Multimodal Interactions of IgG, Fab, F(ab')$_2$, Fc, and Aggregates with Hydroxyapatite

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Introduction

Hydroxyapatite (HA) has demonstrated valuable utility in the field of IgG purification for its ability to remove aggregates, to achieve single-step IgG purity approaching levels achieved by affinity methods, and to purify Fab and other fragmentary constructs. This emphasizes the need for a detailed understanding of how IgG and related architectures bind to HA, since such an understanding is necessary to support both purification process development and validation. It equally emphasizes the need for a set of tools to characterize retention.

HA is a multimodal solid phase adsorbent with the structural formula $(Ca_5(PO_4)_3OH)_2$. Crystal surface calciums each bear a single positive charge capable of mediating electrostatic interactions with protein residues, including both attraction of negatively charged residues and repulsion of positively charged residues. Protein polycarboxyl domains in which individual residues are appropriately spaced may participate in chelating interactions with HA calcium. Histidyl coordination with calcium has been evaluated but found not to contribute to retention of IgG. Crystal surface phosphates each bear two negatively charged oxygen atoms capable of mediating electrostatic attraction of protein amino residues or repulsion of carboxyl residues. Electrostatic interactions are influenced by pH and conductivity without respect to the identity of the salt. Calcium affinity becomes weaker at alkaline pH, but is resistant to conductivity as indicated by persistent binding of IgG at 1-2 M sodium chloride in the absence of phosphate. Elution of chelating interactions usually requires the presence of ions with high affinity for calcium, such as phosphate.

The obvious potential for complex multimodal interactions is enhanced by the arrangement of calcium and phosphate on the crystal surface. Individual calcium residues are surrounded by an inner perimeter of 3 negatively charged oxygen atoms, about 120 pm distant, and an outer perimeter of 3 more at about twice that distance (Figure 1). This configuration reveals a 6:1 dominance of negative to positive charges per unit, but one negatively charged oxygen from each unit is shared by the neighboring unit, reducing the cumulative surface charge ratio to 5:1. This disagrees with the charge ratio implied by the structural formula because the majority of calcium and phosphate groups are involved in the crystal structure. The simultaneous influence of the many potential interactions, compounded by differences among proteins with respect to the number of charges, isoelectric point (pI), and conformational distribution of charges, creates selectivites that are highly distinctive from single-mode adsorptive methods such as anion or cation exchange chromatography.

Materials and methods

Antibody fragments were generated from a humanized monoclonal IgG_1 by digestion with immobilized papain or pepsin. Tetra-aggregates were purified from another monoclonal IgG_1 by HA chromatography. CHT™ type I, 40μm was obtained from Bio-Rad Laboratories and packed in 5 x 50 mm MediaScout® columns by ATOLL. A series of experiments to evaluate the combined effects of phosphate and calcium was conducted with sodium chloride gradients over a range of phosphate concentrations. A 1 mL column of CHT type I 40 μm was equilibrated with 20 mM Hepes, pH 7.0, at a linear flow rate of 600 cm/hr. 100 μL of sample was injected, and the column washed with equilibration buffer. The sample was eluted with a 20 column volume (CV) linear gradient to 50 mM Hepes, 1.0 M sodium chloride, pH 7.0. In a subsequent experiment, the equilibration, wash, and elution buffers contained 5 mM sodium phosphate, and in subsequent experiments, both buffers contained 10, 20, 40, 80, or 160 mM sodium phosphate. This was followed by a series of phosphate gradients at different chloride concentrations. The column was equilibrated with 20 mM Hepes, pH 7.0, loaded, washed, and eluted with a 20 CV linear gradient to 160 mM sodium phosphate, pH 7.0. It was then cleaned with 500 mM sodium phosphate, pH 7.0. A second experiment was run with 10 mM sodium chloride in both gradient buffers. Subsequent runs were conducted in the presence of 20, 40, 80, and 160 mM sodium chloride.All chromatography experiments were conducted on an AKTA™ Explorer 100 from GE Healthcare.

Results and Discussion

Figure 2 plots retention of IgG in sodium chloride gradients at different phosphate concentrations; Figure 3 in phosphate gradients at different chloride concentrations. Retention of lysozyme and BSA are plotted to represent the behavior of calcium affinity-dominated and cation-exchange-dominated dominated proteins. BSA is dominated by calcium affinity, lysozyme by cation-exchange. Figure 3 is notable because elution conductivities ascend with increasing sodium chloride concentration. This highlights the conductivity resistance of calcium affinity. If binding was purely electrostatic for any solute, then its retention curve would be horizontal (also in Figure 2). The fact that the BSA curve so closely paralells the line of conductivity at gradient start shows that it is almost completely insensitive to conductivity. Despite the different contexts, both figures demonstrate that cooperative binding between calcium affinity and cation exchange dominate retention of IgG. Varations in curve shapes in the early parts of the gradients of some solutes suggest weak electrostatic contributions by other interactions.

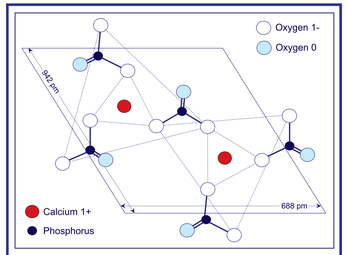

Figure 1. Distribution of charges on the surface of hydroxyapatite. Modified from Kawasaki, 1991, J. Chromatogr. 544 147

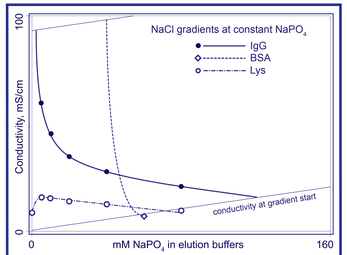

Figure 2. Retention of IgG, BSA, and lysozyme in sodium chloride gradients at different phosphate concentrations.

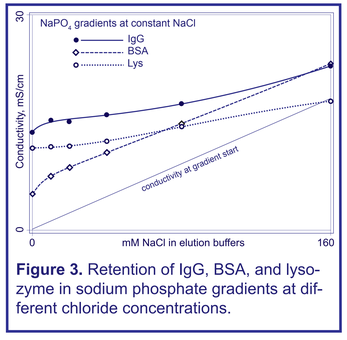

Figure 3. Retention of IgG, BSA, and lysozyme in sodium phosphate gradients at different chloride concentrations.

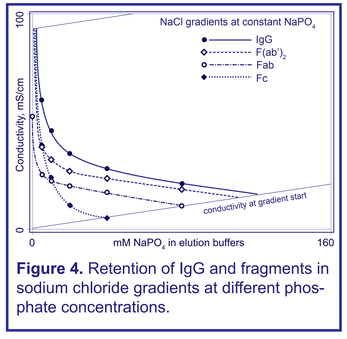

Figure 4. Retention of IgG and fragments in sodium chloride gradients at different phosphate concentrations.

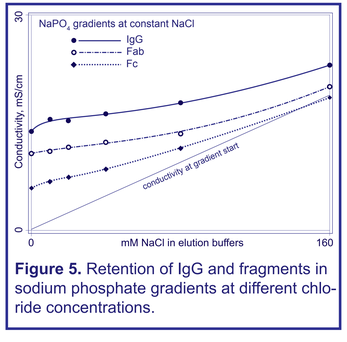

Figure 5. Retention of IgG and fragments in sodium phosphate gradients at different chloride concentrations.

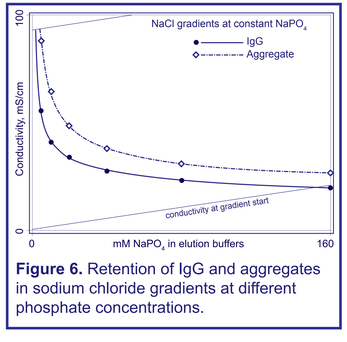

Figure 6. Retention of IgG and aggregates in sodium chloride gradients at different phosphate concentrations.

Acknowledgements

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Figure 4 compares retention of fragments with IgG in sodium chloride gradients at different phosphate concentrations; Figure 5 in phosphate gradients at different chloride concentrations. Like, IgG, retention curves for antibody fragments demonstrate cooperative binding, but each exhibits different relative contributions by calcium affinity and cation exchange. Fc retention was dominated by calcium affinity, while retention of Fab was dominated by cation-exchange. F(ab)'2 exhibited a curve shape similar to Fab, but stronger retention. Note that the retention curve for intact IgG incorporated the distinctive elements of its fragments but stronger retention.

Tetra-aggregates bound more strongly than IgG, by both calcium affinity and cation exchange, but not by a factor of four (Figure 6). This is logical since associations among the individual IgG components are certain to block some interactions with HA. This provides an example of negative cooperativity.

Results from studies such as this have direct practical value for identifying the most effective conditions to achieve a particular separation. The greater the vertical offset between the elution conductivities for a given pair of solutes at a specific phosphate concentration, the greater the separation. Figure 6 thus demonstrates that a sodium chloride gradient applied at 5 mM phosphate supports the most effective aggregate removal. Figure 4 shows that the same conditions also support the best removal of Fab. Fab contamination tends not to be an issue in IgG purification because it it does not bind well to protein A, but Fc fragments are frequently a challenge. Fc would be removed at the same conditions, though more effectively at 10 mM phosphate, and even more at higher phosphate concentrations. Concentrations higher than 10 mM phosphate however would compromise aggregate removal. Comparison with Figure 2 demonstrates that 10 mM phosphate would also support removal of BSA and other acidic contaminants, as well as small alkaline contaminants.

The phosphate-chloride grid approach also provides a systematic characterization tool that can be used to support process validation. With sodium chloride gradients for example, the variation in the vertical offset between two solutes over a specified range of phosphate concentration illustrates the sensitivity of the separation to variations within that range. Grid results can thus be used to document the quantitative response of the process to any pertinent variable, and to document that process specifications lie within a range where routine variations in such variables will not adversely affect process control.