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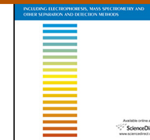

Conformational plasticity of IgG during protein A affinity chromatography

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ABSTRACT

Single step elution of a protein A column with 100 mM acetate pH 3.5 produced a curvilinear gradient with pH dropping steeply at first then more gradually as it approached endpoint. IgG with a native hydrodynamic diameter of 11.5 nm began to elute at pH 6.0 with a size of 9.4 nm. IgG size continued to decrease across the peak, reaching a minimum of 2.2 nm at pH 3.9. Secondary structure of early eluting IgG was only mildly affected but later eluting fractions became increasingly non-native with the 2.2 nm population exhibiting the highest proportion of β-sheet and lowest random coil of all conformations. Size reduction and structural change of IgG through this portion of the elution peak were attributed dominantly to a pre-existing tendency of highly concentrated IgG to adopt reduced size conformations at low pH and conductivity, facilitated by the known conformational relaxation of IgG by its interaction with protein A. IgG size increased to 10.4 nm as elution pH approached 3.5 across the tailing fractions. Major loss of β-sheet and increase of α-helix and random coil were observed in parallel. Late elution of this population was attributed to it being eluted from interactions with 2 distinct protein A domains, one bound to each side of the Fc region, creating a higher dissociation constant than single-site Fc-protein A interactions, and requiring more severely disruptive conditions for elution. The high degree of conformational disruption was attributed to simultaneous interaction of both heavy chains with protein A.

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1. Introduction

The conformation of IgG purified by protein A affinity chromatography has been long assumed to be fully native. This was confirmed in a recent study [1], where titration of protein A-eluted IgG to physiological conditions revealed size and secondary structure identical to IgG before its application to protein A. Surprisingly however, the antibody experienced transient conformational change during elution, accompanied by a reduction of average hydrodynamic diameter from about 11.5 nm to 5.5 nm. These results were interpreted to reflect the known effect of protein A destabilizing the upper portion of IgG's second constant domain [2,3], compounded by denaturating effects of low pH [4–9].

Despite its intuitive appeal, this interpretation might represent an oversimplification, and it could further represent a case of correlation not reflecting causality. Modeling based on ultracentrifugation and X-ray/neutron scattering has shown concentration-dependency of IgG4 size [10]. Formulation studies have shown that 100 g/L solutions of IgG1 in water adopted reduced size conformations at low pH [11,12]. Elevated concentration at low pH and conductivity are also features of IgG eluted from protein A. This suggests that concentration- and condition-dependent variability of IgG size probably contributed to the results reported in the previous protein A study [1].

The present study revisits IgG size and conformation during protein A affinity chromatography at a higher level of detail. Where the initial study analyzed eluted IgG as a single pool, the present study analyzes size and secondary structure at increments across the elution peak. It also considers the influence of how individual IgG molecules interact with protein A on columns. IgG's primary protein A binding site resides at the hydrophobic cleft between the second and third constant domains of IgG's Fc region [2,3]. There is one on each heavy chain. This creates potential for IgG to bind protein A domains at one or both sites [13], with potential to influence its elution characteristics, and potential for different elution characteristics to expose eluting subpopulations to different conditions.

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2. Materials and methods

2.1. Equipment, reagents, and preparation of experimental materials

Buffers, salts, and reagents were obtained from Sigma–Aldrich (St. Louis, MO), except allantoin, which was obtained from Merck Millipore (Darmstadt, Germany). Toyopearl AF-rProtein A-650F was obtained from Tosoh Bioscience (Tokyo). UNOsphere™ Q was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Capto™ adhere was obtained from GE Healthcare (Uppsala, Sweden). Chromatography media were packed in XK or Tricorn™ series columns (GE Healthcare). Chromatography experiments were conducted on an ÄKTA™ Explorer 100 or Avant 25 (GE Healthcare).

A prospective biosimilar IgG1 monoclonal antibody (Herceptin®) was expressed by mammalian cell culture in Chinese hamster ovary (CHO, DG44, Life Technologies, Carlsbad, CA) cells using a tricistronic vector developed by Ho et al. [14]. Antibody was produced in 5 L BIOSTAT® B stirred-tank glass bioreactor (Sartorius Stedim Biotech) fed-batch cultures using protein free medium consisting of an equal ratio of CD CHO (Life Technologies) and HyQ PF (GE Healthcare). Cultures were harvested at 30–50% viability. Pumps were avoided during harvest to minimize potential cell disruption.

Cells were removed by centrifugation and microfiltration, then the harvest clarified further by addition of caprylic acid to a final concentration of 0.4% and allantoin to a final concentration of 1%. pH was adjusted to 5.3 with 1 M acetic acid and the mixture was stirred for 2 h. UNOsphere Q pre-equilibrated with 50 mM MES, 150 mM NaCl, pH 5.3 was added at a proportion of 5% (v/v) and mixing continued for at least 4 h. Solids were removed by centrifugation and/or microfiltration.

IgG used to conduct the study was purified by protein A affinity chromatography with 20 mL of media packed in a XK 16/20 column (10 cm bed), run at linear flow rate of 300 cm/h. The column was equilibrated with 5 column volumes (CV) of 50 mM HEPES, 120 mM NaCl, pH 7.0 (HBS). 500 mL of chromatin-extracted cell culture supernatant was loaded and the column washed with 8CV HBS followed by a second wash with 50 mM Tris, pH 8. Antibody was eluted with a single step to100 mM acetic acid, pH 3.5. Protein was collected from the point where UV absorbance at 280 nm reached 50 mAU to the point where it descended below that value. The column was cleaned with 20CV of 0.1 M NaOH. Aggregates, antibody fragments, DNA and residual host cell proteins were further removed by titrating the protein A eluate to pH 8.0, adding NaCl to 1 M, and loading 1.5 g IgG onto a 150 mL Capto adhere column (XK 26/40, linear flow rate 280 cm/h) equilibrated to 50 mM Tris, 1 M NaCl, pH 8.0. The column was washed with 10CV of equilibration buffer and antibody was eluted with a step to 50 mM MES, 0.35 M NaCl, pH 6.0. Antibody was collected from the point where UV absorbance at 280 nm reached 50 mAU to the point where it dropped below that value. The column was cleaned with 10CV of 100 mM acetic acid, pH 3.0, then 20CV of 1 M NaOH.

2.2. Experimental methods

Experiments were conducted with purified IgG loaded onto 12 mL columns (XK 16/20 column, 6 cm bed height), run at linear flow rate of 300 cm/h. The column was equilibrated, washed, and eluted as above. 3 mL fractions were collected from the point where UV absorbance at 280 nm reached 50 mAU to the point where it descended below that value. Most experiments were conducted with IgG loaded to dynamic capacity at 5% breakthrough, which was about 25 g/L under the conditions employed.

Acetate buffers were prepared from acetic acid and titrated with NaOH to achieve the lowest possible conductivity. The starting buffer for linear pH gradient elution was 20 mM HEPES, 20 mM MES, 20 mM acetate, pH 7.0. The endpoint buffer was 20 mM HEPES, 20 mM MES, 20 mM acetate, pH 3.0. These gradient buffers were chosen specifically because of their low conductivity contributions. MES and HEPES are both zwitterionic and confer no conductivity. 20 mM acetate confers less than 0.2 mS/cm.

IgG concentration at different column strata was determined after loading a column to 50% of its dynamic capacity then washing; or after loading, washing, and elution at pH 4.4. Stratification was determined by removing the column endpieces then extruding the chromatography media in increments through the bottom of the column. The individual increments were collected and incubated in 100 mM acetic acid, pH 3.0 to release the IgG for quantitation.

2.3. Analytical methods

Purity of the IgG used to conduct the study was determined as described in Ref. [1]. In brief, HCP content was estimated by ELISA with a Generation III CHO HCP kit from Cygnus Technologies Inc. (Southport, NC). DNA was measured using a QX100™ Droplet Digital™ PCR System (Bio-Rad Laboratories). Aggregate content was measured by analytical size exclusion chromatography

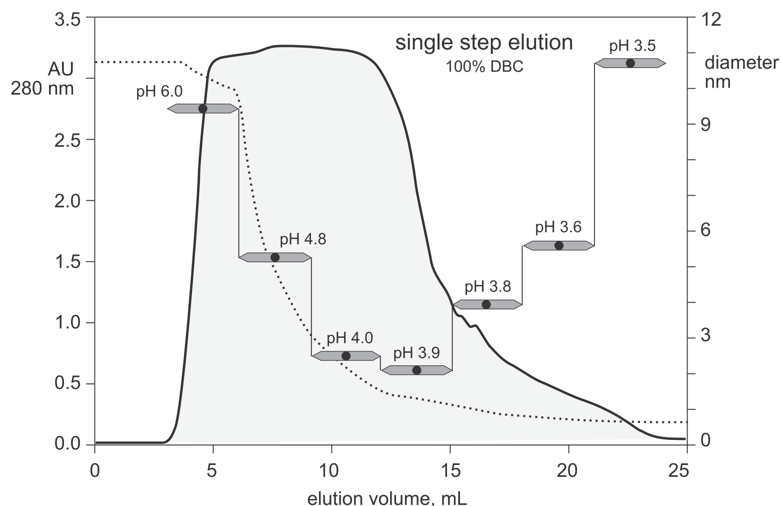

Fig. 1. IgG size across protein A elution by a single pH step. The horizontal bars represent the volume of each fraction taken for analysis. Listed pH values represent off-line measurements with a calibrated meter. The dotted trace represents pH measured by AKTA.

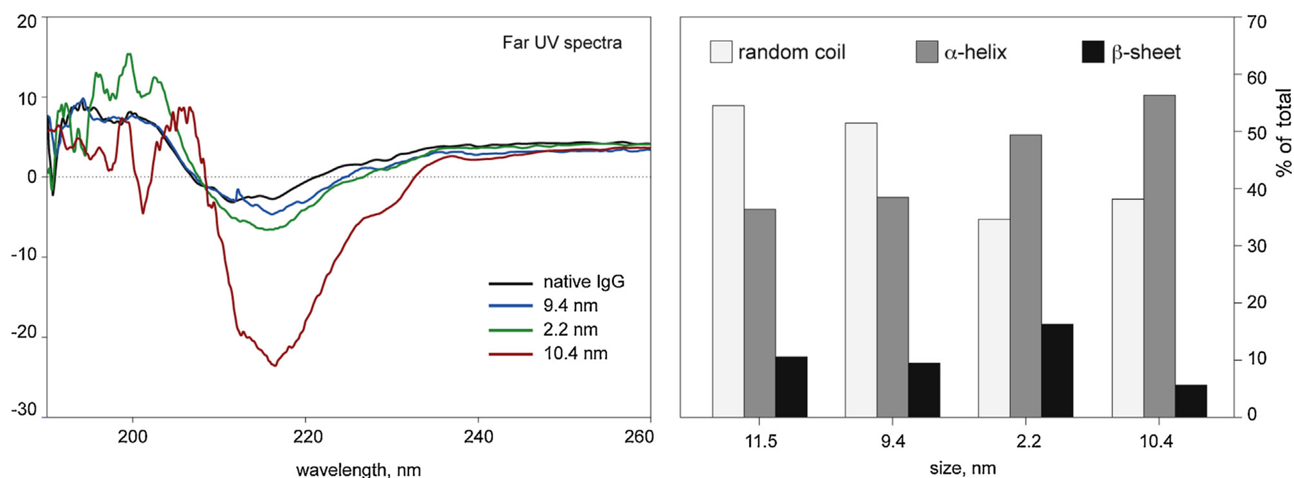

Fig. 2. CD spectra of different IgG sizes eluted from protein A in a single step. Native IgG profile in HBS. Indicated IgG sizes correspond to fractions shown in Fig. 1.

(SEC) with a G3000SWxl column (Tosoh Bioscience) on a Dionex UltiMate™ 3000 LC system (Thermo Scientific).

Solute size distributions in free solution were characterized by dynamic light scattering (DLS) using a Zetasizer ZS (Malvern Instruments, Worcestershire, UK). The sample (200 μL) was mixed gently for 10 s on a vortex before being placed into a quartz cuvette (ZEN2112, Malvern Instruments) using a gel loading tip to avoid bubbles. Viscosity of the carrier solution was determined using a SV-10 viscometer (A&D Company, Tokyo). The backscattered light at 173° was measured and 3 measurements were averaged. Attenuation index was maintained at a value of 7–8. Analysis of the data was performed using version 7.02 of the Dispersion Technology Software provided by the manufacturer.

Circular dichroism (CD) spectroscopy was performed with a JASCO J-810 spectropolarimeter (JASCO Corp., Tokyo). Far-UV spectra (190–260 nm) were obtained at an IgG concentration of 200 μg/mL using a quartz cuvette with a path length of 0.1 cm. Diluents were formulated to match the composition, pH and conductivity of the individual test samples, except lacking IgG, to minimize potential for inadvertant modification of secondary structure. 32 scans were accumulated with a scan rate of 100 nm/min and time constant of 0.125 s. All Spectra were corrected by subtracting the buffer baseline and averaged 32 times. All experiments were conducted at room temperature. Relative amounts of random coil, α-helix, and β-sheet were calculated using K2D2 software [15].

Other experimental details are described or reiterated for clarity in the following section.

3. Results and discussion

The present investigation was conducted with the same antibody used in the study that originally reported elution of reduced-size IgG from protein A [1]. As in that study, experiments were conducted using an IgG1 monoclonal antibody with a native hydrodynamic diameter of 11.5 nm, containing < 1 ppm host cell protein, <1 ppb host DNA, and <0.1% aggregates to minimize potential analytical interference. IgG has been shown to form stable associations with contaminants during elution from protein A [16]. In addition, large contaminating species are known to cause artifacts with dynamic light scattering (DLS) that inflate apparent size of smaller species in the same sample [1,17–19]. DLS was relied

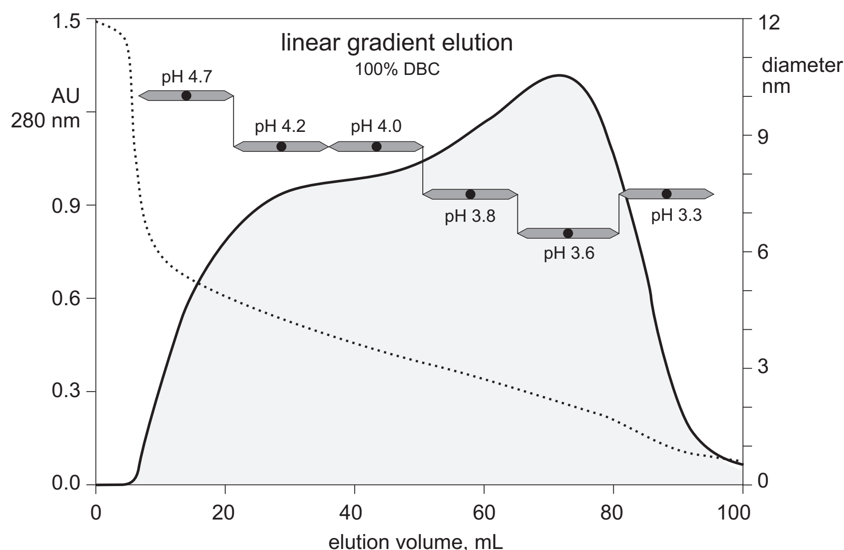

Fig. 3. IgG size across protein A elution by linear pH gradient. The horizontal bars represent the volume of each fraction taken for analysis. Listed pH values represent off-line measurements with a calibrated meter. The dotted trace represents pH measured by AKTA.

upon exclusively to provide IgG size data because antibody transport through size exclusion chromatography media was found to be retarded or prevented by non-specific interactions at low pH and conductivity [1].

3.1. Modification of IgG size and conformation by single step elution

Fig. 1 illustrates heterogeneity of IgG size across a protein A elution peak. Antibody was loaded to 25 mg/mL, representing its approximate dynamic capacity at 5% breakthrough. Elution was performed with a single step to 100 mM acetate, pH 3.5. The pH reduction curve during elution conformed with expectations based on the buffering characteristics of protein A (pI ~5.0 [20,21]) and acetate (pK_a 4.75). pH dropped quickly as the eluent displaced the wash buffer, then diminished more gradually approaching endpoint pH. The first IgG fraction eluted at pH 6.0 with a hydrodynamic diameter of 9.4 nm. Size decreased with diminishing pH, reaching a minimum diameter of 2.2 nm at pH 3.9. IgG size increased steadily thereafter to 10.4 nm at pH 3.5.

Fig. 2 compares circular dichroism (CD) spectra and secondary structural proportions. IgG collected from the leading fraction showed a slight increase in the proportion of α-helix accompanied by minor decreases in random coil and β-sheet. These results were interpreted to indicate native antibody architecture was substantially conserved.

Conformation of the 2.2 nm population surprisingly showed an increase in secondary structure, compared even with the native antibody. β-sheet achieved its highest value among all conformations, increasing from 10.5% to 16.2%. α-helix increased about a quarter from 36.2% to 49.1%. Random coil was reduced by roughly a quarter compared to the native molecule, from 53.4% to 34.5%. These results were interpreted to indicate that although the structure was far from native, it represented a stable intermediate.

Secondary structure of 10.4 nm IgG from the tail fraction was heavily modified. Its dominant feature was reduction of β-sheet content to its lowest proportion among all conformations, at 5.7%. This represented a reduction of nearly half from the native molecule, and nearly two thirds from the 2.2 nm conformation. In contrast, the proportion of α-helix increased to its highest value among all conformations, 56%. The proportion of random coil increased to 38.1% from its 34.5% in the 2.2 nm conformation, but remained much lower than its 52.4% in the native molecule. These results were collectively interpreted to indicate gross loss of native structure approaching pH 3.5, with the size increase caused by excess α-helix extending the hydrodynamic axis of the protein.

3.2. Modification of IgG size by linear gradient elution

Fig. 3 illustrates the profile from linear pH gradient elution of the same column loaded in a speratate experminent with the same amount of the same IgG preparation. IgG began to elute at pH 4.7, as opposed to pH 6.0 in the step elution. The difference in apparent IgG behavior was attributed to differences of in-column pH titration between the elution formats. It was considered that the step elution would have eluted IgG from the top of the column immediately following its introduction. Since unretained proteins are transported more rapidly through porous particle columns than ions, that IgG would exit the column ahead of the conditions that dissociated it from the ligand. Linear gradient elution would provide a more accurate indication of the conditions for dissociation of IgG from protein A since the gradual change of conditions would reduce the differential between the column inlet and outlet, compared to the inlet-outlet differential at the early stages of the step gradient.

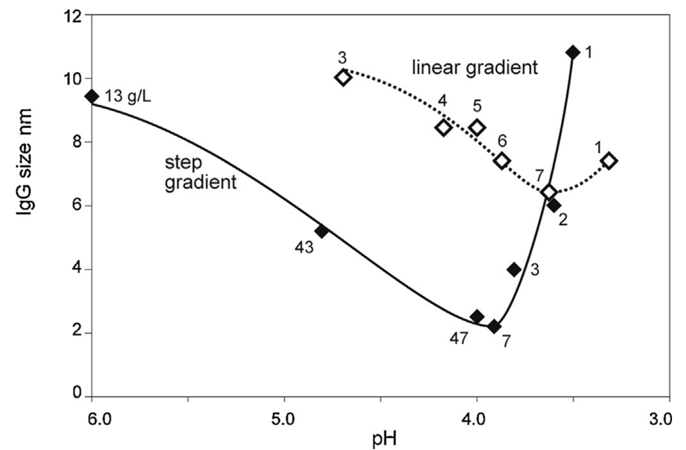

Fig. 4. pH dependency of IgG size as a function of pH for step and linear elution gradients. Numerical values associated with each data point represent IgG concentration in g/L.

Variations in IgG size as a function of pH during linear gradient elution followed the same trends as the single step. IgG size diminished across the main body of the peak and increased across the tailing fractions, but with significant distinctions from the step format. Amplitude of size variation with respect to pH was much less in the linear format while absolute size was much higher. The discrepancy between formats indicated signficant influence by one or more factors beyond pH. The previous study suggested that protein A-mediated denaturation cooperatively enhanced pH effects [1] but that should have affected both elution formats equally.

3.3. The influence of IgG concentration on IgG size

Fig. 4 highlights a major offset of IgG concentration between the two elution formats. Concentration values were determined off line after elution because they were obscured during elution by the chromatograph's UV monitor being saturated during passage of the main body of the peak. There seemed to be no rationale or precedent to suggest that IgG concentration should particularly affect IgG size during protein A chromatography, but emerging research in other areas has shown that antibody size can vary significantly with concentration under certain conditions.

Recent formulation studies have shown dramatic reduction in the size of purified IgG1 monoclonal antibodies at concentrations up to 100 g/L in water [11,12]. The effect is enhanced at lower pH values, causing reductions of IgG size to as low as 2 nm at pH 4.5. Similar size reduction of IgG4 monoclonal antibodies has been described, but beginning at concentrations as low as 2 mg/mL [10]. Detailed size and structural characterization based on analytical ultracentrifugation and neutron/x-ray scattering led the investigators to suggest the effect was mediated by a diffusion-collision mechanism in which higher frequency of collisions would promote adoption of a more compact conformation.

Despite their common element of increasing antibody concentration, the mechanisms for IgG1 and IgG4 differ at least in degree. Where the IgG4 phenomenon was observed to be independent of NaCl concentration [10], size of IgG1 was strongly dependent on low conductivity [11,12]. The previous study reporting reduced IgG1 size in conjuction with protein A also described dependency on low conductivity [1]. Elevation of NaCl increased IgG1 size, and IgG in physiological formulations retained its native size even at concentrations greater than 20 mg/mL.

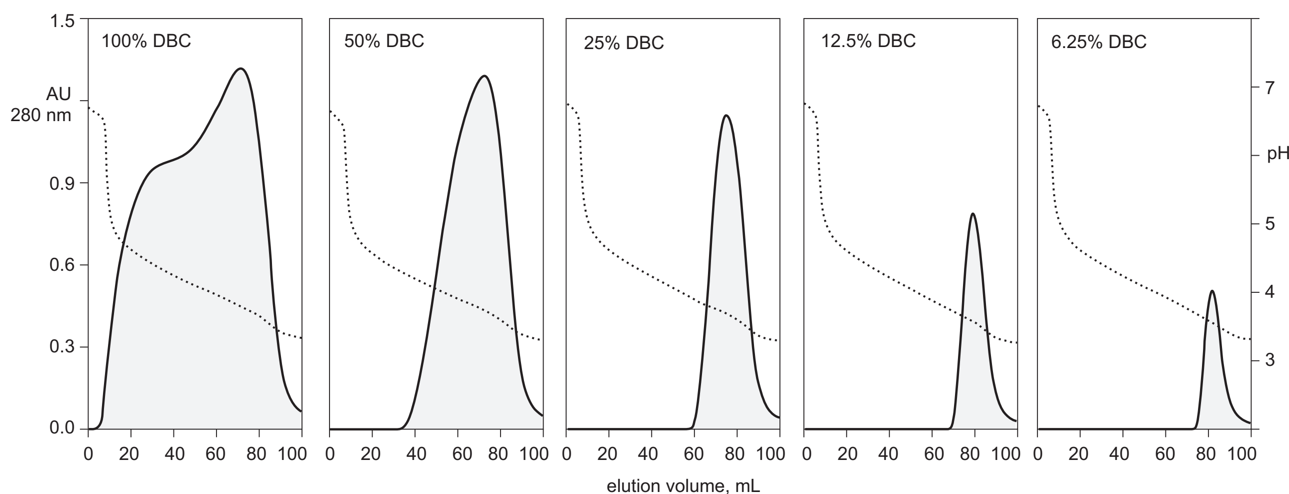

Fig. 5. Linear pH gradient elution from protein A at different column loads.

Given that elution from protein A creates largely the same conditions observed to reduce IgG size in formulation studies, it seems reasonable to suggest those conditions would impose the same effect during protein A. This seems to explain the size reduction across the main peak fractions in both elution formats. It would also explain the size offset between the step and linear gradient curves in Fig. 4. The lower concentration of IgG eluting in the linear gradient would have caused IgG size to be reduced to a lesser degree than the more highly concentrated antibody eluted in the step format.

An apparent weakness of this interpretation is that the 2.2 nm conformation eluted at only 7 g/L. This would seem to be below the threshold required to promote size reduction for IgG1 [11,12], but the IgG4 study identified a phenomenon that reconciles the discrepancy. IgG4 is more flexible conformationally than IgG1. Even at low concentrations, it easily adopts configurations with the Fab regions in contact with the Fc, covering and blocking access to the complement-activation sites [10]. The interaction of protein A with IgG1 is known to create disorder in the upper third of the second constant region of IgG and extending up into the hinge, also with the effect of increasing the flexibility of the mid-region of the molecule [2,3]. This suggests a hypothesis whereby excess flexibility induced by IgG-protein A interactions cooperatively reduces the concentration threshold for size reduction.

3.4. Increasing IgG size across tailing fractions

The increase in IgG size across tailing fractions highlights that it must derive from a distinct mechanism. Its secondary structure suggests it involves severe denaturation, but denaturation does not explain why these fractions elute late. Higher levels of denaturation are generally understood to support more effective dissociation of bioaffinity interactions. This would favor earlier elution, not later. Interestingly, the structure of IgG itself suggests a hypothesis that harmonizes these apparent conflicts.

The primary binding site for protein A is situtated at the hydrophobic cleft between the second and third constant domains of the heavy chain [2,3]. Since the molecule is bilaterally symetrical, there are two such sites per molecule, one on each heavy chain. This creates a possibility for a single IgG molecule to participate in one or two binding events. Dual-site binding has been suggested to create a cooperative effect that would increase the net dissociation constant and cause later elution of that population from protein A columns [13]. It also seems evident that if binding to one protein A molecule conformationally disrupts IgG [2,3], binding to two protein A molecules must disrupt it more.

Ligand presentation on modern protein A media facilitates dual-site binding [13]. Protein A naturally exists in a tentacle format and most commercial media immobilize it by an engineered terminus so it extends from the surface of the solid phase like bristles of a

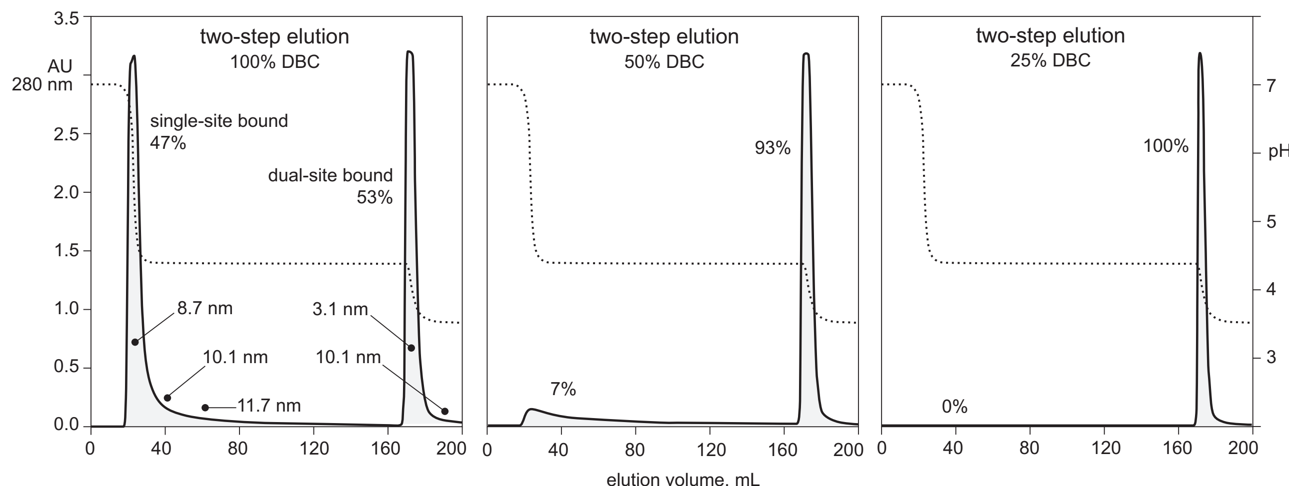

Fig. 6. The effect of column load on elution of IgG at pH 4.4 and pH 3.5.

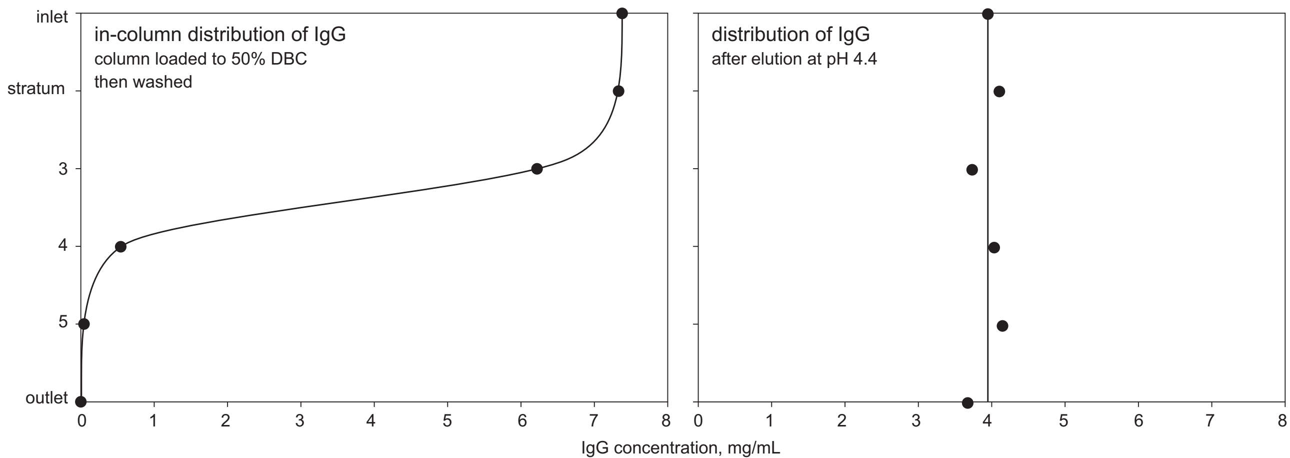

Fig. 7. IgG concentration across protein A column strata before and after elution at pH 4.4.

brush. The individual bristles are understood to be flexible owing to the random coil linkages between the 4–5 IgG-binding domains on each protein A molecule. Their extension into the mobile phase and their inter-ligand spacing is understood to be mediated by mutual electrostatic repulsion among individual IgG-binding domains due to their negative charge at physiological pH [20,21].

This creates an expectation that IgG first entering a column would most likely become bound by both of its protein A-binding sites; stepwise, initially by one, then the other. Each such event would cross-link the participating protein A ligands. Later-entering IgG would encounter fewer potential binding sites and need to overcome cross-link-restricted access to contact them. Dual-site binding should therefore become less frequent in proportion with column load, resulting in an increasing proportion of single-site bound IgG as the column approaches saturation.

Fig. 5 supports this hypothesis. IgG loaded to 6.25%, 12.5%, and 25% DBC produced roughly symmetrical elution peaks at the end of the gradient. These peaks were interpreted to dominantly contain dual-site-bound IgG. Loading to 50% DBC produced strong asymmetry with a shallower slope to the leading side suggestive of incipient bimodality. Loading to 100% DBC produced obvious bimodality, with early eluting single-site bound IgG overlapping with later eluting dual-site bound IgG.

These findings were strongly indicative of dual-site bound IgG manifesting a larger dissociation constant that should enable its fractionation from single-site bound IgG in a two-step gradient. This was confirmed in Fig. 6, showing results from columns loaded at 100%, 50%, and 25% DBC. Initial elution at pH of 4.4 released a large peak from the column loaded to 100% DBC. This was interpreted to represent IgG bound by single-site interactions. The same treatment released very little IgG from the column loaded to 50% DBC, suggesting most of the IgG was bound by dual-site interactions. pH 4.4 elution released nothing from the column loaded to 25% DBC, suggesting all of the IgG was bound by dual-site interactions. The balance of the IgG, presumed to represent dual-site bound IgG, was eluted in all cases by a step to pH 3.5.

Characterization of IgG distribution vertically through the column permitted deeper insight. IgG was concentrated near the top of the column after loading and washing (Fig. 7). After the pH 4.4 elution step, it was distributed evenly at a lower concentration over the entire length of the column. This was interpreted to indicate that single-site bound IgG became dissociated from protein A at pH 4.4, and some re-bound through dual-site interactions as it passed into the unsaturated regions of the lower column strata. These results suggested the 25% DBC load in Fig. 6 might initially have included some proportion of single-site-bound IgG, all of which was re-captured by dual-site interactions after elution at pH 4.4. At

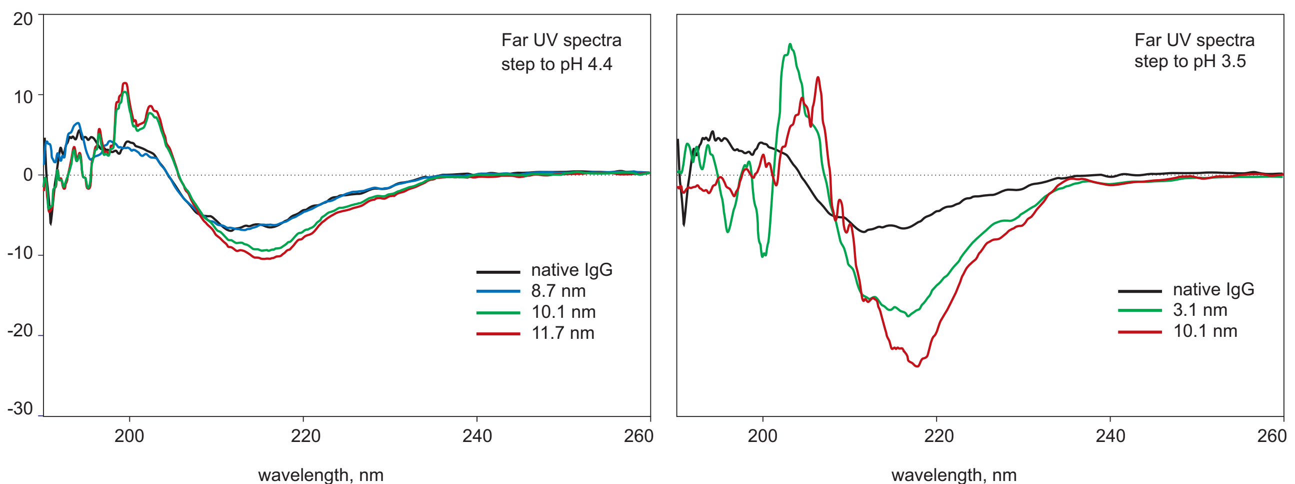

Fig. 8. CD spectra of IgG eluted from protein A by successive steps to pH 4.4 and pH 3.5. Native IgG profile in HBS. Indicated IgG sizes correspond to fractions shown in Fig. 6.

higher IgG loads, the greater amounts of dual-site-bound IgG would have left less opportunity to re-bind single-site-eluted antibodies, and they would have exited the column as shown (Fig. 6).

3.5. Denaturation or facilitation?

Protein A affinity chromatography, especially with acidic elution, has had a long and controversial association with antibody denaturation [2–9,13,22]. This tends to imply that protein A imposes a destructive influence on antibodies, and the present results could be interpreted to support that view. In context with other recent studies however, the present data suggest instead that protein A facilitates already-established conformational behavior patterns that are inherent to IgG. A previous study noted a parallel between protein A and enzyme-substrate interactions [1]. Conformational modification of IgG by contact with protein A could be viewed as an example of induced fit. The ability of elution at pH 3.5 to induce conformational changes normally associated with much lower pH values (independent of protein A) could be viewed as an example of protein A lowering the activation energy required to produce those particular conformations [1]. The apparent ability of protein A to facilitate reduction of IgG1 size at low antibody concentrations appears to be another example of reducing the activation energy required to produce those conformations (Fig. 8).

Gross loss of native secondary structure among tailing elution fractions seems more directly suggestive of protein A imposing a destructive influence, but the effect is only temporary and it is entirely reversible by exposure to physiological conditions, even after holding the antibody at pH 3.5 for 7 days [1]. This seems a fair indication that even the late-eluting grossly modified conformations reside within the normal range of IgG's structurally programmed behaviors.

3.6. Practical ramifications

Transient conformational changes nevertheless remain a potential concern. Besides reduced size and non-native secondary structure, protein A-eluted IgG still under elution conditions has a highly elevated tendency to participate in non-specific interactions [16], and the technique involves many variables beyond just the interaction of IgG with protein A. Protein A co-retains chromatin heteroaggregates that leach diverse host contaminants during elution, enabling IgG-contaminant interactions during elution that inflate contamination and promote aggregation [1,16,23,24]. Conformationally modified IgG eluted from protein A is also more vulnerable to aggregation than native IgG, forming persistent aggregates under conditions where native IgG does not [1,16]. Mazzer et al. [25] showed that protein A-purified IgG4 suffered disproportionate aggregation when it was held at low pH for virus inactivation. Shukla et al. [26] reported that protein A accelerated aggregate formation at low pH.

Specifically how the present observations may contribute to these phenomena remains to be investigated, but one valuable contribution is already apparent: protein A can be used as a tool to generate IgG size and conformational variants for experimentation. This offers hope of better understanding many aspects of protein A affinity chromatography and may lead to worthy practical solutions. Beyond protein A, the study's most important contribution may lie in promoting awareness that IgG conformation is much more plastic than has been generally appreciated. Any purification technique that concentrates IgG and exposes it to low pH and conductivity seems likely to be affected but, as with protein A, stresses created by IgG-ligand interactions could broaden the scope. Concentration-dependent conformational variability could also prove to be a feature of many proteins beyond IgG.

4. Conclusions

Size and conformation of an IgG1 monoclonal antibody eluted from protein A were influenced by protein A-mediated denaturation of IgG, by IgG concentration, by pH and conductivity. IgG on the leading side of the elution profile exhibited a hydrodynamic diameter of 9.4 nm and relatively minor disruption of secondary structure. Size diminished across the main body of the peak to a minimum of 2.2 nm with a highly non-native secondary structure. Size reduction dominantly derived from a pre-existing tendency of IgG to adopt smaller conformations at elevated concentrations, low pH and conductivity. Conformational disruption of the antibody upon initial contact with protein A facilitated the transition and enabled it to occur at modest IgG concentrations. Dramatic loss of secondary structure across tailing fractions was accompanied by an increase in IgG size upto 10.4 nm, apparently caused by excess α-helix extending the hydrodynamic axis of the protein. Late elution of tailing fractions resulted from the antibodies in that fraction being bound simultaneously to two protein A ligands, one on each side of the Fc region, creating a higher dissociation constant and requirement for more severe conditions to achieve elution. Their high degree of structural modification appeared to result from simultaneous interaction of both heavy chains with distinct protein A domains.

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References

[1] P. Gagnon, R. Nian, D. Leong, A. Hoi, Transient conformational modification of IgG during purification by protein A affinity chromatography, J. Chromatogr. A 1395 (2015) 136–142.

[2] J. Deisenhofer, Crystallographic refinement and atomic models of a human Fc fragment ant its complex with fragment B of protein A from Staphylococcus aureus at 2.9 and 2.8 Å resolution, Biochemistry 20 (1981) 2361–2370.

[3] J. Deisenhofer, T. Jones, R. Huber, J. Sjödahl, J. Sjöquist, Crystallization, crystal structure analysis and atomic model of the complex formed by a human Fc fragment and fragment B of protein A from Staphylococcus aureus, Hoppe-Seylars Z. Physiol. Chem. 359 (1978) 975–985.

[4] T. Arakawa, J. Philo, K. Tsumoto, R. Yumioka, D. Ejima, Elution of antibodies from a protein-A column by aqueous arginine solutions, Protein Expr. Purif. 36 (2004) 244–248.

[5] B. Baynes, D. Wang, B. Trout, The role of arginine in the stabilization of proteins against aggregation, Biochemistry 44 (2005) 4919–4925.

[6] R. Latypov, S. Hogan, H. Law, H. Gadgil, D. Liu, Elucidation of acid-induced unfolding and aggregation of human immunoglobulin IgG1 and IgG2 Fc, J. Biol. Chem. 287 (2012) 1381–1396.

[7] M. Thies, R. Kammermeier, K. Richter, J. Buchner, The alternatively folded state of the antibody C_H3 domain, J. Mol. Biol. 309 (2001) 1077–1085.

[8] J. Buchner, M. Renner, H. Lilie, H.J. Hinz, R. Jaenicke, T. Kiefhabel, R. Rudolph, Alternatively folded states of an immunoglobulin, Biochemistry 30 (1991) 6922–6929.

[9] S. Martsev, Z. Krachuk, A. Vlasov, G. Lyakhnovich, Thermodynamic and functional characterization of a stable conformer obtained by renaturation from a partially structured low pH-induced state, FEBS Lett. 361 (1995) 173–175.

[10] L. Rayner, G.K. Hui, J. Gor, R. Heenan, P. Dalby, S. Perkins, The Fab conformations of human immunoglobulin G4 (IgG4) restrict access to its Fc region: implications for functional activity, J. Biol. Chem. 289 (2014) 20740–20756.

[11] W., Fraunhofer, A., Bartl, H.J., Krause, M., Tschoepe, K., Kaleta, Antibody formulations and methods of making same, United States Patent US 8,420,081 B2, 2013, p. 1–119.

[12] D. Dong, Impact of ion-mAb interactions on the UFDF process: towards process and product design, in: Oral presentation at the Bioprocess International Conference and Exhibition, Boston, 2015.

[13] P. Gagnon, Purification tools for monoclonal antibodies, validated biosystems, Tucson (1996) 155–198.
[14] S.C. Ho, M. Bardor, H. Feng, Mariati, Y.W. Tong, Z. Song, M.G.S. Yap, Y.S. Yang, IRES-mediated Tricistronic vectors for enhancing generation of high monoclonal antibody expressing CHO cell lines, J. Biotechnol. 157 (2012) 130–139.
[15] C. Perez-Iratxeta, M. Andrade-Navarro, K2D2: estimation of protein secondary structure from circular dichroism spectra, BMC Struct. Biol. 8 (2005) 25.
[16] P. Gagnon, R. Nian, Y.S. Yang, Q. Yang, C.L. Lim, Non-immunospecific association of immunoglobulin G with chromatin during elution from protein A inflates host contamination aggregate content, and antibody loss, J. Chromatogr. A 1408 (2015) 151–160.
[17] K. Stulik, V. Pacakova, M. Ticha, Some potentialities and drawbacks of contemporary size-exclusion chromatography, J. Biochem. Biophys. Met. 56 (2003) 1–13.
[18] T. Arakawa, D. Ejima, T. Li, J. Philo, The critical role of mobile phase composition in size exclusion chromatography of protein pharmaceuticals, J. Pharm. Sci. 99 (2010) 1674–1692.
[19] K. Ahrer, A. Buchacher, G. Iberer, A. Jungbauer, Analysis of aggregates of human immunoglobulin G using size exclusion chromatography, static and dynamic light scattering, J. Chromatogr. A 1009 (2003) 89–96.
[20] I. Björck, B. Petersson, J. Sjöquist, Some physicochemical properties of protein A from Staphylococcus aureus, Eur. J. Biochem. 29 (1972) 579–584.
[21] J. Langone, Protein A of Staphylococcus aureus and related immunoglobulin receptors produced by streptococci and pneumococci, Adv. Immunol. 32 (1982) 157–252.
[22] S. Vunnum, G. Vedantham, B. Hubbard, Protein A-based affinity chromatography, in: U. Gottschalk (Ed.), Process Scale Purification of Antibodies, J. Wiley and Sons, Hoboken, 2009, pp. 79–102.
[23] P. Gagnon, R. Nian, J. Lee, L. Tan, S.M. Abdul-Latiff, C.L. Lim, C. Chuah, X. Bi, Y.S. Yang, W. Zhang, H.T. Gan, Nonspecific interactions of chromatin with immunoglobulin G and protein A, and their impact on purification performance, J. Chromatogr. A 1340 (2014) 68–78.
[24] R. Nian, P. Gagnon, Advance chromatin extraction inproves capture performance by protein A affinity chromatography, J. Chromatogr. A (2015), http://dx.doi.org/10.1016/j.chroma.2015.12.044.
[25] A. Mazzer, X. Perraud, J. Halley, J. O'Hara, D. Bracewell, Protein A chromatography increases monoclonal antibody aggregation rate during subsequent low pH virus inactivation hold, J. Chromatogr. A 1415 (2015) 83–90.
[26] A. Shukla, P. Gupta, X. Han, Protein aggregation kinetics during protein A chromatography: a case study for an Fc fusion protein, J. Chromatogr. A 1171 (2007) 22–28.