Storing an Ion-Exchange Chromatography Gel in Dilute Alkali During Recycling Improves Cleaning

Chromatography gels used in pharmaceutical production are often recycled, so they must be cleaned effectively between uses. Sodium hydroxide (NaOH, usually at concentrations of 0.5–1 M) has been used successfully as a cleaning and sanitizing agent (1,2). For ion-exchange media, a combination of NaOH and sodium chloride (NaCl) is most effective, the NaCl removing ionically bound material from the gel (1).

Chromatography media should be stored in solutions that inhibit microbial growth, such as 20% ethanol or 0.01 M NaOH (1). Because alkali is an effective cleaning agent and long contact times are involved, we anticipated that storing chromatography gel in dilute alkali might enhance cleaning. We amended a column recycling procedure we have used at small scale (3) to include storage in 0.01 M NaOH (Table 1), as recommended in the literature (4).

Our recycling procedure includes two NaCl washes: one before and one after the hydroxide washes. Although the majority of material is removed during the first sodium chloride wash, the purpose of the second wash is to remove material solubilized by the hydroxide and also to convert the (anion-exchange) gel to the chloride form. We studied the effect of storing gels in 0.01 M NaOH on both the removal of material during recycling and on residual material remaining on the gel following recycling, which would be removed with process buffers in the next cycle.

This work was carried out using DEAE-Sepharose CL-6B anion-exchange gel (Amersham Biosciences, www.abiotech.com) used in the purification of prothrombin-complex concentrate from cryoprecipitate-depleted human plasma. However, the findings from our study should be relevant to the cleaning and storage of any ion-exchange gel.

**Materials and Methods**

**Gel.** In this study, we used postprocess gel from manufacturing. The gel had been batch-adsorbed with cryoprecipitate-depleted human plasma and washed with process buffers as described in the literature (5). Following product elution, the gel was washed with 2 M NaCl (the first recycling step) before being removed from the process column in water-for-injection (WFI).

**Recycling.** We collected samples of gel and continued recycling at small scale using Amicon G16 chromatography columns (Millipore, www.millipore.com/amicon) with an internal diameter of 16 mm and a bed height of 15–17 cm. Gel was packed into the column with 2 M NaCl and washed with additional 2 M NaCl to bring the trace back to baseline. Recycling was then continued (from the 0.5 M NaOH wash onwards) as in Table 1.

**Blank chromatography runs.** Following recycling, the gel was washed with carbon-free versions of the process buffers (as in the process but without protein application) to determine whether residual material remained on the gel after cleaning that would be removed in the next cycle. We used carbon-free versions of the process buffers so that we could measure trace amounts of residual material by total organic carbon (TOC) analysis. We omitted the citrate (normally present at 10 mM in the process buffers) and replaced it with additional sodium phosphate buffer (increasing the concentration from 10 mM to 20 mM). This resulted in buffers with the same pH, ionic strength, and NaCl concentration as the normal process buffers.

**Jane Turton and Zain Moola**

This case study shows that storing an ion-exchange chromatography gel in dilute alkali, followed by removal of the solubilized material, enhances gel cleaning. More material is removed from the gel during column recycling, and carry over of material from one cycle to the next is substantially reduced.

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Table 1. Summary of column recycling procedure with storage in 0.01 M NaOH: The procedure was carried out after washing with elution buffer at a flow rate of 0.64 cm min⁻¹ (1.3 mL min⁻¹ in a 16 mm internal diameter column).

<table>
<thead>
<tr>
<th>Recycling Stepa</th>
<th>Volume or Duration of Wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 M NaCl</td>
<td>two BVs[2]</td>
</tr>
<tr>
<td>0.5 M NaOH</td>
<td>60 min. from pH &gt;12</td>
</tr>
<tr>
<td>WFI[3]</td>
<td>two BVs</td>
</tr>
<tr>
<td>0.01 M NaOH</td>
<td>two BVs</td>
</tr>
<tr>
<td>Storage</td>
<td></td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>30 min. from conductivity as NaCl solution (~80 mS)</td>
</tr>
<tr>
<td>WFI</td>
<td>to pH &lt;7.7, conductivity &lt;100 μS</td>
</tr>
</tbody>
</table>

*aSteps added to the recycling procedure to include storage in dilute alkali are shown in bold type.

*bBV = bed volume  cWFI = water-for-injection

For example, the process wash buffer contains 10 mM citrate, 10 mM phosphate, and 190 mM NaCl at pH 6.5. The carbon-free version (buffer 1) was prepared by combining 20 mM Na₃HPO₄, 190 mM NaCl with 20 mM Na₂HPO₄, and 190 mM NaCl to a pH of 6.5. Similarly, the carbon-free elution buffer (buffer 2) was prepared by combining 20 mM Na₃HPO₄, 310 mM NaCl with 20 mM Na₂HPO₄ and 310 mM NaCl to a pH of 6.5. Previous studies in our laboratory have shown that results obtained using carbon-free versions of the process buffers are similar to those obtained from normal process buffers. Bed volumes (BV) of washes used were as in the process. (These were three BVs of wash buffer, four BVs of elution buffer, and finally two BVs of recycling 2 M NaCl.) Similarly, bed height of the gel (approximately 15 cm, with 12–18 cm limits) and linear flow rate (0.64 cm min⁻¹) were the same as that used in the process. The entire wash from the blank run was collected in 10 defined portions, and each wash portion was analyzed for TOC.

In blank runs, wash applies to all the buffers used, including the elution buffer. Because carbon-free wash buffer for buffer 1 could be ambiguous, we called that wash the first buffer wash. During this wash, the column effluent was collected in BV portions. During washing with carbon-free elution buffer (buffer 2), wash was collected on either side of, and corresponding to, where product fractions would normally be eluted.

**Monitoring**

Absorbance changes at 280 nm during recycling and blank chromatography runs were followed by Uvicord (an ultraviolet monitor from Amersham Biosciences) at high sensitivity, using an absorbance unit (AU) range of 0.1. TOC analysis was performed on a Shimadzu (www.shimadzu.com) TOC-5050 analyzer following the manufacturer’s instructions.

**Removal of material during recycling.** During recycling, a large peak appeared during the first (2 M) NaCl wash (off the AU scale at range 2). A large peak of material was also removed during the 0.5 M NaOH wash (off the AU scale at range 0.1). However, the peak removed during the subsequent NaCl wash was small when we carried out the recycling procedure without the storage steps (that is, without the steps shown in bold in Table 1) as shown in Figure 1a. The following observations refer to peaks removed during this second sodium chloride wash, which took place after the hydroxide wash or washes. When recycling was carried out that included washing with 0.01 M NaOH (as in Table 1) but no storage, a larger NaCl peak was seen (Figure 1b). However, when gel was subjected to both washing and storage in 0.01 M NaOH, the size of the NaCl peak increased further, increasing with the time of storage (Figure 1c–f).

Larger NaCl peaks appeared when the gel was stored at room temperature than at 4 °C. When gel was stored at 4 °C for seven days, the NaCl peak (not shown) was only slightly larger than that seen after three days’ storage at 4 °C (Figure 1d). However, after storage at room temperature for six days, the size of that peak increased greatly (Figure 1f). The 0.01 M NaOH itself removed little, if any, material from the gel; the trace remained at the baseline even when the gel was further washed with 0.01 M NaOH after storage (data not shown).

**Effect of storage on residual material.** A small amount of material was removed in carbon-free blank chromatography runs carried out on gel that was recycled without storage or with only overnight storage at 4 °C in 0.01 M NaOH. Residual material was removed by the first buffer wash (that precedes washing with elution buffer) and could be seen as a very small, broad increase in absorbance on chart recorder traces (Figure 2a–c). This material was removed during the second BV of that wash, and we have called this part of the effluent the blank run buffer 1 peak region (BR1P), in all blank runs (Figure 2, Table 2).

No other increases in absorbance appeared on the traces other than minimal changes associated with solution fronts. In particular, material did not appear to be removed in the region of the trace where product would normally be eluted. The amount of material removed in the first

![Figure 1](image-url) (left) Peaks removed during washing with NaCl following treatment with NaOH: In trace (a), gel was recycled without the storage steps shown in bold in Table 1. In traces (b–f), gel was recycled with the full washes shown in Table 1. Lengths of time stored in 0.01 M NaOH before the NaCl was (b) no storage, (c) overnight (18.5 hours) at 4 °C, (d) three days (66 hours) at 4 °C, (e) overnight (18 hours) at room temperature, and (f) six days (138 hours) at room temperature.
Effects of cycle number of gel used. We considered the possibility that the number of cycles the gel had been used could affect our results. The amount of material removed from gel recycled with storage might have increased with the number of previous cycles in which gel had been recycled without storage. The experiments were carried out in two series: on gel collected during cycles eight and nine (Figure 1a and f and Figure 2a) and on gel collected during cycles 15 and 16 (Figures 1 and 2b–e). Cycle numbers of gel samples used at the start of the following cycle are included in Table 2.

Results can be compared with those obtained for gel of the same (or differing by only one) cycle number. This clearly shows a relationship between storage in dilute alkali and the subsequent size of the NaCl peak and on the amount of material removed in the following blank run. Increasing the number of cycles that gel had been used did not markedly affect the results. The largest NaCl peak seen following treatment with hydroxide was obtained after six days of storage in dilute alkali at room temperature (Figure 1f), despite this experiment having been carried out on the earliest cycle (cycle eight) gel used. Because the number of cycles appears to be relatively unimportant compared with the time and temperature of storage in dilute alkali, the results of all our experiments have been considered together.

Our observations are based on results from a number of experiments. Although only representative traces are shown here (Figures 1a and 2a), the experiments on gel recycled without the storage steps have been carried out a number of times (five as described here and additionally, similar experiments have been carried out in a previous study (3). All experiments consistently provided results similar to those shown here. The results included here (Figures 1a, 2a, and Table 2) are those of experiments that were controls for this study. Results of five experiments in which gel was recycled with storage are included here (Figures 1, 2, and Table 2). In all of these, the same effect was seen (an increase in the amount of material removed during the second recycling NaCl wash and a decrease in the amount of A\textsubscript{280} absorbing material removed during blank runs).

Table 2 shows also TOC results for the part of the wash corresponding to the main product fraction (collected in blank runs with a smaller, initial fraction of 0.2 BV and referred to as product fractions i and ii, a combined fraction of one BV). The table also shows results for the appropriate buffer blanks.

Results of the absorbance changes during washing of recycled gel with carbon-free versions of the process buffers: Removal of material (where seen) took place during the first buffer wash (in the region labeled BR1P). Traces (a–e) show results obtained when gel had been recycled (see Table 1): (a) omitting the storage steps shown in bold in Table 1, (b) with washing but without storage, (c) with overnight (18.5 hours) storage at 4 °C, (d) with storage for three days (66 hours) at 4 °C, and (e) with overnight storage (18 hours) at room temperature.

Buffer wash decreased as the time increased that the gel had been stored in 0.01 M NaOH during recycling. This is in parallel with the increase in peak size seen in the second recycling NaCl wash. After recycling with storage in 0.01 M NaOH for three days at 4 °C or overnight (18 hours) at room temperature, virtually no material was removed from the gel during blank runs (Figure 2d,e). Increasing the storage time further, therefore, had no detectable effect on the blank run results.

TOC analysis. Results of the absorbance traces were confirmed by TOC analysis of the wash portions collected during blank runs. In nearly all cases, TOC results of the wash portions were low and comparable with TOC analysis of the buffer used. Small increases in TOC were seen in the BR1P of blank runs carried out on gel recycled without the storage steps and on gel recycled with washing but without storage in 0.01 M NaOH, and to a lesser extent on gel recycled with only overnight storage at 4 °C in 0.01 M NaOH (Table 2). Note, however, that the results obtained on buffers used for washes (the buffer blanks) sometimes exceeded those of the wash portion collected; increases seen at those low levels may not be significant.

TOC results (in µg mL\textsuperscript{-1}) of wash portions from blank runs: Results shown are for the buffer one peak (BR1P) bed volume (BV) and for wash corresponding to the initial and main product fractions (product fractions i, ii) in a normal process run. Gel had been recycled (Table 1) with storage in 0.01 M NaOH for 0–7 days, or without the steps in bold in Table 1 (method without storage). Results for the corresponding buffer blanks assayed in parallel are shown in italics.

<table>
<thead>
<tr>
<th>Gel Cycle #</th>
<th>Storage in 0.01 M NaOH</th>
<th>BR1P (1 BV\textsuperscript{a})</th>
<th>Buffer One</th>
<th>Product fractions i,ii (1 BV)</th>
<th>Buffer Two</th>
<th>TOC (µg mL\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>no storage</td>
<td>3.6</td>
<td>0.8</td>
<td>1.2</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>16</td>
<td>overnight (18.5 h), 4 °C</td>
<td>2.6</td>
<td>1.1</td>
<td>1.5</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>17</td>
<td>three days, 4 °C</td>
<td>1.6</td>
<td>1.4</td>
<td>0.3</td>
<td>1.3</td>
<td>0.6</td>
</tr>
<tr>
<td>10</td>
<td>seven days, 4 °C</td>
<td>3.4\textsuperscript{b}</td>
<td>3.3</td>
<td>1.0</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>17</td>
<td>overnight (18 h) room temperature</td>
<td>&lt;0.1</td>
<td>3.4</td>
<td>0.8</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>six days room temperature</td>
<td>1.1</td>
<td>0.6</td>
<td>0.6</td>
<td>0.4</td>
<td>0.6</td>
</tr>
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<table>
<thead>
<tr>
<th>Method Without Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
</tr>
<tr>
<td>9</td>
</tr>
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\textsuperscript{a} BV = bed volume
\textsuperscript{b} This result is similar to that obtained for the buffer blank.
has been a consistent finding whenever gel has been recycled with storage (additional results not shown). Moreover, the extent of that effect increased with time and with storage temperature, and this was not attributable to a cycle number effect. We, therefore, consider the improvement in cleaning seen when gel was recycled with storage to be a significant and reproducible effect.

**Discussion of Results**

Here we have described the effect of introducing storage into a recycling procedure we have used previously (3). The gel was stored after it had been washed with 2 M NaCl and with 0.5 M NaOH (Table 1), so that most of the residual material had already been removed by that stage. However, after gel was stored in 0.01 M NaOH, we found an increase in the size of the peak removed during the subsequent NaCl wash. That increase was not merely a consequence of most of the more concentrated alkali being washed from the gel before the washing with NaCl, because the size of that peak increased with the time and temperature that the gel had been stored in the dilute alkali. The 0.01 M NaOH storage solution itself removed little material from the gel. However, extended contact with the dilute alkali appears to have solubilized material from the gel, which remained bound by ionic interactions and was later displaced by the following NaCl wash.

**Recycling with addition of storage** in 0.01 M NaOH, therefore, leads to more material being removed during recycling (in the sodium chloride wash). The blank run results showed that less material remained on the gel which would be removed during the next process cycle. Therefore, amending the recycling procedure to include storage in dilute alkali is beneficial for cleaning.

**Storage times.** We obtained almost clear blank runs on gel that had been recycled with storage in dilute alkali overnight at room temperature or for three days at 4 °C. Increasing the storage time further had no detectable effect on the blank run results (results not shown). However, it did result in a further increase in the size of the NaCl peak (compare Figures 1e and 1f), showing that additional material had been removed from the gel during recycling. Therefore, longer storage is desirable. Whereas the times we used can provide guidance, presumably, the time required to achieve optimal cleaning will depend on the particular process involved.

Storing gel in 0.01 M NaOH is unlikely to harm it. Studies in our laboratory have shown that keeping gel in 0.5 M NaOH for 70 hours does not adversely affect its performance (3). Other studies have also shown that performance of Sepharose gels is maintained following long-term exposure (7.5 months) to cleaning solutions (0.2 M NaOH, 1 M NaCl) containing concentrations of NaOH above 0.01 M (6). According to Amersham Biosciences, most gels (including Sepharose ion-exchange media) can be stored in 0.01 M NaOH for up to one year without significant change in function (1).

**Removing solubilized material.** It is important that material solubilized during storage in dilute alkali be cleared from the gel to prevent it from being removed during the next cycle, and in particular, to prevent the possibility of it being eluted with the product. In our recycling procedure, that was achieved by the second (1 M) NaCl wash. However, many recycling procedures do not include washing with a high concentration of NaCl after hydroxide treatment. Gel may be prepared for the next cycle by washing it with an equilibration buffer. For ion-exchange gels, that buffer usually contains a low concentration of NaCl and may be effective in removing solubilized material. However, we have not investigated the effect of solutions of lower ionic strength on removal of material solubilized during storage.

**TOC analysis** is often considered the best method for detecting low levels of residual protein (7). However, in this study, the absorbance traces (obtained by following absorbance changes at 280 nm with a Uvicord at high sensitivity during washing of recycled gel) gave a better indication of residual material removal than did the TOC results. Although the TOC method can detect very low levels of material (0.1 ppm of carbon, which corresponds to approximately 0.2 ppm of most blood proteins) (8), our results showed considerable variation so that only levels substantially higher than those could be considered significant.

As far as we are aware, this is the only study reporting beneficial cleaning effects of storing chromatography gels in dilute hydroxide. Seely et al. found that trace amounts of material were removed from ion-exchange gels during storage in mild caustic solutions between cycles (6). They did not, however, report looking at residual material removed during subsequent washes, other than those corresponding to where product would normally be eluted. Our study indicates that most of the material removed as a result of storage in dilute hydroxide was not removed in the dilute hydroxide itself, but during the subsequent NaCl wash.

**Acknowledgments**

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**References**

(8) J. McPhee, personal communication (Bio Products Laboratory, 2001).