A 3-mechanism model for adsorption of IgG on CHT™ ceramic hydroxyapatite

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Introduction

Previous work with IgG discussed retention on CHT ceramic hydroxyapatite by a combination of two mechanisms: phosphoryl cation exchange involves the interaction of positively charged amino groups on proteins with the negatively charged phosphate groups on CHT, and calcium metal affinity, which involves the protein carboxyl groups and the positively charged calcium groups on CHT. Although the initial binding of the latter mechanism is also electrostatic, they eventually form much stronger coordination bonds.

A third mechanism which has not been explored previously is metal-histidine interaction. Metal affinity ions such as calcium, as presented in CHT, can act as electrophiles, seeking the possibility of sharing electron pairs with other atoms so that a bond or charge-charge interaction can be formed. Such an interaction has been demonstrated between metal ions and the imidazole groups of histidyl side chains in a variety of enzymes. Extension of this observation to IgG which carries six histidine residues on its FC region and other histidine residues on its CDR suggests the possibility of histidine-calcium interaction as contributory to IgG retention.

The present study attempts to determine the reactive constituents of the three mechanisms: phosphoryl cation exchange, carboxyl chelation and calcium-histidine interaction. Results will be presented demonstrating the effect of pH, phosphate and imidazole on purification performance.

Experimental

Chemicals

CHT Type I 20 µm and 40 µm were obtained from Bio-Rad Laboratories (Hercules, Ca). All chemicals were purchased from VWR (Brisbane, Ca). The phosphate buffers were prepared with sodium phosphate monobasic in nanopure water and were titrated to target pHs with 1M NaOH.

Proteins

Protein A eluate: Clarified tissue culture fluid obtained from Avid Biosciences (Tustin, Ca) was applied to a Millipore ProSep-A column. IgG was obtained by elution using 0.1M glycine, 0.05M NaCl, pH 3.8. The eluate was demonstrated to contain monomer and up to 40% aggregate by size exclusion chromatography on a Bio-Sil® SEC 400-5 column (Bio-Rad Laboratories (Hercules, Ca).

Polyclonal human IgG was purchased from Sigma-Aldrich (St. Louis, Mo).

IgG1 and IgG4: Protein A eluate containing >95% monomer.

Chromatography

The experiments were conducted on a Bio-Scale™ MT2 column obtained from Bio-Rad Laboratories (Hercules, Ca). The column dimensions were 0.7 x 2.6 cm. The column was dry packed using a density of 0.60 gm/mL. Each column was wetted in ten column volumes of 20% ethanol before equilibration. All chromatography experiments were automated and executed using the Biologic DuoFlow™ System and software. To determine the elution profile, chromatography was carried out with a sodium chloride at target pH. As an example, the column was equilibrated in 0.005M Na phosphate pH 6.5 (buffer A). After IgG injection, the column was washed with ten column volumes of equilibration buffer. The IgG was eluted using a gradient in NaCl concentration to 1.5M (buffer B). This was achieved by programming the chromatography system to go from 0 to 100% B in 40 column volumes. The column was cleaned with 0.5M Na phosphate pH 7 prior to equilibration before each run. For imidazole containing buffers, imidazole was added to both buffers A and B and pH titrated with 6N HCl.

Resolution factor (R)

The resolution factor reflects the quality of separation between the monomer (A) and the aggregates (B). It is determined according to the equation, R=2[tB-tA]/(WA+WB), where t and W correspond to the retention time and peak width at baseline respectively. A resolution value of 1.5 implies a complete separation of the two adjacent peaks.

Peak of monomer

It is located by determination of % B in which the monomer's eluted peak maximum appears.

Frontal dynamic binding capacity (DBC)

Frontal loading studies to 10% breakthrough for CHT was performed at 600 cm/hr. After equilibration, each column was set off-line at the beginning of the run to obtain the absorbance at 100% breakthrough. OD values were determined at 280 nm for a polyclonal antibody solution with an OD value of 1.2. The antibody solution was loaded onto the column through the injector valve after attaining 100% breakthrough. DBC at 10% breakthrough was calculated by multiplying the antibody concentration in the load by the frontal volume.

Statistical Factorial Design (SFD)

With limited amount of feed material, SFD is employed to help predict what results are expected when process parameters are changed. The screening experiment was designed with two levels of pH: 6.5 and 7.5; two levels of phosphate concentrations: 0.005M and 0. 02M; and two levels of imidazole concentrations: 0M and 0.3M. The experiments were arranged in 23 statistical factorial design with a total of eight runs. Three runs using the center point conditions were also carried out. These runs are important to establish linearity trends and to estimate errors in measurement. Conditions for the eleven experiments are shown in Figure 1. The effects of pH (factor 1), phosphate concentrations (factor 2) and imidazole concentrations (factor 3) on resolution and peak elution were explored using Umetrics MODDE 7 software package.

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Figure 1: DBC optimum at pH 6.7 to 6.8

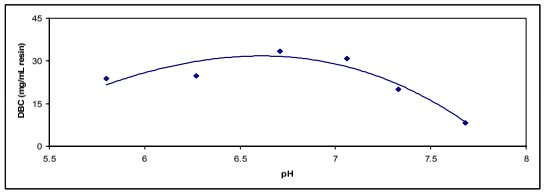


Figure 2: Inverse relation between DBC and PO_4 concentration

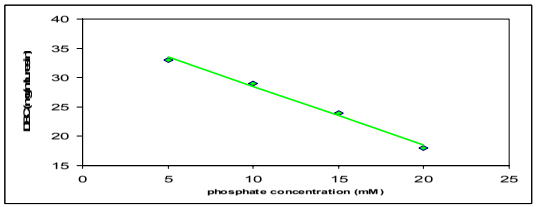


Figure 3: DBC decreases as imidazole concentration increases

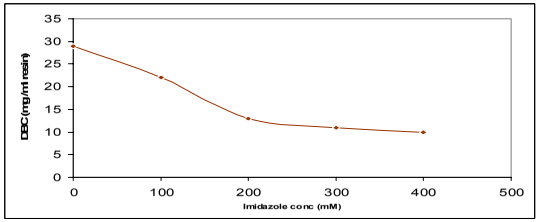


Figure 4: DBC dramatically decreases at 150 mM NaCl

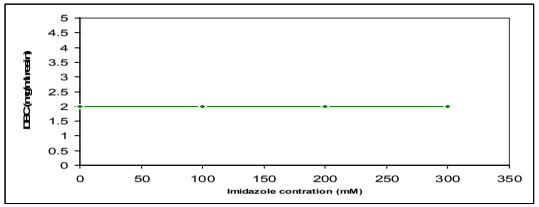
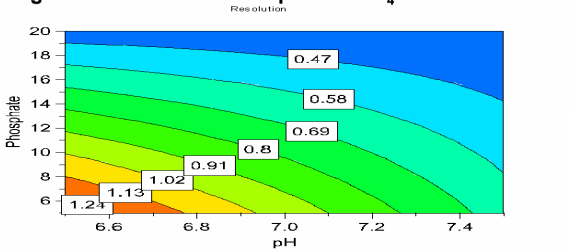


Figure 5: Inverse effect of pH and PO_4 on resolution

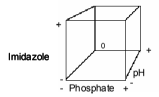


Factorial Design Analysis

Variable Ranges

	pH	Phosphate (mM)	Imidazole (M)
+	7.5	20	0.3
0	7.0	12.5	0.15
-	6.5	5	0

Resolution = f (pH, phosphate, imidazole)
Peak location = f (pH, phosphate, imidazole)



Factorial Design Experiments

Run #	pH	Phosphate (mM)	Imidazole (M)
1	7.5	20	0.3
2	6.5	20	0.3
3	7.5	5	0.3
4	6.5	5	0.3
5	7.5	20	0
6	6.5	20	0
7	7.5	5	0
8	6.5	5	0
9	7.0	12.5	0.15
10	7.0	12.5	0.15
11	7.0	12.5	0.15

Table 1: Effect of imidazole &NaCl on migration of peak maximum

		% B at peak maximum	Shift index (%)
IgG_1	Control	43	0
	0.12M NaCl	34	-21
	0.3M imidazole	31	-28
IgG_4	Control	48	0
	0.12M NaCl	42	-13
	0.3M imidazole	32	-33

Table 2: SFD Results and Conclusions

	Resolution	Location of peak maximum
pH 6.5 vs 7.5	Improved at pH 6.5	Higher eluent concentration at pH 6.5
Phosphate 0.005M vs 0.02M	Improved at 5 mM phosphate	Higher eluent concentration at 5 mM phosphate
Imidazole 0M vs 0.3M	No improvement in majority of the cases containing 0.3M imidazole	Higher eluent concentration without imidazole