to the other resins. The domain contributions for mAb B on Prototype 5 behavior were also very different than those observed for mAbs A and C in all resin systems [19]. In the current work, the domain contributions for mAb B were evaluated at pH 5 and 5.5 to interrogate the unique elution behavior observed in this range, particularly on Prototype 5.

As described in the methods section, $(Fab)_2$, Fab, and Fc fragments were produced using enzymatic digests and domain retention in linear salt gradients was compared to the intact mAb behavior. Figure 4 shows the retention of mAb B and the constituent domains at several pH for Prototype 5 and Nuvia cPrime. As can be seen in the figure, a dramatic shift was observed for the

278 relative domain contributions between pH 5.5 and 6 for mAb B on Prototype 5. At pH 6, the
279 intact mAb was more retained than either the $(Fab)_2$ or the Fc domains, which co-eluted [19]. On
280 the other hand, at pH 5.5 the $(Fab)_2$ domain was significantly more retained than either the Fab
281 or the Fc domains and this was strongly correlated with the increased retention for the intact
282 antibody. The fact that the single Fab domain was not as dramatically affected by the decrease in
283 pH suggested that interactions in the hinge region and/or avidity effects were likely playing a
284 role in the increased retention of the $(Fab)_2$ domain.

285 Figure 4 also shows the retention of mAb B and associated domains on Nuvia cPrime. As
286 can be seen, at both pH 5.5 and 6, the Fc domain and intact mAb co-eluted while the Fab and
287 $(Fab)_2$ domains were both less retained. As the pH was further decreased to 5, the $(Fab)_2$ domain
288 became slightly more retained than the Fc domain but still eluted earlier than the intact antibody.
289 (Similar trends were also observed on Capto MMC as shown in the supplementary information,
290 Supplemental Figure S3.) This domain behavior qualitatively agreed with the experimental
291 trends shown in Figure 3, in that mAb B did not show as dramatic an increase in retention on
292 Nuvia cPrime or Capto MMC below pH 6 as compared to Prototype 5. Domain contributions
293 were also evaluated for mAbs A and C (Supplemental Figure S4) in this pH range and the trends
294 at pH 5.5 were qualitatively similar to those observed at pH 6 for both of these mAbs [19]. The
295 results with mAb B are significant because they indicate that pH, in addition to ligand chemistry,
296 can be used to tune the relative importance of the $(Fab)_2$ and Fc domains. This opens up opportu-
297 nities to potentially use pH as a powerful parameter to enable the removal of product related
298 variants and clipped forms on various regions on the antibodies using appropriate salt and pH
299 changes during the chromatography. Clearly these results demonstrate that optimizing operating
300 pH should be an important consideration in process development. Furthermore, evaluating

domain contributions, including understanding the impact of pH, has potentially important implications beyond this work to even more challenging separations such as the purification of bispecific antibodies.

3.3 Protein surface property patches

Previous work in our lab with model proteins [34-36] and Fab variants [17, 18] has demonstrated that proteins can have preferred binding regions in multimodal systems. Furthermore, the local distribution of surface charge and hydrophobicity has been shown to be important for determining preferential interaction sites [34-36]. To provide insight into the experimental behavior described above, surface property patches were calculated for each of the antibodies in the range of pH 5 to 7.

Protein surface properties are commonly evaluated using spatial aggregation propensity (SAP) [39] and electrostatic potential (EP) property maps [40, 41]. Recently, several groups have demonstrated that descriptors based on local and regional properties (e.g. surface patches) are important for predicting protein behavior in multimodal [15, 16, 42], HIC [43] and ion exchange systems [44, 45]. In this work, surface patches were considered rather than conventional property maps to allow a quantitative analysis of how these patches change with pH. A potential advantage of this approach as compared to SAP and EP maps is that the patches have clearly defined size and strength cutoffs that could potentially minimize noise and focus attention on regions of importance for ligand interactions.

Visualizing these clusters on the protein surface can also provide qualitative insights into the distribution and importance of various protein surface properties. Figure 5 shows the ribbon structure for each mAb with the positive (blue), negative (red), and hydrophobic (green) patches identified at pH 5 and 7. As can be seen in the figure, the mAbs have qualitatively different

distributions of surface property patches in the Fab domains and the distributions changed with pH. Since the mAbs shared a common Fc domain, this analysis focused on the Fab region. As illustrated in Figure 5A, mAb A had predominantly positive and hydrophobic patches in the V_L and V_H domains at pH 5. In addition, several negative patches were identified at the interface of the variable and constant domains. The hinge region also contained two strong positive patches at pH 5. Increasing the pH to 7 resulted in the disappearance of positive patches at the top of the V_L and V_H domains near the CDR loops as well as in the hinge region. Additional negative patches appeared near the CDR loops as well as at the variable/constant domain interface. In contrast, Figure 5B shows that mAb B had a high concentration of negative patches in the V_L and V_H domains near the CDR loops at both pH 5 and pH 7, and that this region also contained several hydrophobic patches. mAb B lacked strong positive patches in the hinge region that were observed for mAb A. Figure 5C shows that mAb C had fewer hydrophobic patches in the Fab domain as compared to mAbs A and B. As observed for mAb A, positive patches were identified in the hinge region for mAb C at pH 5, which were no longer present at pH 7. mAb C showed the least change in the charged patches when going from pH 5 to 7 which was expected since it had the highest pI. In contrast to mAbs A and B, these patches were not co-localized and were scattered throughout the Fab domain.

Figure 6 shows how the charge and hydrophobic patch areas varied across pH where the values plotted on the y-axis are the sum of all of the patch areas for a specified property. The sum of positive patch areas for each mAb is shown in Figure 6A. As can be seen in the figure, mAb A had the largest value below pH 5.5 while mAbs B and C had similar positive patch areas. Above pH 5.5, mAbs A and C showed similar positive patch areas while mAb B, with an intermediate pI, exhibited higher values. Figure 6B shows the sum of negative patch areas for each

mAb. mAb A had the highest total area for all negative patches across the entire pH range considered while mAbs B and C showed similar areas. The sum of the hydrophobic patch areas for each mAb are presented in Figure 6C. These values remained relatively constant across this pH range with mAb A having the highest sum, followed by mAbs B and C. The fact that the hydrophobic patch areas were greater than the charged surface patch areas is likely due to the cutoff strength and size parameters (Table 2) employed to calculate these patches.

The total surface patch areas showed some correlations with the experimental trends. For example, an elution order transition was seen experimentally for mAbs A and C (Figure 2) and also was observed for the total positive patch areas (Figure 6A). On the other hand, the experimental behavior for mAb B could not be explained based on this global patch analysis. mAb B eluted first in most of the experiments, despite having the largest positive patch area (for pH > 5.75), similar negative patch area to mAb C, and intermediate hydrophobic patch areas. This result highlights the fact that the regional distribution of these patches, rather than the total patch areas is an important factor in determining retention in multimodal systems, in agreement with previous studies [15-19].

3.4 CDR surface property patches

The chromatographic results indicated that the $(Fab)_2$ domain was important for all three mAbs below pH 6. In addition, we had previously hypothesized that the CDR loops could be important interaction sites within this domain for mAbs A and C at pH 6 based on experimental and *in silico* analysis described in detail in [19]. Accordingly, we were interested in evaluating the distribution of patches in the CDR loops at low pH. Figure 7 shows how the sum of the patch areas in the CDR changed as a function of pH. Different trends were observed for the CDR patches as compared to patches for the entire mAb (Figure 6). As can be seen in Figure 7A, the

sum of the positive patch areas in the CDR loops for mAbs B and C remained constant across the experimental pH range. In contrast, mAb A had the highest positive CDR patch area at pH <5.5 and this area rapidly decreased between pH 5.5 and 7. This decrease can be attributed to the titration of Histidine residues in the CDR of mAb A, as will be demonstrated below. Figure 7B plots the negative CDR patch surface area. mAb B had the highest negative surface patch area, which remained constant throughout this pH range. In addition, mAb C had a higher negative patch area in the CDR compared to mAb A between pH 5.5- 6.5, despite having a higher pI. Finally, as can be seen in Figure 7C, mAb B had the highest sum of the hydrophobic positive patch areas in the CDR loops followed by mAbs A and C.

The CDR loop patches qualitatively agreed with the experimental behavior observed for the three mAbs. For example, mAb A was more retained than mAb C below pH 6 in all resin systems and also had a higher positive patch area in the CDR loops. The decrease in positive patch area for mAb A between pH 6-7 coincided with the pH range in which elution order reversals were observed between mAbs A and C on the different resin systems. This analysis indicates that the CDR loops could indeed be important interaction sites for mAbs A and C in these multimodal systems.

At pH 6 we had previously observed negative and hydrophobic regions in the CDR of mAb B and had indicated that this may be partially responsible for the reduced binding of the Fab and $(Fab)_2$ domains as compared to the Fc region [19]. In the current work, $(Fab)_2$ B domain binding increased at lower pH, particularly on Prototype 5. The patch analysis presented in Figure 7 shows that the mAb B CDR was strongly negative and hydrophobic across the entire pH range of 5-7 indicating that this pH dependence of $(Fab)_2$ B binding is not explained by the CDR patch

392 data. Thus, the increased retention of the $(Fab)_2$ domain of mAb B is likely due to interactions
393 with other regions on the $(Fab)_2$.

394 Interestingly, the CDR loop hydrophobic patch area for mAb A appeared to increase slightly
395 with pH while that for mAbs B and C remained relatively constant across pH. To evaluate this
396 trend in more detail, patches are plotted graphically in Figure 8A and visualized on the mAb
397 surface. Figure 8B shows the top view of mAb A as a ribbon structure with the four CDR His
398 residue side chains and pKa values (reported as the average value from the two Fab domains)
399 shown explicitly. In Figure 8C, the positive and hydrophobic CDR loop patches are overlaid on
400 top of the ribbon structure for several pH values. As seen in Figure 8A, the decrease in positive
401 patch area and increase in hydrophobic patch area were somewhat correlated between pH 5.5 -
402 7.0, which was the range of the theoretical Histidine (His) residue pKa values (Figure 8B).
403 Figure 8C illustrates that the increase in hydrophobic and decrease in positive patch areas shown
404 in Figure 8A corresponded to the de-protonation of Histidine residues. At pH 5.5, His 2, 3, and 4
405 contributed to positive patches and served as bridges between the positive and hydrophobic
406 clusters, leading to a strong network of hydrophobic and positive patches. At pH 6, His 1 was un-
407 protonated and the associated positive charge patch disappeared, disrupting the patch network
408 and isolating the hydrophobic and charge patches indicated by the circle. Further increasing the
409 pH, deprotonated His 2 and 3, leaving a large hydrophobic patch without the strong positive
410 component observed at lower pH. The increase in hydrophobic patch area likely resulted from
411 additional contributions from Histidine residues as they were deprotonated at the higher pH.

412 Fewer Histidine residues were in the CDR loops for mAbs B and C compared to mAb A.
413 Figure 9 presents the top views of mAbs A, B, and C at pH 6 with the Histidine residue side
414 chains shown explicitly. The patches were visualized at pH 6 because the charge and hydropho-

bic patches for mAbs B and C remained relatively constant across pH 5.5-7 and pH 6 was representative of the patches in this range. As can be seen in Figure 9, the Histidine residues for mAbs B and C contributed to hydrophobic rather than positive patches even at low pH, which eliminated the positive to hydrophobic transition that was observed for mAb A (Figure 8C).

4. Conclusions

This work investigated how pH impacted mAb retention in MM CEX systems. The retention of three mAbs with different pI and surface hydrophobicities was evaluated using linear salt gradient experiments between pH 5-7. mAb retention showed an inverse, non-linear correlation with pH. Changing pH affected the elution order, creating unique windows of selectivity in each of the MM CEX resins. Three resins showed showed similar selectivity trends while a fourth resin with an aliphatic linker exhibited different selectivities and enabled the design of separations that were not possible in the other MM CEX resins. The unique behavior of mAb B on Prototype 5 in the range of pH 5-6 motivated the evaluation of domain contributions under these conditions. mAb B showed a spectrum of domain contributions, demonstrating that pH can be used to tune the relative importance of the $(Fab)_2$ and Fc domains for some mAbs.

Protein surface patches were calculated between pH 5-7 and connected to the experimental trends. Interestingly, differences in the titration of CDR loop patches for each mAb were shown to be a result of Histidine titrations and patches in this region were qualitatively correlated with experimental trends including the observed elution order reversals. The present patch analysis lays the foundation for bridging the gap between molecular level MD simulations and column-scale chromatography experiments to understand how these patches change with pH and the implications for identifying preferred binding regions under different mobile phase conditions.

These results indicate that pH can potentially be used as a lever for the strategic design of multimodal steps to create flow through, bind and elute, or weak partitioning operations with important implications for the design of integrated and/or continuous downstream purification processes. Furthermore, the ability to tune domain contributions using pH creates intriguing possibilities for current downstream challenges such as the removal of product-related impurities, as well as purification of bispecific mAbs.

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600 **Figure Captions**

601 **Figure 1.** Multimodal cation exchange ligands.

602 **Figure 2.** mAb retention in linear salt gradient experiments at pH 5-7 on (A) Capto MMC (B)
603 Nuvia cPrime (C) Prototype 4 and (D) Prototype 5. Refer to materials and methods section in
604 text for gradient details.

605 **Figure 3.** mAb B retention in linear salt gradient experiments on four different MM CEX resins.
606 Refer to materials and methods section in text for gradient details at each pH condition.

607 **Figure 4.** Domain contributions for mAb B at different pH conditions.

608 **Figure 5.** Visualization of positive (blue), negative (red), and hydrophobic (green) patches on
609 mAb surface for (A) mAb A (B) mAb B and (C) mAb C at pH 5 (top) and pH 7 (bottom).

610 **Figure 6.** Dependence of surface patch area on pH for (A) positive (B) negative and (C)
611 hydrophobic patches. At each pH, the y-axis represents the sum of the areas for all patches of the
612 specified type.

613 **Figure 7.** Dependence of CDR surface patch area on pH for (A) positive (B) negative and (C)
614 hydrophobic patches. At each pH, the y value represents the sum of the areas for all patches of
615 the specified type.

616 **Figure 8.** Analysis of mAb A CDR surface patches. (A) Dependence of patch area sum on pH.
617 (B) Top view of Fab A with His side chain residues shown in teal, light chain CDR loops in
618 purple, and heavy chain CDR loops in orange. His pKa values estimated in MOE are indicated in
619 parentheses. (C) Visualization of mAb A CDR patches from pH 5.5-7.

620 **Figure 9.** Top view of (A) mAb A (B) mAb B and (C) mAb C at pH 6 with His side chain
621 residues shown in green, light chain CDR loops in purple, and heavy chain in orange. CDR
622 surface patches are shown with positive patches in blue, negative patches in red and hydrophobic
623 patches in green.

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