Antibody Aggregate Removal by Hydroxyapatite Chromatography in the Presence of Polyethylene Glycol

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Abstract

This paper reports a method for improving the effectiveness of aggregate removal from monoclonal antibodies by the use of hydroxyapatite (HA) in the presence of polyethylene glycol (PEG). PEG preferentially enhances aggregate retention, thereby increasing the degree of separation between aggregated and non-aggregated antibodies. PEG has a similar effect on ion exchangers but only half the enhancement observed with HA. The HA method is suitable for preparation of aggregate-free IgG and IgM monoclonal antibodies for research, diagnostic, or therapeutic applications.

Keywords: hydroxyapatite, antibody purification, IgG, IgM, aggregate removal, polyethylene glycol

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1. Introduction

Hydroxyapatite (HA) has been reported frequently to support good fractionation of antibody aggregates and polymers. Simple phosphate gradients have proven adequate

for fractionation of IgA polymers (Aoyama and Chiba, Luellau et al), fractionation of IgM monomers from fully assembled pentamers (Yamakawa and Chiba, Aoyama and Chiba, Coppola et al), and removal of aggregates from some IgG monoclonals (Josics et al, Franklin). Phosphate gradients in the presence of chloride ions have recently been demonstrated to extend the range of IgG monoclonal antibodies from which aggregates can be removed (Sun, Gagnon et al, Sun). Even aggregate levels as high as 40-60% are reduced to less than 0.1%. Chloride/phosphate gradients simultaneously support up to 2 logs reduction of host cell protein contaminants, more than 3 logs DNA reduction, more than 4 logs endotoxin reduction, 2-3 logs leached protein A reduction, and 3-4 logs reduction of enveloped virus (Giovannini and Freitag, Schubert and Freitag, Gagnon et al, Sun, Ng et al).

The superior purification performance of chloride/phosphate gradients resides in their ability to independently control HA's two primary antibody retention mechanisms: phosphoryl cation exchange, and calcium metal affinity. The differential effects of chlorides and phosphates on the elution of antibodies were first recognized by Hjerten in 1959. Subsequent publications defined hydroxyapatite's interactions with proteins (Gorbunoff, Gorbunoff and Timasheff) leading to recommendations for routine screening with phosphate and chloride (Kawasaki, Gagnon). In the absence of phosphate, most IgG monoclonal antibodies will not elute from HA even at 2 M sodium chloride, thereby demonstrating their retention by calcium affinity. The majority however, can be eluted in sodium chloride gradients at phosphate concentrations as low as 5-15 mM. In contrast, BSA requires about 100 mM phosphate for elution, even in the presence of 2 M NaCl, and DNA requires more than 200 mM phosphate. This demonstrates that despite its resistance to sodium chloride, the calcium-binding component of IgG is relatively weak. Setting a constant low level of phosphate suspends weak calcium affinity interactions but leaves strong ionic interactions intact. A sodium chloride gradient can then dissociate ionic bonds, eluting non-aggregated IgG. Aggregates elute at higher sodium chloride concentrations. Leached protein A-IgG complexes and phosphorylated contaminants such as DNA, endotoxin, and lipid enveloped viruses all exhibit strong calcium affinity and remain bound until the column is cleaned with 500-600 mM phosphate.

Despite their utility, the effectiveness of chloride/phosphate gradients appears to be inversely related to the concentration of phosphate required support elution of a particular antibody within a sodium chloride gradient. Aggregate removal is generally excellent with IgG monoclonal antibodies that can be eluted in NaCl at phosphate concentrations of 5-15 mM but declines with antibodies that require more than 20 mM phosphate to elute within a gradient of NaCl. Removal efficiency of other contaminants declines in parallel. Present experience suggests that more than half of IgG clones fall into the low-phosphate group, but this leaves a large number of antibodies potentially in need of an effective method for aggregate removal. The chloride/phosphate approach also fails to accommodate IgM monoclonal antibodies evaluated to date.

Previous investigations have shown that PEG increases protein retention on ion exchangers (Milby et al, Gagnon et al). PEG is preferentially excluded from protein surfaces, leaving them surrounded by a PEG-free exclusion zone (Arakawa and Timasheff). The discontinuity between this exclusion zone and the high-PEG mobile phase is thermodynamically unfavorable. PEG is likewise excluded from the surface of chromatographic stationary phases (Arakawa). When proteins bind to a stationary phase such as HA in the presence of PEG, they are able to share hydration water, allowing some water to transfer to the mobile phase, thereby lowering the bulk PEG concentration. This reduces the magnitude of the discontinuity in PEG concentration between the exclusion zone and the mobile phase. In addition, the hydrated surface area of the bound protein is lower than the additive surface areas of the protein and stationary phase separately. Both phenomena are thermodynamically favorable and stabilize the association of the protein with the stationary phase. Proteins consequently elute at higher eluant concentrations than in the absence of PEG. This model assumes that PEG does not perturb water structure or the native hydration characteristics of the proteins or stationary phase.

Results with ion exchangers demonstrate a linear correlation between the log of protein molecular weight and the degree to which PEG enhances retention. This suggests a possibility of using PEG to differentially enhance retention of aggregates, perhaps

sufficiently to improve the effectiveness of their removal from antibody preparations. This paper challenges that hypothesis, and extends it to a comparison with the effects of PEG on aggregate removal by HA. Experimental results demonstrate that PEG does enhance aggregate removal on ion exchangers, but the degree of enhancement is at least 2-fold greater with HA. Practical ramifications of the technology are discussed.

2. Materials and methods

2.1. Materials

Protein A purified IgG monoclonal antibodies and unpurified monoclonal IgM were provided by Avid Bioservices (Tustin, CA). Buffer components were purchased from Sigma/Aldrich (St. Louis, MO). A 5 x 300 mm Bio-Sil™ 400-5 size exclusion column and ceramic hydroxyapatite CHT™ Type I, 20 μm and 40 μm, were used for IgG (Bio-Rad Laboratories, Hercules, CA). CHT Type II, 40 m was used for IgM. Hydroxyapatite was packed into 5 x 50 mm (1 mL) and 11.3 x 100 mm (10 mL) MediaScout® columns by Atoll GmbH (Weingarten, Germany). A 7.8 x 300 mm TSK-gel™ G4000SWXL column was employed for size exclusion analysis of IgM (Tosoh Bioscience, Montgomeryville, PA). 1 mL ReSOURCE® Q and S columns were obtained from GE Healthcare (Piscataway, NJ). All experiments were performed on an AKTA® Explorer 100 (GE Healthcare).

2.2. Procedures

2.2.1. Preparation of IgG aggregate reference material

A protein A-purified monoclonal IgG_1 antibody (MAb1) containing more than 40% aggregates was fractionated by HA in a chloride/phosphate gradient. A 10 mL MediaScout column of CHT Type I, 40 micron was equilibrated with 5 mM sodium phosphate, 20 mM MES, pH 6.5. The column was loaded with 250 mg of protein A purified antibody, and eluted with a 30 column volume (CV) linear gradient to 5 mM sodium phosphate, 20 mM MES, 2.0 M sodium chloride, pH 6.5. The aggregate fraction was collected and analyzed by size exclusion on the Bio-Sil column. About 65% of the aggregate population comprised a 4-antibody form, with lesser amounts of larger forms. Conditions and representative chromatograms are given in Gagnon et al (2006). An

additional IgG_1 (MAb2) with about 10% total aggregates was prepared and analyzed in the same manner. These samples were used to characterize aggregate distribution in the following chromatography methods.

2.2.2. Ion exchange chromatography in the presence and absence of PEG. ReSOURCE Q was equilibrated with 10 mM sodium phosphate, pH 7.5 at a linear flow rate of 565 cm/hr (3 mL/min). 50 µL of protein A purified MAb1 or aggregate reference sample were injected, and the column was washed with equilibration buffer. The column was eluted with a 20 CV linear gradient to 500 mM sodium phosphate, pH 7.5. The experiments were repeated with 7.5% PEG-4600 added to both buffers. ReSOURCE S was equilibrated with 10 mM sodium citrate, pH 6.0 at a linear flow rate of 565 cm/hr. 50 µL of protein A purified MAb1 or aggregate reference sample were injected, and the column was washed with equilibration buffer. The column was eluted with a 20 CV linear gradient to 200 mM sodium citrate, pH 6.0. The experiments were repeated with 7.5% PEG-4600 added to both buffers. After use, both columns were cleaned with 1 M sodium chloride, sanitized with 1 M sodium hydroxide, and stored in 20% ethanol.

2.2.3. HA chromatography of IgG with phosphate gradient elution in the presence and absence of PEG. A 1 mL column of CHT Type I, 20 µm was equilibrated with 10 mM sodium phosphate, 20 mM MES, pH 6.5, at a linear flow rate of 300 cm/hr (1 mL/min). 100 µL of protein-A purified MAb1, MAb2, or the respective aggregate reference was injected, the column was washed with equilibration buffer, then eluted in a 30 CV linear gradient to 500 mM sodium phosphate, pH 6.5. The experiments were repeated in the presence of 3.75%, 5.625%, and 7.5% PEG-4600 in the gradient buffers. The HA column was cleaned with 500 mM sodium phosphate, pH 6.5, sanitized with 1 M sodium hydroxide, and stored in 0.1 M sodium hydroxide. A third protein A purified IgG monoclonal antibody (MAb3) was loaded and eluted under the same non-PEG conditions. The experiment was repeated with 10% PEG-1000 added to the wash and elution buffers. The ReSOURCE Q column was equilibrated to 50 mM Tris, pH 8.5. The IgG peaks from the respective HA experiments were titrated to pH 8.5, then loaded by in-line dilution at a proportion of 10% sample, 90% equilibration buffer. The column was

washed with equilibration buffer and eluted in a 15 CV linear gradient to 50 mM Tris, 0.5 M sodium chloride, pH 8.5. Aggregate content of the two preparations was evaluated on the Bio-Sil column.

2.2.4. Purification of IgM in the absence of PEG during HA chromatography. An IgM monoclonal antibody was purified as described in Gagnon et al (2008). In brief, a 10 mL column of CHT type II 40 µm was equilibrated with 10 mM phosphate, 20 mM MES, pH 6.7, at a linear flow rate of 200 cm/hr (3.33 mL/min). 1 L of supernatant was loaded, the column washed to baseline with equilibration buffer, then eluted with a 30 CV linear gradient to 500 mM sodium phosphate pH 6.7. The IgM peak from the CHT step was applied to a cation exchange column and eluted in a linear gradient from 10 to 500 mM sodium phosphate. Aggregate content was evaluated on the TSK-gel column.

2.2.5. Purification of IgM in the presence of PEG during HA chromatography. The method described in section 2.2.4 was repeated except that the wash and elution gradient of the HA step were conducted in the presence of 10% PEG-600. Aggregate content was evaluated on the TSK-gel column.

2.2.6. HA chromatography of IgG with chloride/phosphate gradient elution in the presence and absence of PEG. A 1 mL column of CHT type I, 20 µm was equilibrated with 10 mM sodium phosphate, 20 mM MES, pH 6.5, at a linear flow rate of 300 cm/hr (1 mL/min). 100 µL of protein-A purified IgG MAb1 or aggregate reference was injected and the column was washed with equilibration buffer, then eluted in a 30 CV linear gradient to 10 mM sodium phosphate, 20 mM MES, 1 M NaCl, pH 6.5. The experiments were repeated with 3.75% and 7.5% PEG-4600 in the gradient buffers. After use, the column was cleaned with 500 mM sodium phosphate, pH 6.5, sanitized with 1 M sodium hydroxide, and stored in 0.1 M sodium hydroxide.

2.2.7. Analytical SEC. The Bio-Sil 400-5 and G4000SWXL columns were equilibrated to 0.2 M arginine, 20 mM MES, pH 6.5. 50µL of IgG was applied to the Bio-Sil column at a

linear flow rate of 150 cm/hr (0.5 mL/min). 50µL of IgM was applied to the TSK-gel column at a linear flow rate of 62 cm/hr (0.5 mL/min).

3. Results

3.1. Performance comparison, ion exchange gradients in the presence and absence of PEG. Figure 1 illustrates elution profiles of the MAb1 on a high performance anion exchanger in the absence and presence of 7.5% PEG-4600. Figure 2 illustrates the corresponding profile on a high performance cation exchanger. Aggregate distribution is highlighted in gray. No aggregate separation was apparent on either exchanger in the absence of PEG. Aggregate retention was enhanced on the anion exchanger but not sufficiently to achieve baseline separation. Resolution was better on the cation exchanger, but still insufficient to achieve baseline separation.

3.2. Performance comparison, phosphate gradients in the presence and absence of PEG. Figure 3 illustrates elution profiles of MAb1 on HA eluted in a phosphate gradient, at a series of PEG-4600 concentrations ranging from 0 to 5.625%. Profiles are aligned at the point where non-aggregated antibody begins to elute to facilitate comparison of PEG-mediated effects. Aggregate distribution is highlighted in gray. No aggregate separation was apparent in the absence of PEG. Aggregate separation at 3.75% PEG was superior to anion exchange at 7.5%, and roughly equivalent to cation exchange at 7.5%. Baseline separation of the aggregate population was achieved by HA at 5.625% PEG-4600.

Figure 4 illustrates the relative enhancement of aggregated and non-aggregated antibody as a function of PEG concentration. Data points indicate phosphate concentration at peak center. As expected, the aggregate population is more responsive. Figure 5 plots chromatographic resolution as a function of PEG, according to the formula $R = \Delta V/((w_1+w_2)/2)$ where ΔV = the buffer volume in mL between peak centers, w_1 = the width in mL of the non-aggregated IgG peak at 10% peak height, and w_2= the width of the aggregate peak. The horizontal dashed line indicates a value of R=1.5, which is generally accepted to correspond to baseline resolution.

Figures 6 illustrates an overlay plot of aggregate separation for MAb1 and MAb2. Solid plots illustrate aggregate behavior. Dashed plots illustrate behavior of non-aggregated antibody. The vertical offset between plots results from the earlier elution of MAb2. The most striking feature however is the parallelism of response to PEG. Figure 7 illustrates an overlay plot of aggregate resolution for the two antibodies, with the dashed plot illustrating MAb2. In the context of Figure 6, the results shown in Figure 7 suggest that earlier eluting antibodies require slightly more PEG to achieve equivalent aggregate resolution, but as in Figure 6, the most striking feature of the data is the parallelism between the two antibodies. This was interpreted to indicate that PEG dominates the native selectivity of the HA, regardless of the retention characteristics of individual antibodies. These data suggest that PEG response should be similarly uniform with other IgG monoclonal antibodies.

Figure 8 compares the analytical SEC profiles of IgG MAb3 prepared in the presence and absence of 10% PEG-1000. No aggregates are observed in the sample processed with PEG. In the corresponding HA experiment, aggregate did not elute until the 500 mM phosphate cleaning step (w/o PEG, data not shown). These data illustrate the efficacy of smaller molecular weight PEG preparations.

Figure 9 compares the analytical SEC profiles of IgM purified in the presence or absence of PEG-600 at the HA step. No aggregates are observed in the sample processed with PEG. As with IgG MAb3, IgM aggregates failed to elute in the presence of PEG and were removed from the column by the cleaning step in 500 mM phosphate (w/o PEG, data not shown). These data demonstrate that the use of PEG is at least as effective for IgM monoclonal antibodies, and illustrate the efficacy of yet lower molecular weight PEG. The fact that both IgG and IgM aggregates were retained by HA until it was cleaned with 500 mM phosphate suggest that lower PEG concentrations and/or lower molecular weights may be effective.

3.3. Performance comparison, phosphate/chloride gradients in the presence and absence of PEG. Figure 10 illustrates a chloride/phosphate gradient of MAb1 and the effects of

added PEG. Resolution is close to baseline even in the absence of PEG, but clearly better in 3.75% PEG, and dramatically superior in 7.5% PEG. Figure 11 illustrates the relative enhancement of aggregated and non-aggregated antibody as a function of PEG concentration. Retention of non-aggregated IgG increased only modestly at higher PEG concentrations. Aggregate retention increased more than non-aggregated IgG, but to a lesser degree than observed in the phosphate gradient. Nevertheless, the compound effect of chloride, phosphate, and PEG provides roughly the same separation at 3.75% PEG as is obtained at 5.625% PEG in the phosphate gradient (Figure 3). Figure 12 plots chromatographic resolution in the chloride/phosphate/PEG system. Two noteworthy differences from phosphate gradients are apparent. The magnitude of improvement from 0 to 7.5% PEG is less than obtained in phosphate gradients, and the response is essentially linear.

4. Discussion

PEG enhances separation of IgG aggregates on ion exchangers as expected, but enhancement is at least 2-fold greater on HA. This has important process ramifications. PEG increases buffer viscosity, and viscosity reduces diffusivity (Chicz and Regnier). Porous particle based chromatography media such as ion exchangers and HA rely on diffusion for mass transport, so elevated viscosity may depress capacity, increase eluted peak width, and/or require reduced flow rate to compensate (Afeyan et al). Thus, even though PEG levels could conceivably be increased sufficiently for ion exchangers to offer separation comparable to HA, the compromises would likely be intolerable. Operating pressure would likely become a limiting factor as well, and it is questionable whether such PEG concentrations would be usable in any case since PEG is a well-known antibody precipitating agent.

The shape of the retention enhancement and resolution curves offer clues as to why HA is more responsive. As previously discussed, the purpose for the low level of phosphate in sodium chloride gradients is to weaken calcium affinity interactions. This leaves HA dominantly a phosphoryl cation exchanger, and as illustrated in Figure 2, the degree of enhancement on the cation exchanger was modest compared to HA in phosphate

gradients. The implication is that PEG acts cooperatively with both the calcium affinity and phosphoryl cation exchange mechanisms to achieve a greater enhancement than single-mode methods such as ion exchange. This may also explain why HA in phosphate gradients offers better separation of IgA and IgM polymers than ion exchangers.

The dominance of PEG over the foundation selectivity of HA simplifies method development. If the objective is to quickly obtain aggregate-free antibody for research purposes, the following template will likely serve without modification for IgG or IgM antibodies. Equilibrate the HA column with 10 mM sodium phosphate, 20 mM MES, pH 6.7; apply the sample; wash with 10 mM sodium phosphate, 20 mM MES, 10% PEG-1000, pH 6.7; then elute in a 30 CV linear gradient to 500 mM sodium phosphate, 10% PEG-1000, pH 6.7. CHT Type I is recommended for IgG and Type II for IgM.

Although less broadly applicable, chloride/phosphate/PEG gradients offer more effective removal of host cell proteins, leached protein A, DNA, endotoxin, and virus from IgG monoclonal antibodies. Users with access to high throughput robotic systems may elect to explore the full scope of phosphate/chloride/PEG combinations, but the following template provides a good starting point for the majority of IgGs. Equilibrate with 10 mM sodium phosphate, 20 mM MES, pH 6.7; apply the sample; wash with 10 mM sodium phosphate, 20 mM MES, 10% PEG-1000; elute with a 30 CV linear gradient to 10 mM sodium phosphate, 20 mM MES, 10% PEG-1000, 2.0 M sodium chloride, pH 6.7. If the IgG fails to elute, increase the phosphate concentration.

High molecular weight PEG preparations are effective at lower concentrations, but low molecular weight PEGs are more advantageous overall. Many PEG polymers with average molecular weights of 1000 or less are approved inactive ingredients in parenteral formulations (US Food and Drug Administration). Such polymers can be removed from final product by diafiltration or dialysis, whereas PEG-6000 has roughly the same hydrodynamic radius as a globular protein with a molecular weight of 50-100 kD (Gagnon) and precludes size-based elimination methods. PEG of any size can be removed by binding the antibody in a subsequent ion exchange step and allowing the

PEG to flow through the column. For manufacturing methods, it will also be advantageous to minimize the PEG concentration, since this will minimize viscosity and contribute to better resolution as well as lower operating pressures. Residual PEG can by measured down to 0.5 μg/mg of protein (0.0005% w:w) by complexation with barium iodide (Skoog).

Whether the method is practiced in conjunction with phosphate or phosphate/chloride gradients, the use of PEG contributes to better recovery of non-aggregated antibody. Most chromatography methods suffer loss of resolution when large protein loads are applied to a column. This usually requires that some non-aggregated antibody be sacrificed to ensure complete aggregate removal from the remainder. PEG allows HA columns to be loaded to capacity without compromising aggregate removal. In Figure 8 for example, the broad separation at 7.5% PEG allows complete rejection of aggregates without any peak cutting on the trailing side of the non-aggregated IgG peak; the entire peak can be recovered. PEG can also facilitate high recovery in applications where the objective is for non-aggregated antibody to flow through the column upon application, while aggregates and other contaminants are retained.

In summary, HA in the presence of PEG provides a valuable enabling method for removing aggregates from IgG and IgM monoclonal antibodies. Aggregate-free antibody can be obtained on small HA columns with negligible method development for research or toxicological studies, and without having to resort to the slow, low capacity alternative of SEC. The method can be more thoroughly optimized, scaled up, and integrated with other purification methods to meet manufacturing requirements for antibodies with large scale commercial potential.

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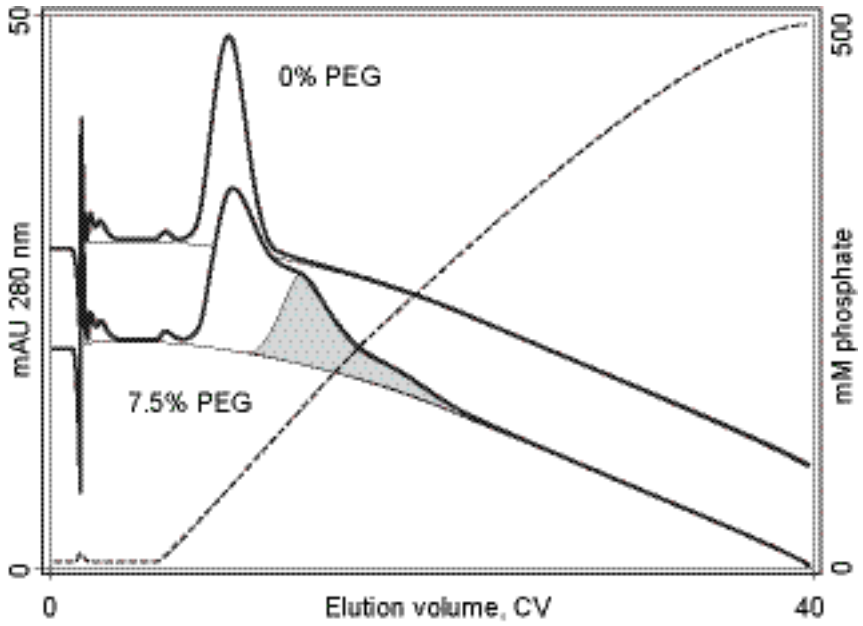


Figure 1. Anion exchange elution profiles of highly aggregated IgG in the presence and absence of PEG. MAb1. PEG-4600. See section 2.2.2 for experimental details.

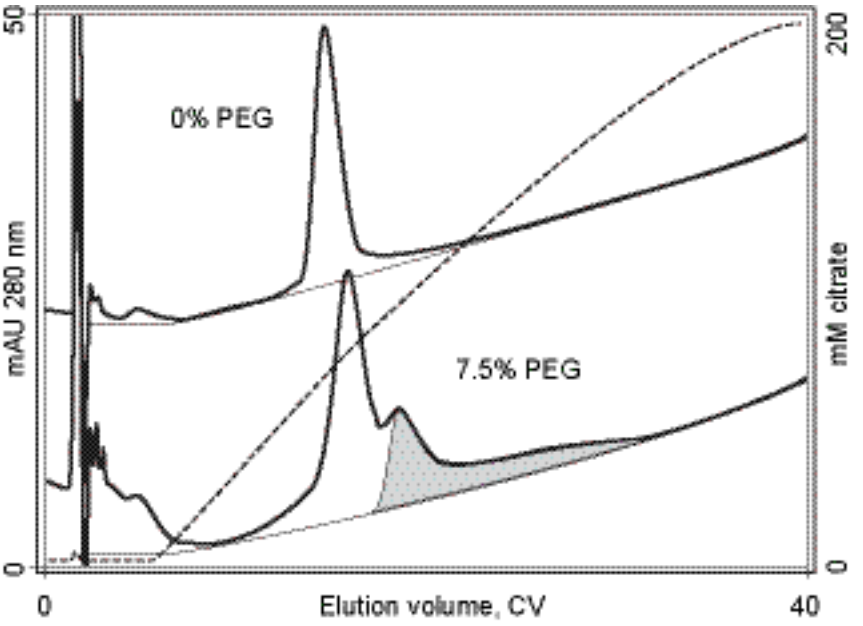


Figure 2. Cation exchange elution profiles of highly aggregated IgG in the presence and absence of PEG. MAb1. PEG-4600. See section 2.2.2 for experimental details.

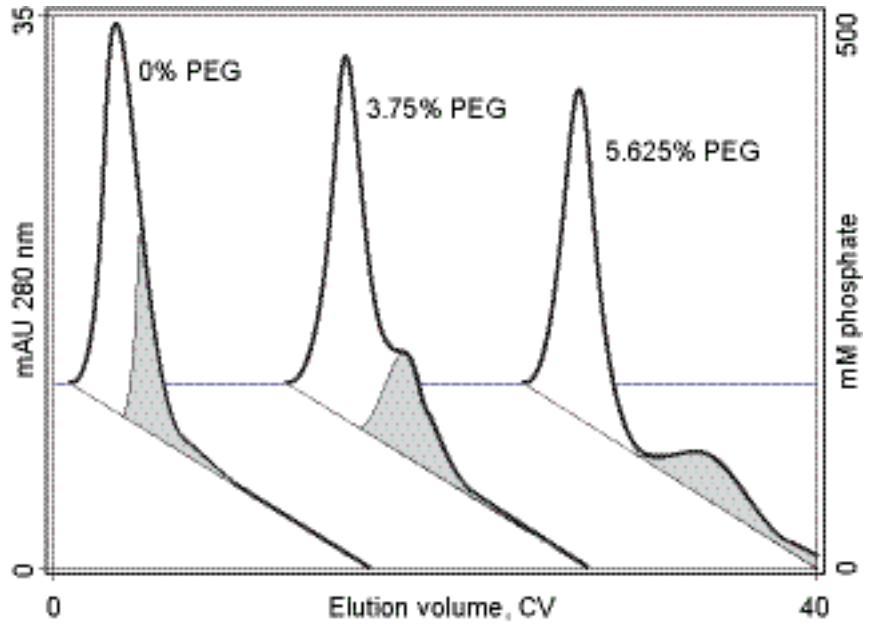


Figure 3. Aggregate separation on HA as a function of PEG concentration in phosphate gradients. MAb1. PEG-4600. See section 2.2.3 for experimental details.

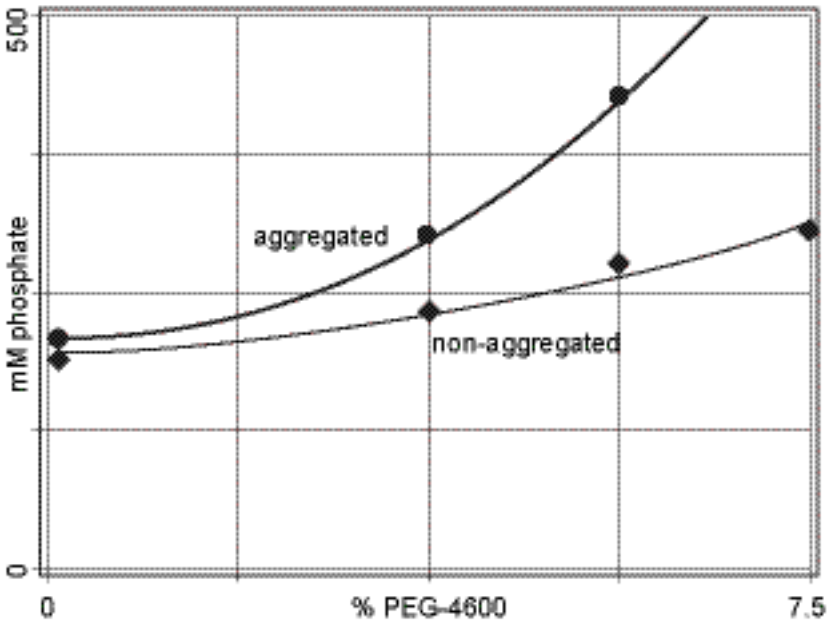


Figure 4. Differential retention of aggregates and non-aggregated IgG as a function of PEG concentration in phosphate gradients. MAb1. See section 2.2.3 for experimental details.

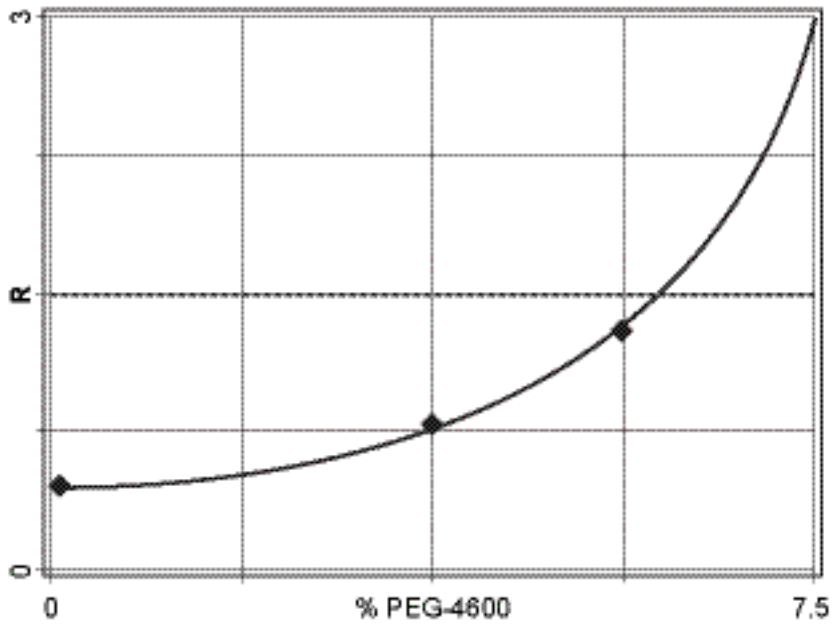
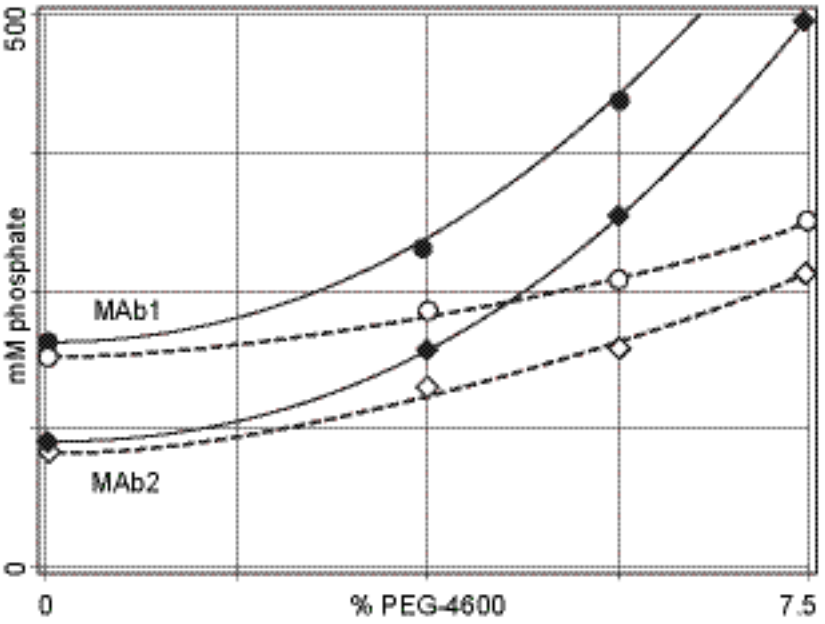
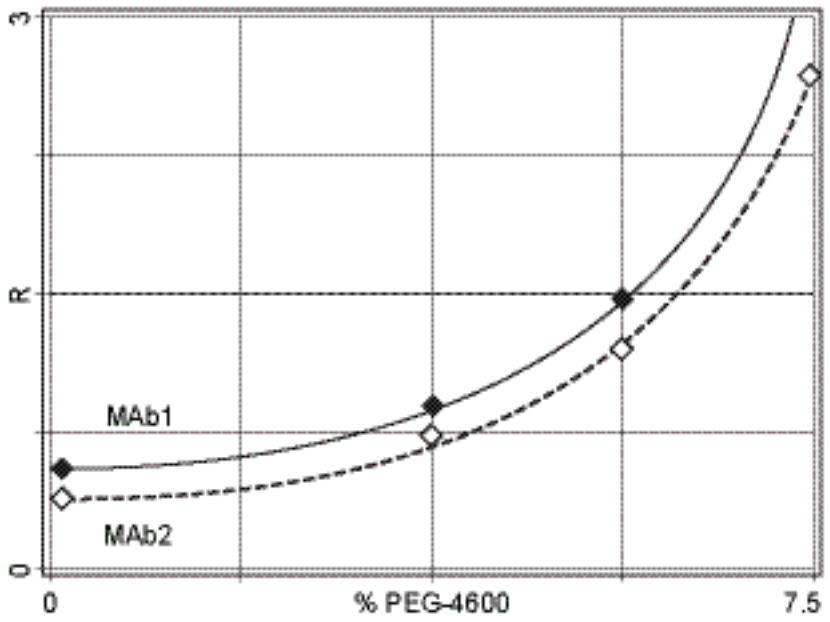


Figure 5. Resolution of aggregates from non-aggregated antibody as a function of PEG concentration in phosphate gradients. MAb1. See section 2.2.3 for experimental details.



Figures 6. Differential retention of aggregates and non-aggregated IgG as a function of PEG concentration in phosphate gradients. See section 2.2.3 for experimental details.



Figures 7. Resolution of aggregates from non-aggregated antibody as a function of PEG concentration in phosphate gradients. See section 2.2.3 for experimental details.

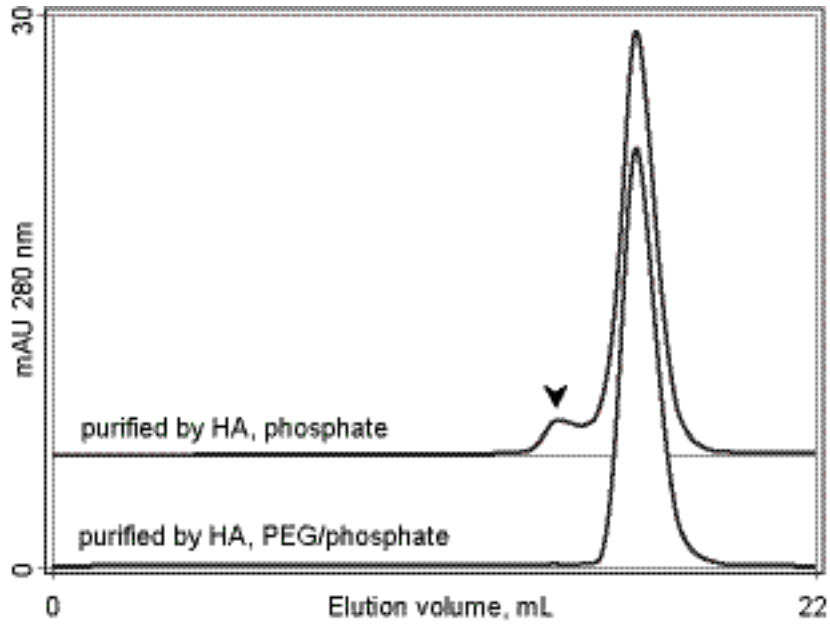


Figure 8. Analytical size exclusion chromatography of purified IgG. MAb3. 10% PEG-1000. See sections 2.2.3 and 2.2.7. for experimental details.

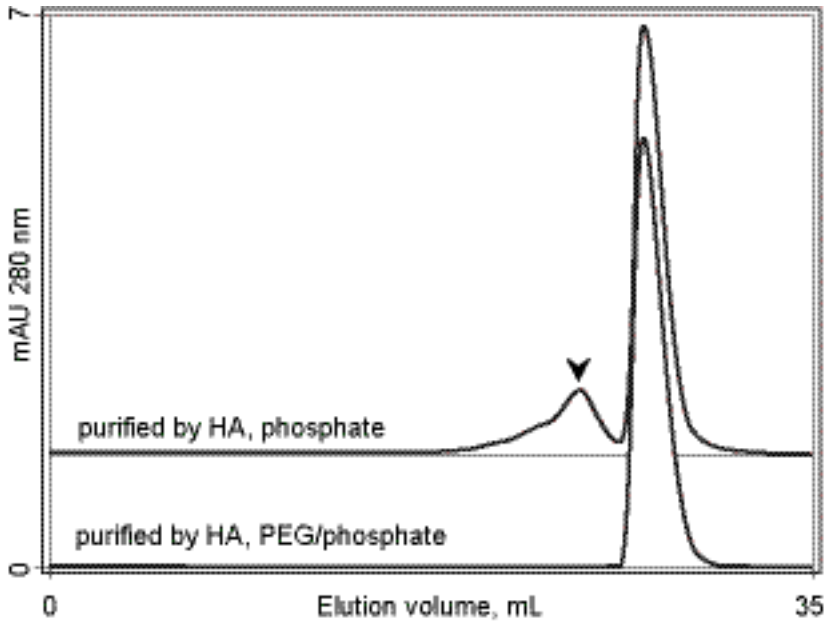


Figure 9. Analytical size exclusion chromatography of purified monoclonal IgM. 10% PEG-1000. See sections 2.2.4, 2.2.5, and 2.2.7 for experimental details.

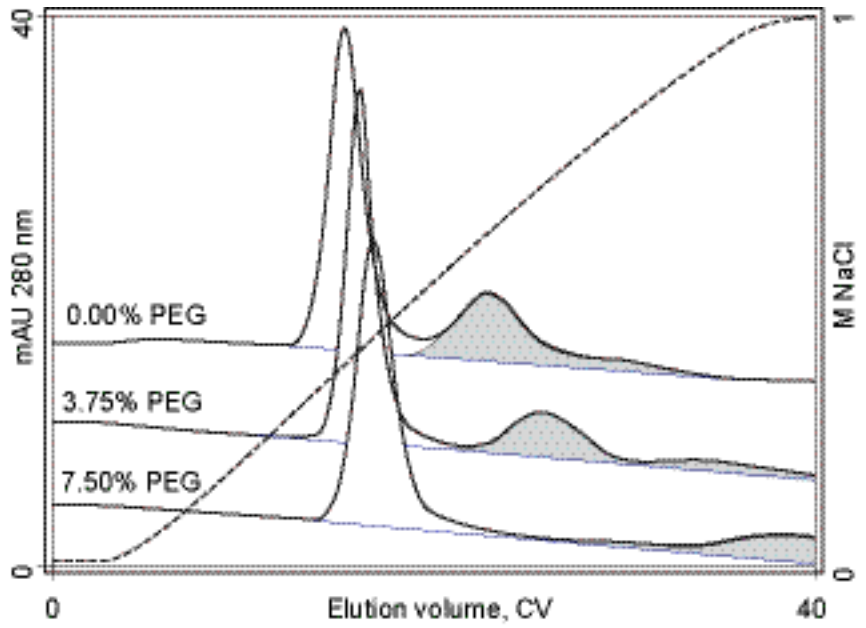


Figure 10. Aggregate separation on HA as a function of PEG concentration in chloride/phosphate gradients. MAb1. PEG-4600. See section 2.2.6 for experimental details.

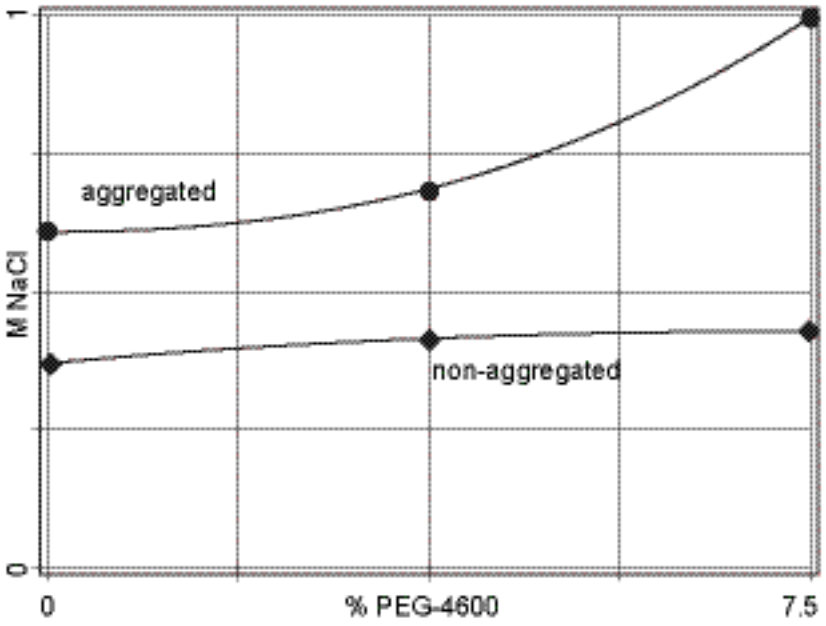


Figure 11. Differential retention of aggregates and non-aggregated IgG as a function of PEG concentration in chloride/phosphate gradients. MAb1. See section 2.2.6 for details.

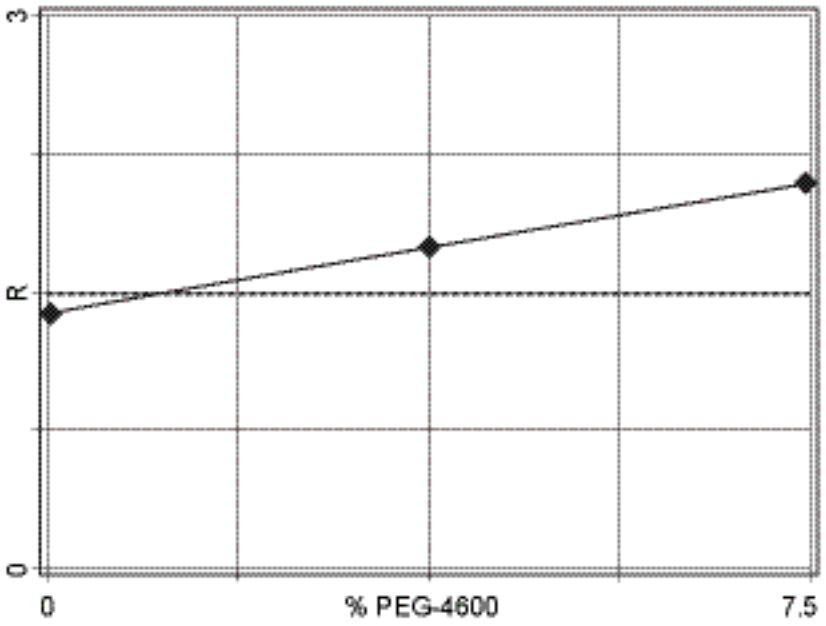


Figure 12. Resolution of aggregates from non-aggregated antibody as a function of PEG concentration in chloride/phosphate gradients. MAb1. See section 2.2.6 for details.