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Removal of Aggregates of Monoclonal Antibodies by Ion Exchange Chromatography

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***Abstract*—**The primary objective of this work was to study the effect of resin chemistry, pH and molarity of binding and elution buffer on aggregate removal using Cation Exchange Chromatography and find the optimum conditions which can give efficient aggregate removal with minimum loss of yield. Four different resins were used for carrying out the experiments: Fractogel EMD SO_3^-(S), Fractogel EMD COO^-(M), Capto SP ImpRes and S Ceramic HyperD. Runs were carried out on the AKTA Avant system. Design of Experiments (DOE) was used for analysis using the JMP software. The dependence of the yield obtained using different resins on the operating conditions was studied. Success has been achieved in obtaining yield greater than 90% using Capto SP ImpRes and Fractogel EMD COO^-(M) resins. It has also been found that a change in the operating conditions generally has different effects on the yields obtained using different resins.

***Keywords*—**Aggregates, cation exchange chromatography, design of experiments, monoclonal antibodies.

I. Introduction

MONOCLONAL antibodies (mAbs) have proved to be a highly successful class of therapeutic products. They have been introduced as therapies to a variety of diseases such as rheumatoid arthritis, multiple sclerosis and different forms of cancer [1], [2]. It is extremely important to develop a reliable purification process that is capable of removing both product related impurities such as aggregates and process related impurities such as host cell protein, nucleic acids and leached Protein A [3], [4]. It is also very important to ensure that the loss of yield of the product during purification is the minimum.

In this work, the primary focus was on the removal of aggregates of monoclonal antibodies using ion exchange chromatography. It is essential to remove aggregates since they can cause loss of activity as well as toxicity and immunogenicity. Because of their toxic potential, aggregates can cause an unwanted response or even overreaction of a patient's immune system.

Chromatography is undoubtedly the workhorse of downstream processing. The various chromatographic techniques commonly used for mAb purification include Protein A chromatography, Ion Exchange Chromatography, Hydrophobic Interaction Chromatography, and Multimodal Chromatography. Protein A Chromatography is generally used as the capture step since it provides >98 % purity in a single step. It is subsequently followed by two polishing steps in most cases to reduce the remaining impurities, particularly high molecular weight aggregates and host cell proteins to acceptable levels [5], [6].

Most mAb purification processes include at least one ion exchange chromatography (IEX) step. Ion Exchange Chromatography separates proteins with differences in charge to give a very high-resolution separation with a high sample loading capacity. Separation by this versatile technique is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. It is ideal for reducing high molecular weight aggregate, charge-variants, residual DNA and host cell protein, leached Protein A and viral particles. The resins used are relatively inexpensive and separation is fairly selective. There are two types of Ion Exchange Chromatography, Cation Exchange Chromatography (CEX) and Anion Exchange Chromatography (AEX). Anion Exchange Chromatography uses a positively charged group (weakly basic such as diethylamino ethyl, DEAE or dimethylamino ethyl, DMAE; or strongly basic such as quaternary amino ethyl, Q or trimethylammonium ethyl, TMAE or quaternary aminoethyl, QAE) immobilized to the resin. On the other hand, Cation Exchange Chromatography uses a resin modified with negatively charged functional groups. It is carried out with either a strong cation exchanger, containing a bonded sulfonic acid group, such as sulfopropyl (SP), or with a weak cation exchanger, containing a weak acid such as carboxymethyl (CM) [7], [8]. Strong ion-exchangers retain their charge over a wider pH range as compared to weak ion exchangers. Weak ion-exchangers often provide slightly different selectivity from strong ion-exchangers.

The ion exchange process can be divided into four basic stages [9]:

- Equilibration
- Sample Application and Wash
- Elution
- Regeneration

A. Equilibration

In this step, the buffer is applied with the desired conditions so that the system is ready for the ion exchange process. When equilibrium is reached, all stationary phase charged groups are associated with exchangeable counter-ions.

B. Sample Application and Wash

The basic objective in this step is to bind the target molecule/s and wash out all unbound material. Only proteins

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carrying a charge opposite to the stationary phase will bind to it while those with the same charge or no charge will not bind.

C. Elution

In this step, the buffer conditions are changed to elute particles that have bound to the stationary phase. This can be done by either changing the pH of the buffer solution or increasing the salt concentration. Proteins which have weaker ion interactions will be released at lower salt concentrations while those with stronger interactions will remain bound to the column longer.

D. Regeneration

The basic purpose of this step is to remove all bound protein from the stationary phase to ensure that the full capacity of the stationary phase is available for the next run.

II. Materials and Experimental Protocols

The basic aim of this work was to study the effect of resin chemistry, pH and molarity of binding and elution buffer, salt type, and salt molarity on aggregate removal using Cation Exchange Chromatography and find the optimum conditions which can give efficient aggregate removal with minimum loss of yield.

Four different resins were used for carrying out the experiments: Fractogel EMD SO_3^-(S), Fractogel EMD COO^-(M), Capto SP ImpRes and S Ceramic HyperD. Two different buffers were used in the experiments: Phosphate buffer and HEPES buffer.

The runs were carried out on the AKTA Avant system by GE Healthcare. It is a preparative chromatography system designed for fast and secure development of scalable methods and processes. It is operated by UNICORN 6 control software.

Four different columns were used each packed with one resin. The dimensions of the column used were 4 mm by 25 mm. Different sets of runs were carried out using all the four columns by varying certain parameters such as pH and molarity of binding and elution buffer. In some of the runs, Phosphate buffer was used while in the others, HEPES buffer was used. The elution salt was changed in a few of the runs, using KCl instead of NaCl. The conditions used for the various runs are summarized in Table II (In Run 8, Phosphate pH 7.5 15 mM was used for washing which would facilitate the comparison between binding at pH 6.2 and at pH 7.5).

For the purpose of analysis, Agilent 1200 Series HPLC was used. HPLC relies on the pressure of mechanical pumps on a liquid solvent to load a sample mixture onto a separation column, in which the separation occurs.

For carrying out the analysis, the following buffers had to be prepared:

- Size Exclusion Chromatography Buffer
  It contains the following:
  1. 100 mM Phosphate pH 7.0
  2. 100 mM Na_2SO_4
  3. 0.05% NaN_3
- Ion Exchange Chromatography Buffer
  It contains the following:
  1. A: 15 mM Phosphate pH 7.5
  2. B: 25 mM Phosphate pH 7.75
  3. 200 mM NaCl
  4. 0.05 % NaN_3

Design of Experiments (DOE) was used for analysis using the JMP software. Analysis of DOE is built on the foundation of the analysis of variance, a collection of models in which the observed variance is partitioned into components due to different factors which are estimated or tested.

The actual by predicted plot indicates the goodness of the fit. It shows actual yield by predicted yield values with a regression line and 95% confidence curves. The sorted parameter estimates table is useful in screening situations. A bar chart shows the t-ratio, with the lines showing the 0.05 significance level. Prediction profiles are useful in multiple-response models to help judge which factor values can optimize a complex set of criteria. It helps us change one variable at a time and look at the effect on the predicted response.

III. Results and Discussion

For Fractogel EMD COO^-(M), using the prediction profiler shown in Fig. 3, salt type, buffer type, elution pH and salt molarity are statistically significant factors. The use of KCl as elution salt gives a higher yield than NaCl under all conditions. At low salt molarity and high elution pH, the use of Phosphate buffer gives a higher yield while under the conditions of high salt molarity and low elution pH, HEPES buffer gives a better yield.

In the case of Capto SP ImpRes, a very good fit was not obtained which may be because there are certain other factors affecting the yield which may not have been considered. Using the prediction profiler shown in Fig. 6, salt molarity and salt type are statistically significant factors. KCl as elution salt gives a higher yield than NaCl in all operating conditions. The yield decreases with increase in salt molarity for this resin.

For the resin Fractogel EMD SO_3^-(S), from Fig. 9, buffer type, salt type, and wash pH and elution molarity are statistically significant factors. HEPES buffer gives a better yield than Phosphate buffer under all conditions. Also, the yield obtained with KCl as elution salt is higher than NaCl irrespective of the operating conditions. With increase in elution molarity, yield increases. Also, with increase in wash pH, a lower yield is obtained. In case of S Ceramic HyperD, the prediction profiler depicted in Fig. 12 indicates that the statistically significant factors are buffer type, salt molarity, wash pH and binding molarity. The use of Phosphate buffer gives a higher yield than HEPES buffer. The yield increases with increase in binding molarity for this resin. There is an increase in yield with increase in salt molarity. The yield decreases with increase in wash pH.

TABLE I
PROPERTIES OF DIFFERENT CATION EXCHANGE CHROMATOGRAPHY RESINS USED

Resin	Type	Vendor	Functional Group	Backbone	Particle Size (in micron)
Fractogel EMD SO_3^-(S)	Strong	Merck	Sulfoisobutyl	Methacrylate	20-40
Fractogel EMD COO^-(M)	Weak	Merck	Carboxyethyl	Methacrylate	40-90
Capto SP ImpRes	Strong	GE Healthcare	Sulfopropyl	High flow agarose	36-44
S Ceramic HyperD	Strong	Pall	Sulfopropyl	Polystyrene shell and hydrogel	50 (average)

TABLE II
CONDITIONS FOR DIFFERENT RUNS

Run Number	Binding Buffer	Elution Buffer
1	Phosphate pH 7 15 mM	Phosphate pH 7 15 mM + 200 mM NaCl
2	Phosphate pH 7 15 mM	Phosphate pH 7.5 15 mM + 200 mM NaCl
3	Phosphate pH 7 15 mM	Phosphate pH 7.5 50 mM + 200 mM NaCl
4	Phosphate pH 7.5 15 mM	Phosphate pH 7.5 15 mM + 200 mM NaCl
5	HEPES pH 7 20 mM	HEPES pH 7 20 mM + 200 mM NaCl
6	Phosphate pH 7.5 15 mM	Phosphate pH 7.5 15 mM + 200 mM KCl
7	Phosphate pH 6.2 15 mM	Phosphate pH 7.5 15 mM + 200 mM KCl
8	Phosphate pH 6.2 15 mM	Phosphate pH 7.5 15 mM + 200 mM KCl
9	Phosphate pH 7.5 15 mM	Phosphate pH 7.5 100 mM (No salt)
10	HEPES pH 7.5 50 mM	HEPES pH 8.75 50 mM + 50 mM NaCl
11	HEPES pH 8 20 mM	HEPES pH 8 20 mM + 200 mM NaCl
12	HEPES pH 8 100 mM	HEPES pH 9 100 mM + 50 mM NaCl
13	HEPES pH 8 200 mM	HEPES pH 9 200 mM + 50 mM NaCl
14	HEPES pH 7 20 mM	HEPES pH 7.75 20 mM + 200 mM NaCl
15	HEPES pH 7 50 mM	HEPES pH 7 50 mM + 200 mM NaCl
16	HEPES pH 7.5 20 mM	HEPES pH 7.75 20 mM + 200 mM NaCl

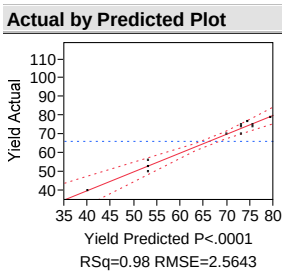

Fig. 1 Actual by predicted plot for Resin 1

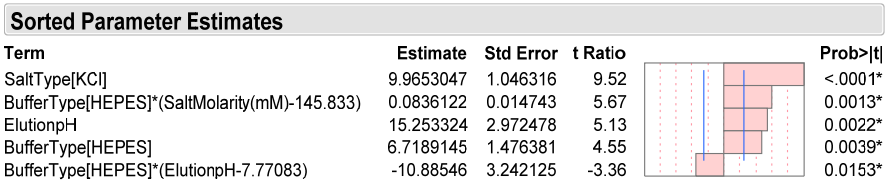

Fig. 2 Sorted parameter estimates for Resin 1

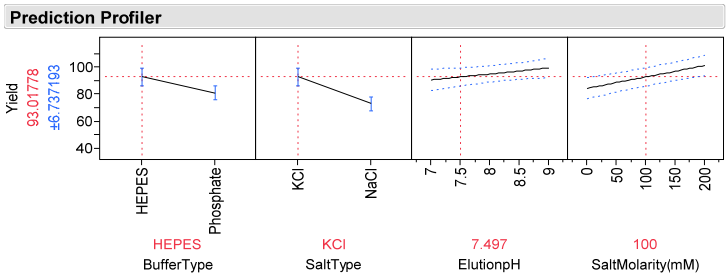

Fig. 3 Prediction profiler for Resin 1

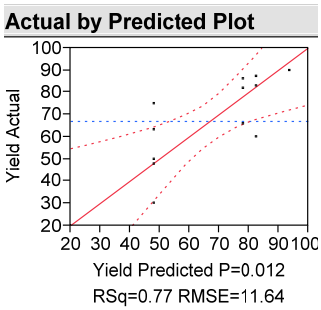

Fig. 4 Actual by predicted plot for Resin 2

Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
SaltMolarity(mM)	-0.227723	0.056743	-4.01	0.0070*
SaltType[KCl]	14.975248	4.435801	3.38	0.0149*

Fig. 5 Sorted parameter estimates for Resin 2

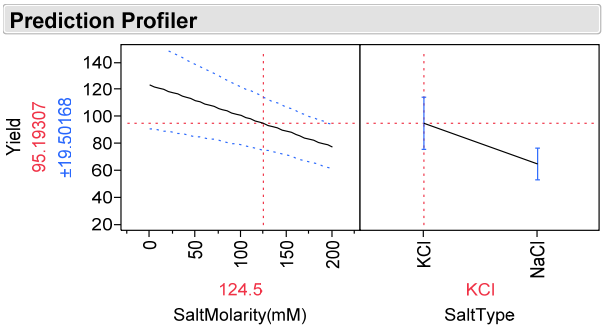

Fig. 6 Prediction profiler for Resin 2

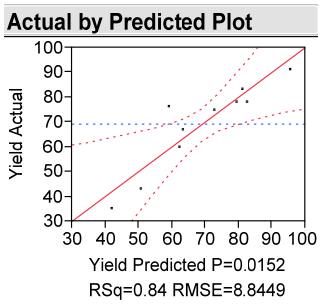

Fig. 7 Actual by predicted plot for Resin 3

Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
BufferType[HEPES]	14.507565	3.340574	4.34	0.0049*
SaltType[KCl]	15.397304	3.65651	4.21	0.0056*
WashpH	-17.63927	6.417412	-2.75	0.0334*
ElutionMolarity	0.239838	0.09652	2.48	0.0475*

Fig. 8 Sorted parameter estimates for Resin 3

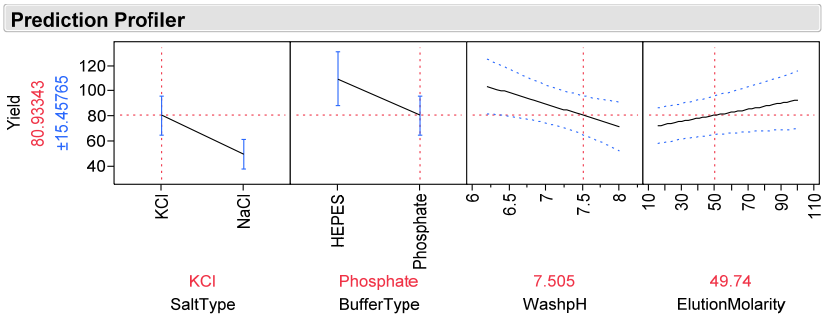

Fig. 9 Prediction profiler for Resin 3

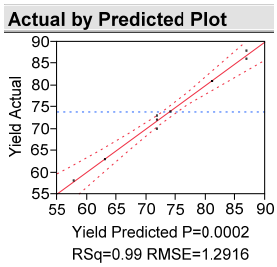

Fig. 10 Actual by predicted plot for Resin 4

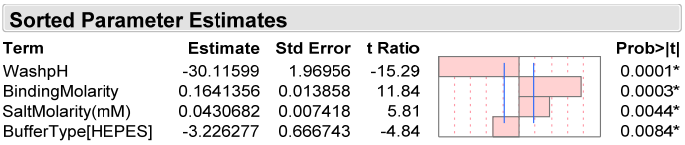

Fig. 11 Sorted parameter estimates for Resin 4

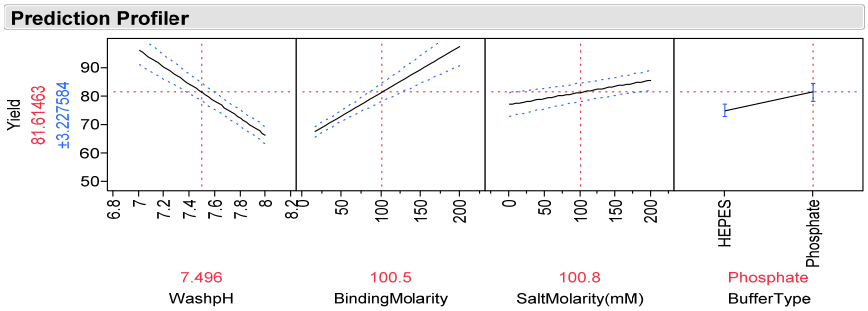

Fig. 12 Prediction profiler for Resin 4

IV. Conclusion

It has been successfully demonstrated that it is possible to remove aggregates using Cation Exchange Chromatography under the operating conditions used in this work. This may help reduce the number of polishing chromatography steps from three to two which would improve the yield, shorten the processing time and thus improve the process economy. Success has been achieved in obtaining yield greater than 90% using Capto SP ImpRes and Fractogel EMD COO^-(M) resins. It has been found that a change in the operating conditions generally has different effects on the yields obtained using different resins and hence, it is difficult to generalize the effects of certain parameters on the yield.

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