Purification of Recombinant Proteins on Nuvia™ cPrime™ Hydrophobic Cation Exchange Media: A Simple Approach to Method Development

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Tech Note

Protein Purification

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Introduction

Mixed-mode chromatography has become an important purification tool for downstream process developers, who are always seeking highly selective yet robust methods for the purification of recombinant proteins. Mixed-mode chromatography matrices are designed to present multiple interaction modes to resolve target proteins and impurities, entities that are themselves complex multimodal molecules. Under specific purification conditions, one or more such interaction modes may be involved in the binding or repulsion between the target protein and the chromatography media. Therefore, the behavior of a protein during purification by mixed-mode chromatography is often not predictable on the basis of its pI or amino acid sequence.

Here, we describe the use of a Design of Experiment (DOE) approach in the initial screening of chromatographic conditions for the purification of a diverse set of protein targets on Nuvia cPrime hydrophobic cation exchange media (Figure 1). The ligand on this media has three major functionalities: a weak carboxylic acid end group, an aromatic hydrophobic ring, and an amide bond serving as a potential hydrogen bond donor/acceptor. The combined effect of these structural elements provides unique selectivity and good conductivity tolerance, allowing protein purifications to be conducted effectively under gentle conditions. Our studies show that, with a limited amount of protein sample and chromatography media, a simple DOE setup can be used to determine the effects of buffer pH and conductivity on selectivity, recovery, and robustness of protein purification on Nuvia cPrime. Working conditions established by such scale-down studies can be used for the purification of a target protein on a preparative scale.

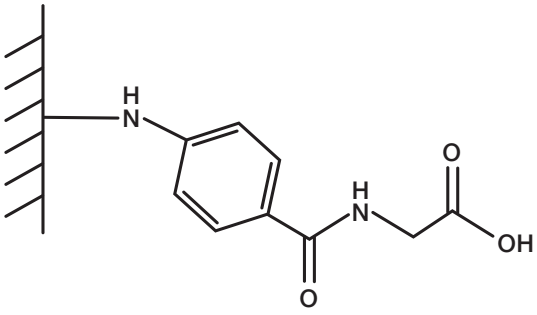

Fig. 1. Mixed-mode ligand for Nuvia cPrime media.

Materials and Methods

General

Bovine serum albumin, bovine carbonic anhydrase, and conalbumin were purchased from Sigma-Aldrich. Lactoferrin was obtained from Glanbia Nutritionals, Inc. Monoclonal antibody, mAbX, was overproduced in a Chinese hamster ovary (CHO) cell culture and previously purified by column chromatographic methods. Protein fractions were analyzed by SDS-PAGE using Criterion™ Tris-HCl 4–20% linear gradient gels (Bio-Rad) stained with Bio-Safe™ Coomassie stain (Bio-Rad), and quantified on a GS-800™ calibrated densitometer (Bio-Rad). The clearance of *E. coli* host cell proteins (HCPs) and double-stranded DNA (dsDNA) were determined by *E. coli* HCP ELISA kit F410 (Cygnus Technologies) and Quant-iT dsDNA High-Sensitivity Assay Kit (Invitrogen), respectively. Protein concentration was determined by UV absorption at 280 nm, using the respective coefficients at 1 mg/ml.

Design of experiments (DOE) setup

JMP software (www.jmp.com) was used in the design of experiment studies to identify optimal binding and elution conditions on Nuvia cPrime for a set of five proteins. These proteins vary in their molecular mass and pI (Table 1). The effects of two parameters, buffer pH, and conductivity, were evaluated by a two-level fractional factorial experimental design, with three center points and a total of 11 experiments (Table 2). They were executed in spin column format (see next section for details). The static binding capacity and recovery of target proteins were plotted against the binding or elution

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buffer pH and sodium chloride concentration. A standard least squares model was employed to obtain the response surfaces and to predict the optimal conditions for maximum target protein binding capacity and recovery.

Table 1. List of proteins used in the study.

Test Protein	pI	Molecular Mass
Bovine serum albumin	4.7	67 kD
Bovine carbonic anhydrase	5.9	29 kD
Conalbumin	6.9	78 kD
Lactoferrin	9.2	78 kD
mAbX	9.5	150 kD

Table 2. Design of experiment setup.

Experiment	Binding pH	Binding [NaCl], mM	Elution pH	Elution [NaCl], mM
1	4.0	400	8.0	10
2	8.0	10	8.0	10
3	**6.0**	**205**	**6.0**	**505**
4	4.0	400	4.0	1,000
5	8.0	400	8.0	1,000
6	8.0	10	4.0	1,000
7	**6.0**	**205**	**6.0**	**505**
8	4.0	10	8.0	1,000
9	4.0	10	4.0	10
10	**6.0**	**205**	**6.0**	**505**
11	8.0	400	4.0	10

Note: Experiments 3, 7, and 10 are the center points.

Developing methods for protein purification on Nuvia cPrime media using design of experiments (DOE)

Mini Bio-Spin® columns (Bio-Rad), each containing 100 µl of pre-equilibrated Nuvia cPrime, were used in the tests. Nuvia cPrime resin was mixed with 500 µl of test protein solution (6 mg/ml in respective binding buffer) at room temperature with constant agitation for 3 min. The unbound protein was collected at the end of the incubation by spinning at 1,000 x g for 1 min. Loading was repeated once. Each spin column was washed once with 5 CV of binding buffer. All unbound proteins collected during column loading and wash were combined. To each spin column, 500 µl of respective elution buffer were added and mixed with loaded resin at room temperature for 3 min with agitation. The eluted protein was collected by spinning at 1,000 x g for 1 min. This was repeated once and eluates from both runs were combined. The protein concentrations in these samples were quantified for the determination of static binding capacity and recovery of each test protein under specific conditions.

Capture of lysozyme from *E. coli* lysate

The same DOE approach was used in the screening of chromatographic conditions for capturing lysozyme (pI 9.3) from an *E. coli* lysate. Approximately 500 µl of preconditioned *E. coli* lysate containing 3 mg of lysozyme, were loaded onto each spin column with 100 µl pre-equilibrated Nuvia cPrime media. The yield and purity of eluted lysozyme from each set of binding and elution condition combinations were chosen as the responses to parameter variations. Eluates were analyzed by reducing SDS-PAGE. The band density of lysozyme in each eluate was used as the indicator for its yield under specific conditions; the abundance of lysozyme among all proteins in each eluate represented the purity of a particular lysozyme preparation. The A_{280}/A_{260} ratio was used to evaluate the efficiency of host cell nucleic acid clearance. A higher A_{280}/A_{260} ratio indicates a relatively lower nucleic acid contamination level. The design space for optimal yield and purity of lysozyme was identified from the response surfaces generated by the JMP software.

For scale-up preparation of lysozyme from *E. coli* lysate, a 1 ml column (0.56 x 4 cm) of Nuvia cPrime media was used. This column was equilibrated with 20 mM sodium acetate and 150 mM NaCl (pH 4.0). *E. coli* lysate containing 3 mg/ml lysozyme was adjusted to pH 4.0 and applied onto the column at a linear velocity of 250 cm/hr. The dynamic binding capacity of lysozyme at 10% breakthrough was determined by inline UV absorption at 280 nm on a BioLogic DuoFlow QuadTec™ 10 system (Bio-Rad). Bound lysozyme was eluted using 10 CV of 20 mM sodium phosphate and 1 M sodium chloride (pH 7.5). Recovery of lysozyme was calculated by comparing the amount of lysozyme eluted from the column and the total amount of lysozyme loaded. The reported value was the average of two runs. This procedure was repeated for determining the 10% breakthrough dynamic binding capacity of lysozyme in the same buffer at pH 4.5. The purity of lysozyme and dsDNA content in eluates were also quantified.

Results and Discussion

Increasingly diverse therapeutic protein candidates are entering drug development pipelines. The pI and molecular mass of these recombinant proteins can be predicted from their encoding DNA sequences. However, information on other aspects of their physiochemical/conformational properties is often incomplete, posing a challenge to downstream purification process developers who are tasked to elucidate a purification strategy. We have explored the possibility of using DOE, a fractional factorial design consisting of 11 experiments, for initial chromatographic conditions screening. A set of five diverse proteins with differing pI value and molecular mass have been included in this study (Table 1). The binding capacity and recovery of these proteins on Nuvia cPrime, in response to changes in buffer pH and sodium chloride concentration, were assessed statistically. The predicted optimal binding and elution conditions are summarized in Table 3. From the data, we can find areas of optimal performance where target protein binding and recovery are maximized. Our results also suggest that these proteins employ dramatically different modes of interaction with Nuvia cPrime under the conditions explored.

Maximum binding of bovine serum albumin and bovine carbonic anhydrase are reached at the lowest tested pH and sodium chloride concentration, while high pH and high sodium chloride concentrations are required for their complete recovery from Nuvia cPrime. Therefore, it can be concluded that the main interaction between these test proteins and Nuvia cPrime is electrostatic in nature. In other words, the cation exchange character of Nuvia cPrime is more dominant in the purification of these bovine proteins.

The best binding condition for conalbumin is the same as that for bovine serum albumin and bovine carbonic anhydrase, indicating that charge-charge interaction is predominant in initial binding as well. Interestingly however, maximum recovery of this protein is achieved at mildly acidic pH (~6.0) in the presence of modest salt concentration (~600 mM). This would indicate that the use of higher salt concentrations may promote hydrophobic interactions between conalbumin and Nuvia cPrime, thus hampering its elution. It is also possible that an increase in buffer pH causes conalbumin to adopt an alternative conformation, which exposes more hydrophobic regions for a stronger association with the chromatography media.

Lactoferrin and mAbX are two basic proteins with a pI of around 9. They are expected to be positively charged in the entire test pH range and interact strongly with Nuvia cPrime via electrostatic interaction. Interestingly, optimal binding capacity for these proteins was observed in the presence of substantial concentration of sodium chloride, which suggests the binding of these proteins is enhanced by the hydrophobic interactions. Both high pH and high salt concentration are needed for the proteins complete elution. Therefore, charge-charge interaction is the main driving force for the association of these proteins with Nuvia cPrime.

Table 3. Optimal binding and elution conditions of various proteins on Nuvia cPrime.

Test Protein	pI	Optimal Conditions Predicted by DOE
Bovine serum albumin	4.7	Binding: 10 mM NaCl, pH 4.0 Elution: 1,000 mM NaCl, pH 8.0
Bovine carbonic anhydrase	5.9	Binding: 10 mM NaCl, pH 4.6 Elution: 1,000 mM NaCl, pH 8.0
Conalbumin	6.9	Binding: 10 mM NaCl, pH 4.0 Elution: 505 mM NaCl, pH 6.0
Lactoferrin	9.2	Binding: 205 mM NaCl, pH 4.0 Elution: 1,000 mM NaCl, pH 8.0
mAbX	9.5	Binding: 300 mM NaCl, pH 4.6 Elution: 800 mM NaCl, pH 8.0

Our data suggest that a simple DOE screening study is sufficient for predicting the chromatographic behavior of these test proteins on Nuvia cPrime, despite the fact that they are very different in size, charge state, and hydrophobicity.

In a real-world protein separation scenario, more exhaustive DOE can be performed to fine-tune the separation condition. An alternative approach is to create buffer gradients on a traditional packed column to understand the impact of buffer pH, conductivity, and additives on column chromatography performance, following the initial DOE screening.

The same DOE strategy was applied to process development for the purification of lysozyme from an *E. coli* lysate. At pH 4.0–5.0, lysozyme can be efficiently captured from *E. coli* lysate in the presence of up to 100 mM of sodium chloride. Under this condition, nucleic acids as well as acidic proteins from the expression host cells are expected to flow through the column. While loading lysate at pH near 8.0 may also increase the selectivity for lysozyme over other protein impurities, higher binding buffer pH does compromise the yield and nucleic acid impurity removal according to the model. The elution of lysozyme from Nuvia cPrime is robust in the entire pH range tested. However, higher sodium chloride concentration in elution buffer is required for maximum recovery of this protein and optimal nucleic acid clearance. The response contours from modeling also define the design space for the purification of target protein (Figure 2). It is worth mentioning that lysozyme is a basic protein, with a pI value very close to that of lactoferrin and mAbX. However, hydrophobic interaction does not seem to play any important role in its interaction with Nuvia cPrime. In other words, the pI of an unknown protein alone is not sufficient for predicting its chromatographic behavior on Nuvia cPrime. This is consistent with observations made on other multimodal chromatography media (Cramer 2013).

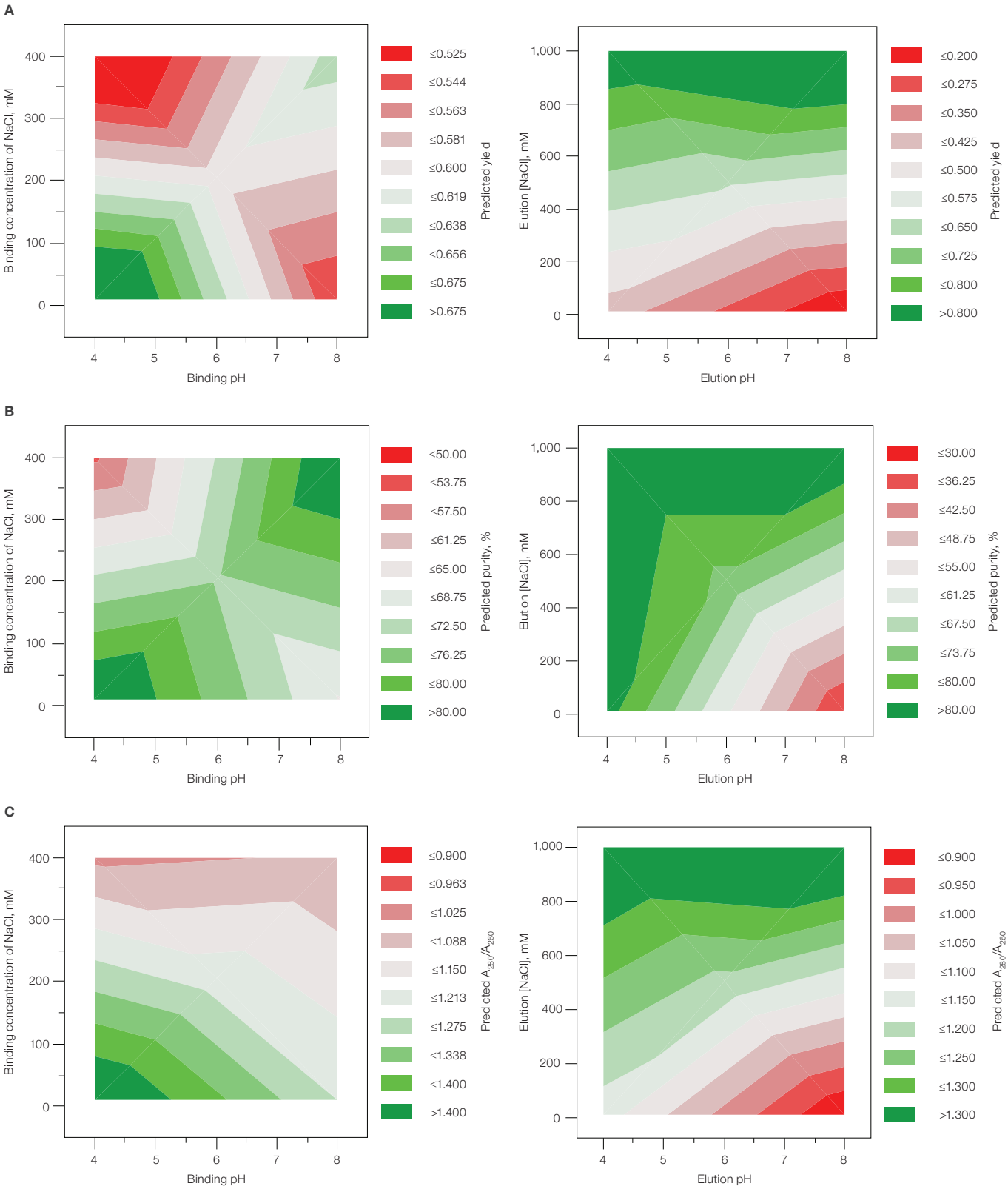

Fig. 2. Capturing lysozyme from *E. coli* lysate using a Nuvia cPrime column. A, effect of buffer pH and sodium chloride concentration on the yield of lysozyme; **B**, effect of buffer pH and sodium chloride concentration on the purity of lysozyme; **C**, effect of buffer pH and sodium chloride concentration on the removal of host cell nucleic acids, as indicated by A_{280}/A_{260}.

A scale-up preparation of lysozyme was prepared on the basis of conditions predicted from the above DOE screening. Feed stream loading was performed at pH 4.0 or 4.5. Such buffer pH variation has no impact on dynamic binding capacity for lysozyme or the clearance of impurities from host cells (Table 4 and Figure 3). The recovery of lysozyme was close to quantitative. These results are in good agreement with the predictions from the DOE screening study.

Table 4. Capture of lysozyme from an *E. coli* lysate.

Binding Buffer	10% DBC, mg/ml	Recovery, %	Purity, %	dsDNA, ppm
20 mM sodium acetate, 150 mM NaCl (pH 4.0)	59	94	91	ND*
20 mM sodium acetate, 150 mM NaCl (pH 4.5)	67	100	92	22 ppm

*ND, not determined.

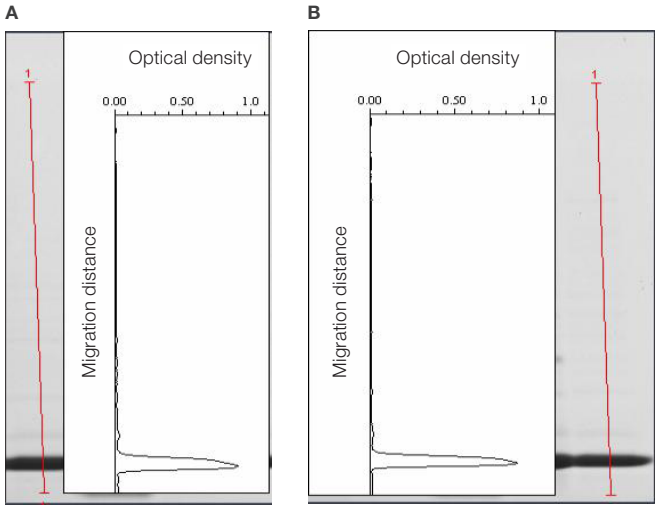

Fig. 3. Electrophoretograms of purified lysozyme. A, *E. coli* lysate was loaded onto the Nuvia cPrime column at pH 4.0 and eluted with 20 mM sodium phosphate and 1 M sodium chloride (pH 7.5); **B**, *E. coli* lysate was loaded onto the Nuvia cPrime column at pH 4.5 and eluted with 20 mM sodium phosphate and 1 M sodium chloride (pH 7.5). The reducing SDS-PAGE gel was scanned with a GS-800 densitometer. The purity of lysozyme in each eluate was illustrated by its optical density trace generated with QuantityOne® software.

Conclusion

We have demonstrated a simple yet effective approach to obtain the optimum binding and elution conditions for a diverse set of proteins on Nuvia cPrime hydrophobic cation exchange media. A total of 11 experiments from a two-parameter, two-level fractional factorial DOE approach was sufficient to assess the effects of buffer pH and conductivity on the chromatographic behavior of a particular protein.

Using the purification of lysozyme from *E. coli* lysate as an example, we have also shown that a design space for maximum target protein purity and yield can be identified from response surfaces generated in the DOE screening. Following the operational conditions suggested by DOE, we are able to scale up the purification of lysozyme from crude expression harvest with remarkable purity.

Finally, we have demonstrated that method development for protein purification on Nuvia cPrime hydrophobic cation exchange media is straightforward. The modes of interaction, as well as the extent of these interactions between feed stream components and Nuvia cPrime can be elucidated early in the process, thus allowing users to quickly and effectively optimize their method for best selectivity and robustness.

Reference

Steven M. Cramer (2013). Fundamental understanding of synergistic interactions in multimodal systems. 8th HIC/RPC Bioseparation Conference, Savannah, GA, USA.

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