Application Note

Separation of PEGylated proteins using MCSGP

Summary

This application note describes the purification of a mono-PEGylated protein from its side-products using the Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) process. MCSGP is suitable for three-fraction chromatographic separations and able to perform solvent gradients. The KNAUER Contichrom® system is the only system on the market that can be used in MCSGP mode for challenging separations. The purification of mono-PEGylated α-lactalbumin from a reaction mixture containing native protein, multi-PEGylated proteins, aggregates and break-down products is a challenging task. Normal batch ion-exchange chromatography led to a yield of only 56% at a purity of 93% for the PEGylated product. In contrast, the MCSGP ion-exchange chromatography process showed an increased yield of 83% at a comparable purity. This shows that MCSGP is superior in terms of yield, purity and productivity due to the countercurrent movement of the liquid and the solid phase.

Category
Countercurrent chromatography, biochromatography, FPLC, bioseparation

Matrix
Proteins, drugs

Method
MCSGP

Keywords
PEGylated protein, MCSGP, continuous chromatography, α-lactalbumin, PEGylation, ion-exchange, FPLC

Analytes
PEGylated proteins

ID
VBS0039N_C_E
Introduction

PEGylation is a process of conjugating drugs or diagnostics, often proteins, with polyethylene glycol (PEG). The PEG is covalently attached to protect the drug against recognition by the immune system and proteases and to increase its hydrodynamic size to reduce renal clearance. In the presented case study, PEGylation is reached by incubating the target molecule with a solution of activated PEG₂₃. The lysine residues of the protein attach to the polyethylene glycol to form the PEGylated protein₂₃₄. The purification of the target protein from the reaction mixture is very challenging as it contains several impurities with very similar adsorptive properties, mainly non-PEGylated and multi-PEGylated proteins. In analytical scale it is possible to separate the proteins by size exclusion (SEC) or ion-exchange chromatography, as the PEGylation increases the molecular size and the PEG-chains shield the electrostatic charges on the protein surface and therefore reduce the strength of interaction with an ion-exchange surface. The higher the PEGylation extent, the weaker are the interactions with a charged surface. However, the differences are not large enough to allow separation in a preparative scale with high yield and purity.⁴

In this application note, the purification of mono-PEGylated α-lactalbumin from a reaction mixture containing native protein, multi-PEGylated proteins, aggregates and break-down products on an ion-exchange column was examined. The research task was to apply the MCSGP process technology and compare the results with the batch process findings.

Experimental:
Sample preparation

α-Lactalbumin was incubated with polyethylene glycol (mPEG) that was activated with N-Hydroxysuccinimide (m-PEG-SPA) in PBS buffer (25 mM phosphate, pH 7.4).

![Fig. 1 PEGylation of proteins](image)

The lysine residues of the protein bind to the PEG. Analysis after the incubation showed that about 80% of the mPEG had reacted with the protein and 20% resulted in break-down products from hydrolysis processes. The half-life of the activated PEG under the reaction conditions is 16.5 min.² An analytical SEC chromatogram (Figure 2) of the reaction mixture in combination with a SDS-PAGE (Figure 3) of the individual fractions showed several impurities in addition to the mono-PEGylated protein.

![Fig. 2 Analytical chromatogram of the reaction mixture](image)

Analytical SEC chromatogram of the reaction mixture after the incubation of α-lactalbumin with PEG. It contains the unreacted protein, mono-, di- and tri-PEGylated protein and some break-down products.
KNAUER recommends to filter the sample through a 0.2 µm syringe filter prior to use.

Preparative method for transfer to MCSGP

The first step in designing a MCSGP process is to perform a batch separation by ion-exchange chromatography. (Fig. 4). The following conditions were used.

<table>
<thead>
<tr>
<th><strong>Column</strong></th>
<th>Poros 50 HQ, 0.5 x 10 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffer A</strong></td>
<td>25 mM Tris, pH 8.5</td>
</tr>
<tr>
<td><strong>Buffer B</strong></td>
<td>25 mM Tris, pH 8.5, 1000 mM NaCl</td>
</tr>
<tr>
<td><strong>Gradient</strong></td>
<td>Time [min]</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
</tr>
<tr>
<td><strong>Flow rate</strong></td>
<td>0.5 ml/min</td>
</tr>
<tr>
<td><strong>Mass injected</strong></td>
<td>22 mg</td>
</tr>
<tr>
<td><strong>Column temperature</strong></td>
<td>25°C</td>
</tr>
<tr>
<td><strong>Detection</strong></td>
<td>UV at 280 nm</td>
</tr>
<tr>
<td><strong>Run time</strong></td>
<td>55 min</td>
</tr>
</tbody>
</table>
Fig. 4
Preparative batch chromatogram

The black line indicates the UV signal at 280 nm. The red line indicates the conductivity measured during the run at the column inlet (linear salt gradient). The red diamonds represent the concentration of the mono-PEGylated protein, the brown squares indicate the impurities, and the blue line the purity of the mono-PEGylated α-lactalbumin. The red transparent rectangular area represents the product collection interval selected for MCSGP using the MCSGP wizard of the ChromIQ® software.
The concentrations of the mono- and di-PEGylated α-lactalbumin were obtained by offline analysis using SEC-HPLC. The results showed that the purity of the mono-PEGylated protein was satisfactory only in a very small elution window, corresponding to only 56% yield.

The generic problem in the chromatographic purification of biomolecules can be simplified to the chromatogram as shown in Figure 5. The chromatogram can be cut into five fractions as indicated by the numbers on the time axis:

1 = weak adsorbing impurities
2 = Product contaminated by weak adsorbing impurities
3 = Product
4 = Product contaminated with strong adsorbing impurities
5 = strong adsorbing impurities.

The aim of an ideal purification process is to collect fraction 3, to drain fractions 1 and 5, and to recover the product contained in fractions 2 and 4.

The definition of the fractions 1–5 and therefore the operating parameters of the MCSGP process are based on the experimental data from offline analysis of the preparative batch chromatogram (Fig. 4). For the MCSGP process the same stationary phase as for the batch experiments is used. The transfer from the results obtained in the batch process to the MCSGP method can be carried out automatically with the ChromIQ® software of the Contichrom® system. For the MCSGP process the two columns are interconnected to recycle fraction 2 and 4 from one column to the other and they are switched to batch mode to elute the impurity fractions 1 and 5 and the pure product fraction 3.
Figure 6 shows the analytical chromatogram of the unpurified feed mixture (blue) compared to the chromatogram of the target compound, after the purification using the Contichrom® system in MCSGP mode. Offline analysis showed that the MCSGP process led to a purity of more than 93% with a yield of 83%.

**Conclusion**

The Contichrom® system in MCSGP mode was applied for the purification of mono-PEGylated α-lactalbumin from the PEGylation reaction mixture using an anion-exchange column. With batch chromatography in linear gradient mode a yield of 56% was obtained. Using the MCSGP process that exploits the advantage of linear gradients and countercurrent movement of the stationary and mobile phase, the difficulty of the separation was overcome and the mono-PEGylated protein was purified with a yield of 83%.

**References**


**Authors**

Dr. Friederike Sander, Columns and Applications Department, KNAUER.
Dr. Thomas Müller-Späth, Chromacon AG.

**Physical properties of recommended columns**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary phase</td>
<td>Poros 50HQ (Applied Biosciences)</td>
</tr>
<tr>
<td>Pore size</td>
<td>50–1000 nm</td>
</tr>
<tr>
<td>Particle size</td>
<td>50 µm</td>
</tr>
<tr>
<td>Matrix</td>
<td>Polystyrene divinylbenzene</td>
</tr>
<tr>
<td>Functional groups</td>
<td>Quaternized polyethylene</td>
</tr>
<tr>
<td>Dimensions</td>
<td>5 mm x 100 mm</td>
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</tbody>
</table>
Recommended instrumentation

The MCSGP process was performed on a KNAUER Contichrom® Lab-10 system in MCSGP mode.

<table>
<thead>
<tr>
<th>Description</th>
<th>Order No.</th>
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<tbody>
<tr>
<td>Contichrom Lab-10 System</td>
<td>C2846.67</td>
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<tr>
<td>Contichrom Prep-100 System</td>
<td>C2647.67</td>
</tr>
</tbody>
</table>

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