Effective Cleaning and Sanitizing of Anion Exchange Chromatography Resins

Paul K Ng[1] and Valerie McLaughlin[2], Process Chromatography Division

[1]Process Applications Group, [2]Marketing Group

Bio-Rad Laboratories, Inc.
2000 Alfred Nobel Drive
Hercules, CA 94547

Abstract

UNOsphere™ Q anion exchange chromatography columns soiled by DNA or endotoxin were decontaminated in situ using common, readily available chemicals. Mixing studies and analysis confirmed that elution fractions contained >99% clearance of DNA with <0.5 M NaCl. Clearance of endotoxin was >10^6-fold. Follow-up gradient separation of model proteins confirmed that the cleaning and sanitization methods reported here do not impair the selectivity of the chromatographic resin. These results are suggestive and promising for a variety of high-throughput chromatographic workflow applications.

Background

Chromatography columns may become contaminated by a variety of protein and nonprotein species during a purification campaign. Consequences of column contamination include an increase in column 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
- Performance requirements

In most cases, the column decontamination method chosen in the laboratory not only forms the basis for 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/SIP procedures is apparent in instructions available from manufacturers of chromatography resins.

Many traditional cleaning solutions are used for CIP and SIP. Target contaminants of these solutions are in Table 1.

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

Unquestionably, the cleaning strategy that has attracted the most attention and produced the most dependable results is the combination of NaCl and NaOH. This combination 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 is applied to base-resistant chromatography resins supplied by various resin manufacturers, it has proven to be highly effective in validation studies for the removal of residual proteins, viruses, and endotoxins (Conley et al. 2005, Dasarathy 1996).

In our investigation, we addressed the clearance of DNA since 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. But 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 post-cleaning DNA recovery and selectivity. Simultaneous clearance of endotoxin and residual proteins will also be discussed.

Methods

DNA

Sheared salmon sperm DNA (catalog #9610-5-D, R&D Systems) was used for DNA recovery studies. 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), which fluoresces on binding to double-stranded DNA. After addition of the working solution of PicoGreen reagent to the sample, and incubation at room temperature for 2 to 5 min, fluorescence was measured using a Cary Eclipse spectrophotometer (Varian, Inc.) 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), a point-of-use test system that uses 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.). The MT2 column dimensions are 0.7 cm in diameter and 2.6–5.2 cm in height. The MT10 column dimensions are 1.2 cm in diameter and 8.8 cm in height.

Endotoxin Concentrate

The endotoxin concentrate consisted of equal amounts of liposaccharides from *Escherichia coli*, *Salmonella enterica* serotype *abortus equi*, and *Pseudomonas aeruginosa* 10. It was assayed with the Endosafe-PTS reader and determined to have 6.64 x 10^6 EU/ml.

Selectivity

A Bio-Rad Laboratories protein standard for anion exchange chromatography (catalog #125-0561), consisting of 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.

Results and Discussion

DNA Recovery

The standard column hygiene sequence developed during this investigation is:

- Clean — 2.0 M NaCl, 3 column volumes
- Sanitize — 1.0 M NaOH, 3 column volumes
- Store — 0.02 M NaOH, 3 column volumes

A study was made of this decontamination sequence 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. The percentage yield (the ratio of A_{260} recovery relative to A_{260} injection) is shown in Table 2.

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

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. Since 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 (Dasarathy 1996).

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

Endotoxin Clearance

Subsequent to soiling with a challenge of 3.3 x 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 3 hr, the column was washed with 0.02 M NaOH. The wash solution was neutralized with phosphate buffered saline prior to LAL assay. The results of the experiment (Table 4) demonstrated excellent clearance of endotoxin. These data are consistent with the most frequently employed CIP/SIP protocols that use NaOH as the sanitizing agent. A clearance factor of more than 6 orders of magnitude was obtained; however, this exceptional efficiency is restricted 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 clearance of the endotoxin is anticipated with NaOH inactivation. The results are consistent with data previously published (Conley et al. 2005).

Table 4. Endotoxin removal from UNOsphere Q column.

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

Residual Contaminant Clearance

Following CIP/SIP and between runs, it is possible that minuscule amounts of residual proteins, microorganisms, and endotoxins could be present. Their concentration will differ among various process applications and is a strong function of the feed streams' concentration. It is necessary to employ 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.

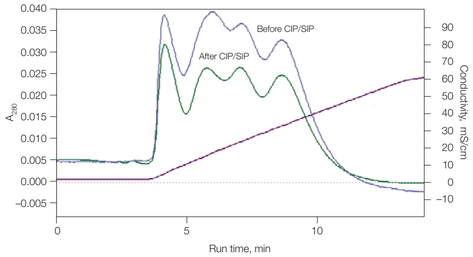

Fig. 1. Selectivity before and after CIP.

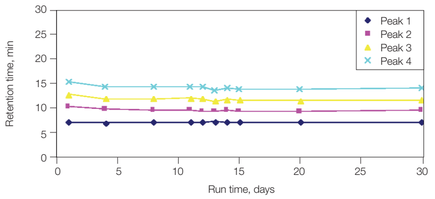

Fig. 2. Selectivity of a 10 ml UNOsphere Q column before and after CIP.

Selectivity of Column Before and After CIP

By using the recommended conditions (see DNA Recovery section), a study was made of the effect of CIP on selectivity. The data in Figure 1 show that the selectivity of the column was unaffected by the decontamination treatment. As would be expected, any change must be carefully monitored over the lifetime of a column. Such a study should be addressed prior to scale-up by the process developers.

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 ≥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 ten cycles in a duration of 30 days. The data are consistent with the superior base stability reported previously (Franklin et al. 2002).

Conclusions

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 UNOsphere Q anion exchange support was evidenced by no change in its selectivity. This CIP/SIP protocol appears to be suitable for both validation and scale-up.

References

Conley L et al., Validation of the ZEVALIN purification process — a case study, pp 469–521 in Process Validation in Manufacturing of Biopharmaceuticals, (Rathore AS and Sofer G, eds), CRC Press, Boca Raton (2005)

Dasarathy Y, A validatable cleaning-in-place protocol for total DNA clearance from an anion-exchange resin, Biopharm 9, 41–44 (1996)

Franklin SG et al., UNOsphere Q support technical data, Bio-Rad bulletin 2729 (2002)