Regeneration Studies of Anion-Exchange Chromatography Resins

Paul K. Ng and Valerie McLaughlin

Chromatography columns can become contaminated by a variety of protein and nonprotein species during a purification campaign. Consequences of column contamination include an increase in backpressure, loss of signal resolution, altered product yield, and medium discoloration. Common chromatographic contaminants include

- residual proteins
- nucleic acids
- lipids
- endotoxins
- viruses and bacteria
- metal ions.

Generally, methods for cleaning-in-place (CIP) and sanitization-in-place (SIP) of chromatographic resins are selected based on the interplay and relevance of three factors: ease of operation, historical experience, and performance requirements. In most cases, a column decontamination method chosen by a laboratory forms the basis not only for process validation, but also for subsequent scale-up. For this reason, an ideal scenario would be development and use of a generic decontamination method. At present, however, even taking into account the varying types of resins available, considerable disparity of CIP and SIP procedures is apparent in instructions available from manufacturers of chromatography resins.

Many traditional cleaning solutions are used for CIP and SIP. Table 1 lists target contaminants of these solutions. Unquestionably, the cleaning strategy that has attracted the most attention and produced the most dependable results is the combination of sodium chloride (NaCl) and sodium hydroxide (NaOH). It has repeatedly proven effective in chromatography column decontamination. The key advantage of NaOH is its bactericidal action: NaOH inhibits the growth of and kills many bacteria and microorganisms. When NaOH–NaCl is applied to base-resistant chromatography resins supplied by various manufacturers, it has proven to be highly effective in validation studies for removal of residual proteins, viruses, and endotoxins (**1, 2**).

In our investigation, we addressed the clearance of DNA because there is limited relevant information or supporting data on mass balance. DNA, being highly negatively charged, has strong affinity for the positively charged surfaces of anion-exchange resins. Any DNA not removed by a cleaning procedure will gradually accumulate over time and diminish column binding capacity and selectivity. Accordingly, we chose to examine chromatography issues such as postcleaning DNA recovery and selectivity, as are discussed here. We also touch upon simultaneous clearance of endotoxin and residual proteins.

Scanning electron micrograph of the UNOsphere Q anion-exchange chromatography resin
(WWW.BIO-RAD.COM)

PRODUCT FOCUS: ALL BIOPHARMACEUTICALS

PROCESS FOCUS: PURIFICATION (CHROMATOGRAPHY, CLEANING)

WHO SHOULD READ: RESEARCH, PROCESS DEVELOPMENT, AND MANUFACTURING

KEYWORDS: ANION-EXCHANGE CHROMATOGRAPHY, NAOH, NACL, ENDOTOXINS, DNA, CIP, SIP, QUANTITATION

LEVEL: INTERMEDIATE

MATERIALS AND METHODS

DNA: For DNA recovery studies we used sheared salmon sperm DNA (catalog #9610-5-D, R&D Systems of Minneapolis, MN, www.rndsystems.com). As indicated in the manufacturer's package insert, the material contains DNA fragments ranging in size from 200 to 500 base pairs.

Quantitation of DNA: Absorbance at 260 nm (A_{260}) was used for monitoring DNA concentration with a conversion factor of 50 µg/mL DNA per absorbance unit. DNA concentration was also measured using a dye-based assay with PicoGreen (Invitrogen of Carlsbad, CA, www.invitrogen.com), which fluoresces on binding to double-stranded DNA. After adding the working solution of PicoGreen reagent to the sample and incubating it at room

temperature for 2–5 min., we measured fluorescence using a Cary Eclipse spectrophotometer (Varian, Inc. of Walnut Creek, CA, www.varianinc.com) with excitation at 480 nm and emission at 520 nm. The detection limit of the PicoGreen assay is 250 pg/mL of double-stranded DNA (75 pg in a 300 µL sample volume). Linearity, with a regression coefficient of >0.99, was routinely obtained in a standard curve spanning 0–500 ng/mL.

Quantitation of Endotoxin: For single sample assays, we used the Endosafe-PTS reader (Charles River Laboratories of Wilmington, MA, www.criver.com), a point-of-use test system that involves existing FDA-licensed *Limulus* amoebocyte lysate (LAL) reagents in a test cartridge with a handheld spectrophotometer. Sensitivity of the assay is 0.05 EU/mL.

UNOsphere Q Chromatography Support: All chromatography experiments were conducted using UNOsphere Q support packed in Bio-Scale MT2 or MT10 columns (Bio-Rad Laboratories, Inc. of Hercules, CA, www.bio-rad.com). The MT2 column dimensions are 0.7 cm in diameter and 2.6–5.2 cm high. The MT10 column dimensions are 1.2 cm in diameter and 8.8 cm high.

Endotoxin Concentrate: The endotoxin concentrate consisted of equal amounts of lipopolysaccharides from *Escherichia coli*, *Salmonella enterica* serotype *abortus equi*, and *Pseudomonas aeruginosa* 10 (all purchased from Sigma-Aldrich, St. Louis, MO, www.sigma-aldrich.com). It was assayed with the Endosafe-PTS reader and determined to have 6.64×10^6 EU/mL.

Selectivity: A Bio-Rad Laboratories protein standard for anion-exchange chromatography (catalog #125-0561), comprising equine myoglobin, conalbumin, chicken ovalbumin, and soybean trypsin inhibitor, was separated using a gradient method (buffer A, 20 mM Tris, pH 8.5; buffer B, 20 mM Tris, 1.0 M NaCl, pH 8.5). The retention time of each protein was determined from the chromatogram.

Chromatography System: All chromatography experiments were automated and performed using a BioLogic DuoFlow Maximizer chromatography system and software (Bio-Rad Laboratories, Inc.). Flow rates of the columns were maintained at 300 cm/hr throughout all experiments.

Table 1: Traditional cleaning solutions for specific contaminants

Cleaning Solutions	Contaminants Removed
1–3 M NaCl, 1–2 M NaOH	Residual proteins, DNA
Guanidine hydrochloride	Residual proteins, lipids
Urea, ethanol, isopropyl alcohol	Residual proteins, lipids
1–2 M NaOH, tri(n-butyl)phosphate/Tween	Viruses, endotoxins
Citric acid, EDTA	Metal ions

Table 2: Effect of wash sequence on DNA recovery

Process Step	Percentage Yield
Flowthrough with wash	0
Eluted fractions at 0.1 M NaCl	0
Eluted fractions at 0.5 M NaCl	0
Eluted fractions at 1.0 M NaCl	35.4
Eluted fractions at 2.0 M NaCl	10.8
2.0 M NaCl with 1.0 M NaOH wash	37.8
1.0 M NaOH wash	7.8
0.02 M NaOH wash	0
Cumulative	91.8

Table 3: Measured DNA clearance using the PicoGreen assay

Sample	Total DNA (ng)	Percentage Remaining
Feed	147,500	100
Fractions from 0 to 0.5 M NaCl	417	0.28
DNA remaining in column	147,083	99.7

Table 4: Endotoxin removal from UNOsphere Q column

Total Challenge Reduction (EU)	Total in Eluate (EU)	Percentage Removal	Log Value
3.3×10^5	<0.16	>99.999	>6

Results and Discussion

DNA Recovery: The standard column hygiene sequence developed during this investigation is

Clean: 2.0 M NaCl, three column volumes

Sanitize: 1.0 M NaOH, three column volumes

Store: 0.02 M NaOH, three column volumes.

This decontamination sequence was studied with fractions collected from stepwise elution of increasing NaCl concentrations up to 2.0 M. A 0.15-µg sample of salmon DNA was injected into a 1-mL UNOsphere Q column. Table 2 shows the percentage yield (the ratio of A_{260} recovery relative to A_{260} injection).

The tested range of NaCl concentrations shown in Table 2 is a commonly used diagnostic elution zone for many proteins of research interest. These data show that insignificant clearance was obtained across the fractions from 0 to 0.5 M NaCl using anion-exchange chromatography. Because there was no detectable absorbance at 260 nm in these fractions, their DNA fractions were determined with the PicoGreen assay. These results (Table 3) indicated significant DNA clearance and agree with data published previously (**2**).

Endotoxin Clearance: Subsequent to soiling with a challenge of 3.3×10^5 units of endotoxin, the column was washed in sequence with 2.0 M NaCl and 1 M NaOH. After holding in 1.0 M NaOH for three hours, the column was washed with 0.02 M NaOH. The wash solution was neutralized with phosphate buffered saline before the LAL assay. Results of the experiment (Table 4) demonstrated excellent clearance of endotoxin resulting from a combination of removal due to charge difference and inactivation of residual endotoxin bound to the column. These data are consistent with the most frequently used CIP–SIP protocols that use NaOH as the sanitizing agent. A clearance factor of more than six orders of magnitude was reached; however, this exceptional efficiency is restricted

Figure 1: Selectivity before and after CIP

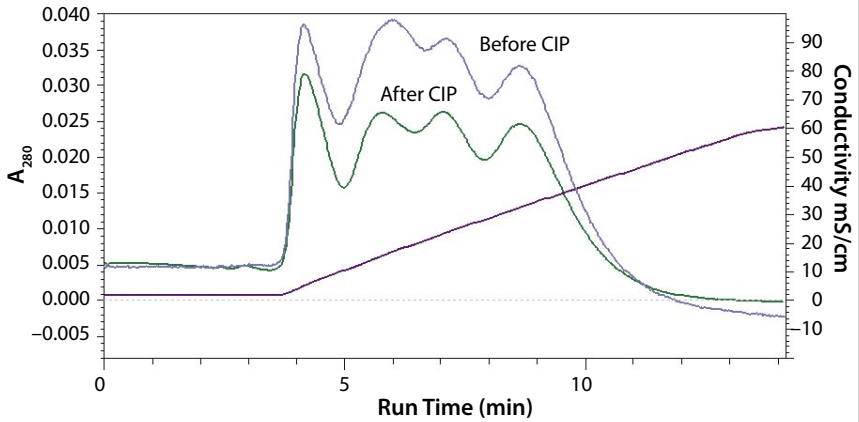
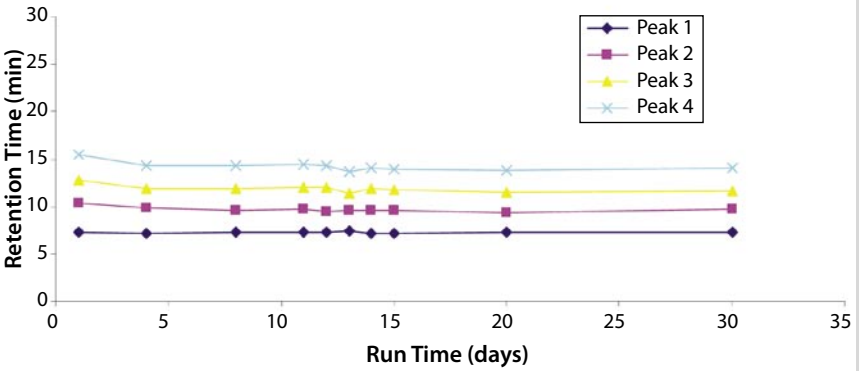


Figure 2: Selectivity of a 10-mL UNOsphere Q column before and after CIP



to artificially high endotoxin feed concentration. At feed concentrations significantly lower than the challenge used in this case, which is a more common endotoxin contamination level, total endotoxin clearance is anticipated with NaOH inactivation. The results are consistent with data previously published (**1**).

Residual Contaminant Clearance: Following CIP/SIP and between runs, small amounts of residual proteins, microorganisms, and endotoxins could still be present. Their concentration will differ among various process applications and is a strong function of feed stream. It is necessary to use practical methods to quantify such residual materials.

Three tests, including A_{280}, microbial load, and LAL, can be used to verify that the resin is consistently meeting necessary and achievable acceptance criteria. The process developer simply compares initial and eluted buffer values to determine the magnitude of residual contaminants.

With regard to column sanitization, NaOH is generally accepted as an effective cleaning agent to provide elevated levels of cleanliness for chromatographic materials and other product contact surfaces. Its ability to inactivate significant levels of commonly found microorganisms has been verified (**3, 4**). Hence, we made no attempt to carry out microbial challenge tests in the current study.

Selectivity of Column Before and After CIP: By using the recommended conditions (see DNA Recovery section), we studied the effect of CIP on selectivity. The data in Figure 1 show that column selectivity was unaffected by the decontamination treatment. The slight difference in A_{280} signal was due to minor variation in the sample load. As would be expected, any change must be carefully monitored over the lifetime of a column. Process developers would address such a study before scale-up.

To evaluate the sanitization–decontamination method further, the 1 mL column was scaled up to 10 mL, and the following cycles were repeated after each run: 2.0 M NaCl wash, 1.0 M NaOH for >3 hr, and storage at 0.02 M NaOH for >16 hr. We then measured the protein's retention time, which is the time between injection and the appearance of the peak maximum. As shown in Figure 2, selectivity remained constant over 10 cycles in a duration of 30 days. The data are consistent with the superior base stability reported previously (**5**).

Good Clearance

A cleaning cycle using 2.0 M NaCl and 1.0 M NaOH has been shown to give good chromatographic clearance of DNA and endotoxin. Excellent base stability of the anion-exchange support was evidenced by no change in its selectivity. This regeneration protocol appears to be suitable for both validation and scale-up.

Acknowledgment

The SEM was provided courtesy of Dr. Danni Wang of Bio-Rad Laboratories.

References

1 Conley L, McPherson J, Thommes J. Validation of the ZEVALIN Purification Process: A Case Study. *Process Validation in Manufacturing of Biopharmaceuticals.* Rathore AS, Sofer G, Eds. CRC Press: Boca Raton, FL, 2005; 469–521.

2 Dasarathy Y. A Validatable Cleaning-In-Place Protocol for Total DNA Clearance from an Anion-Exchange Resin. *BioPharm* 9(8) 1996: 41–44.

3 Jungbauer A, Lettner H. Chemical Disinfection of Chromatographic Resins, Part 1: Preliminary Studies and Microbial Kinetics. *BioPharm* 7(5) 1994: 46–56.

4 Boschetti E, et al. Concerns and Solutions for a Proper Decontamination of Chromatographic Packings. *Chimica Oggi* 11(3–4) 1993: 29–35.

5 Franklin S, et al. UNOsphere Q support technical data, *Bio-Rad Bulletin 2729* (2002).

*Corresponding author **Paul K. Ng** is senior staff scientist of process applications at Process Chromatography Division, Bio-Rad Laboratories, 6000 James Watson Drive, Hercules, CA 94547, 1-510-741-4839, fax 1-510-741-4114, paul_ng@bio-rad.com.*
***Valerie McLaughlin** is senior product manager of marketing at Bio-Rad Laboratories.*