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White Paper

Use of Excipients in Downstream Processing to Improve Protein Purification

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Excipients are used to improve the stability of protein-based therapeutics by protecting the protein against a range of stress conditions such as temperature changes, pH changes, or agitation. Similar stresses are applied to proteins during downstream purification. Shifts in pH during Protein A chromatography, subsequent incubations at low pH for virus inactivation, and changes in conductivity in ion exchange chromatography can lead to aggregation, fragmentation, or other chemical modifications of the therapeutic protein. Given the potential impact on the protein's structural integrity, there is a need for approaches to reduce the risk presented by the conditions during downstream processing. For example, integration of a solution to prevent aggregation of proteins would be a more efficient strategy than implementing steps to remove multimeric forms.

This white paper highlights the results from a recent paper by Stange et. al., in which protein stabilizing excipients such as polyols, sugars, and polyethylene glycol (PEG4000) were used as buffer system additives. Effect of the excipients on elution patterns, stabilization of the monomer antibody, host-cell protein removal, virus inactivation rates and binding capacity of cation exchange chromatography were explored.

Results of the study show that addition of excipients can have beneficial effects on Protein A chromatography and virus inactivation, without harming subsequent chromatographic steps.[1]

Excipient Selection

The impact of excipients on chromatography performance has been evaluated in several studies.[2,3,4] While the addition of excipients in Protein A chromatography buffers can improve column performance, a limited number of excipients and resins have been studied and the impact on subsequent steps was not explored.

The excipients for the study summarized below were selected using a specifically designed screening assay. Polyols, such as sorbitol and mannitol, are typically used as lyoprotectants,[5,6,7] while osmolytes, such as trehalose and sucrose, are well-known stabilizers for protein formulations.[7,8,9] PEG4000 is frequently used either as a partitioning agent in aqueous two-phase systems,[10] as a precipitating agent,[11] or to form covalent adducts (a process known as PEGylation) with proteins to enhance their size and prolong their biological half-life.[12] A concentration of 500 mM or 5% (w/v) in the case of PEG4000 was used for the five excipients based on ideal stabilizing properties at the chosen concentration during the prescreening experiments.

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Excipient Impact on Protein A Chromatography

To determine the impact of different excipients on elution behavior in Protein A chromatography, excipients were introduced into the columns during the second wash step after loading and during elution of the monoclonal antibody (mAb). Normally, Protein A chromatography is performed with a step elution to a low pH; in this study, a linear gradient to low pH was used to more effectively evaluate the impact of excipients. Figure 1A shows representative results of the elution behavior on the Protein A column (Eshmuno® A column) using clarified cell culture harvest mAbB as model protein in the presence of various excipients compared to the control condition without an excipient. The addition of all selected sugars and polyols resulted in a fronting peak similar to the control condition without excipients. In comparison, the addition of PEG4000 led to a sharper and more symmetric elution peak slightly shifted to lower pH and resulted in a comparable or lower elution pool volume (Figure 1B). This was probably caused by interactions of PEG4000 with the bound antibody/Protein A ligand complex, which reduced peak fronting, and led to more symmetrical peak shapes of the eluting antibody. Previous studies showed that the antibody undergoes conformational changes during binding and elution of Protein A chromatography,[13] that the hydration layer of the protein can be penetrated with PEGs through increasing concentration,[14] and that PEGs can interact with nonpolar regions of a protein when exposed during conformational transitions.[15] Therefore, PEG4000 could interact with and stabilize the conformationally altered form of mAbB/Protein A ligand complex during elution, which led to the observed shift in elution pH and narrowing and sharpening of the eluting antibody UV signal peak.

Because Protein A chromatography is commonly used to remove host cell proteins (HCPs) from cell culture harvests, the effect of excipients on their clearance was evaluated. For this purpose, fractions from all excipient conditions were collected during gradient elution and HCP concentrations were determined. The purity of the elution pool relative to HCP content was analyzed by comparison of HCP in the elution pool from collected fractions with total mAb content during the pH gradient.

The elution behavior of HCPs in the presence of PEG4000 differed significantly from control and other excipient conditions (Figure 2A). With PEG4000, more HCPs were eluted in the rear part of the mAb elution peak, meaning the HCP elution was shifted to lower pH conditions and after the antibody desorbed from the column. HCP concentrations of elution pools were then quantified through ELISA for all excipients conditions. The greatest reduction of HCP level in the product pool, down to 0.22 µg HCP/mg mAbB, was achieved with the addition of 5% PEG4000 (Figure 2B).

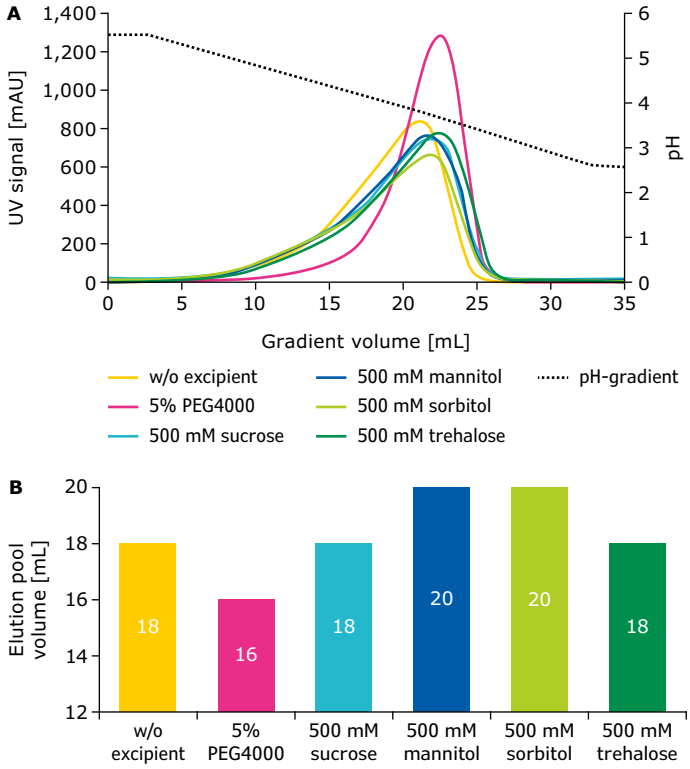

Figure 1.

Elution behavior (A) and elution pool volume (B) of mAbB on Eshmuno® A column without excipient compared with the addition of 500 mM sucrose, 500 mM trehalose, 500 mM sorbitol, 500 mM mannitol, or 5% PEG4000; including the pH trace of the effluent. A gradient from pH 5.5 to 2.75 was applied.

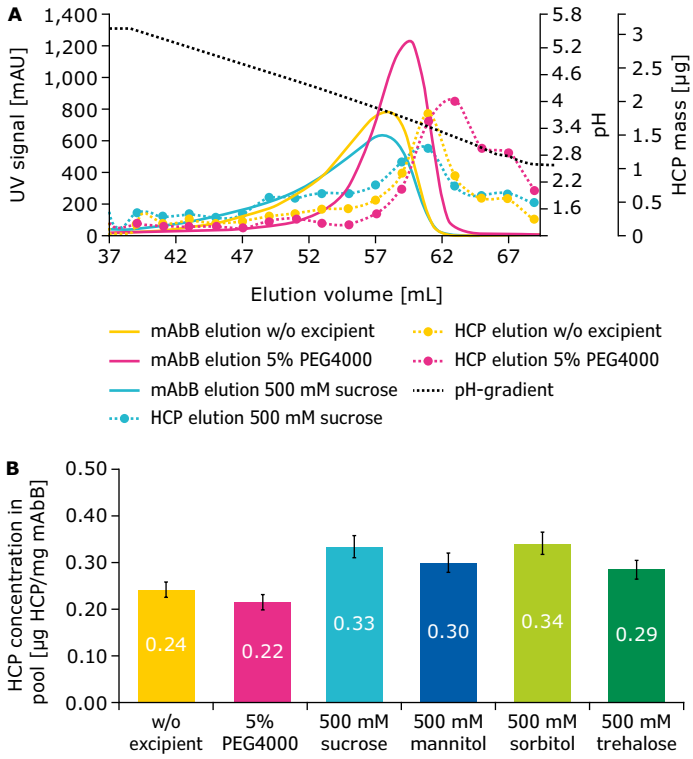

Figure 2.

Influence of excipients on HCP elution (A) and concentration in elution pools (B) with different excipients used for Protein A chromatography with Eshmuno® A column.

Excipient Impact on Low pH Virus Inactivation

The potential stabilizing effects of excipients during the low pH hold used for virus inactivation were also investigated. While virus inactivation is typically performed at pH ≤3.8, in this case, the model antibodies were too stable, and no differences in aggregate level at pH values above 2.8 were observed. For this reason, the pH was adjusted to 2.8 after elution of the antibody from Protein A column to induce stress conditions in order to observe the stabilization effect of the excipients. The aggregate content was measured with size exclusion-HPLC directly after the elution pool was collected and then every 20 minutes over the course of one hour, after the pH was adjusted to 2.8.

Under low pH conditions, the monomer content of mAbB decreased to approximately 93% in the control sample without excipient, while addition of sugars or polyols led to stable monomer content of about 99% within 60 minutes incubation at pH 2.8 (Figure 3). These results demonstrate that these excipients stabilized the protein at low pH condition. In contrast, PEG4000 showed no significant impact on mAb stability under this condition, as indicated by the monomer amount which was similar to the control without excipient.

The effect of the selected excipients on viral inactivation rates was also studied. Viral log reduction factors were studied by using xenotropic *murine leukemia virus* (MLV) spiked into a solution of mAbB; virus inactivation was measured over several time points, up to one hour at pH 3.6. Similar to control experiments without excipients, all selected excipients showed highly effective virus reduction with a log reduction factor >4. Moreover, a slight improvement of the log reduction factors could be achieved by addition of PEG4000, sorbitol, and trehalose in comparison to the result without excipients (Figure 4). This result also demonstrated that the various excipients did not have a negative effect on virus inactivation.

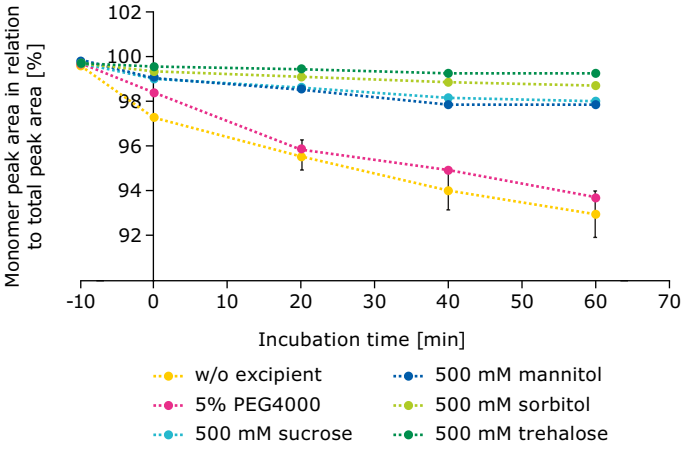

Figure 3.

Formation of aggregates following elution of an mAbB from an Eshmuno® A column during a low pH hold at pH 2.8 in the presence of different single excipients compared to the control condition without excipient.

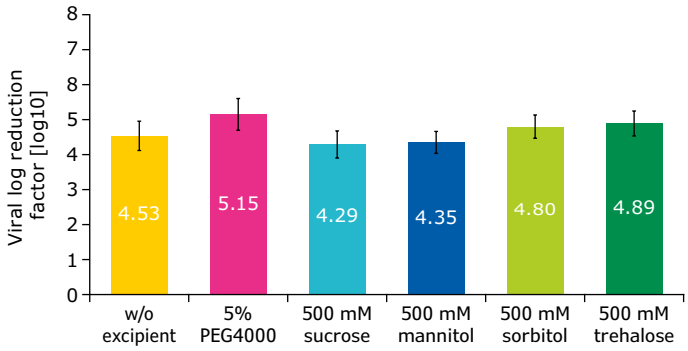

Figure 4.

Viral log reduction factor with and without excipients measured at pH 3.6 after 60 minutes in an infectivity assay with xenotropic *murine leukemia virus*.

Effect of Excipient Combinations on Protein A Chromatography and Low pH Virus Inactivation

Based on the positive effect of PEG4000 on HCP reduction during Protein A chromatography and the protein stabilization effect of sugars and polyols at low pH in viral inactivation, the influence of an excipient combination on both downstream processing steps have been investigated. Figure 5 shows the elution peaks during Protein A chromatography in the presence of either 500 mM sucrose, 5% PEG4000, or a combination of the two excipients. The combination of PEG4000 and sucrose resulted in a sharper peak (A) which was comparable to the peak when only PEG4000 was used.

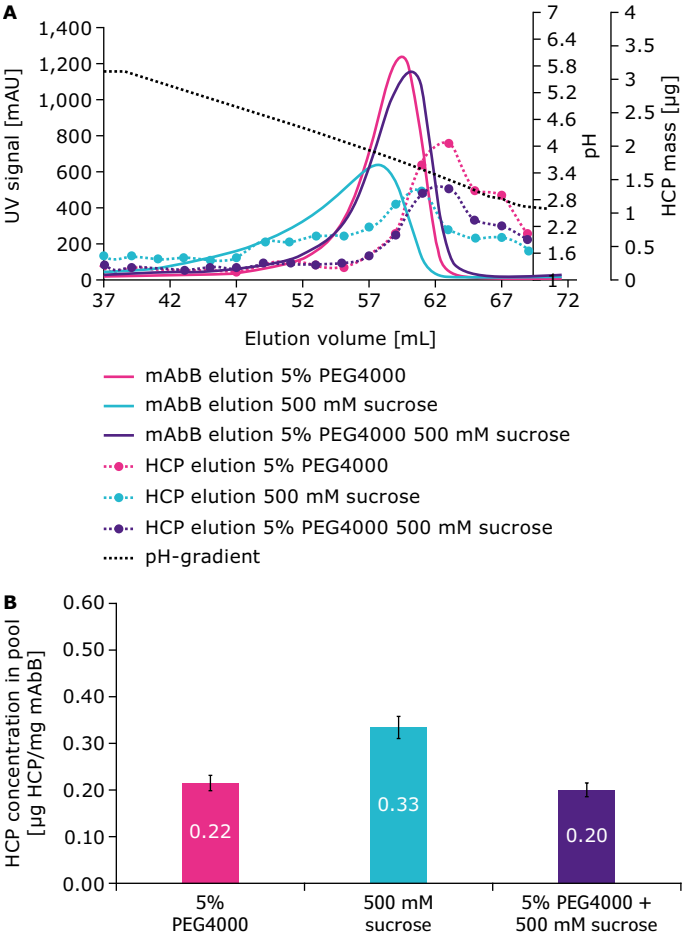

Figure 5.

HCP elution behavior compared to the mAbB elution behavior on an Eshmuno® A column (A) and HCP content in elution pool of the antibody (B) with 500 mM sucrose, 5% PEG4000, and a combination of both excipients.

Similarly, the HCP content was comparable to just PEG4000 (B); in this case, the effect of sucrose was masked during the chromatography performance.

Moreover, the combination of PEG4000 and sucrose showed a comparable protein stabilization effect compared to sucrose alone, with stable monomer content around 99% within 60 minutes at a low pH incubation at pH 2.8 (Figure 6). As such, a combinatory effect of PEG4000 and sucrose could be achieved as evidenced by a sharper elution peak and higher purity of the elution pool of the antibody during Protein A chromatography and higher protein stability during low pH hold in viral inactivation. Other studies confirmed that a combination of these two excipients leads to increased solubility of mAbs, masking the precipitative effects of PEG.[16]

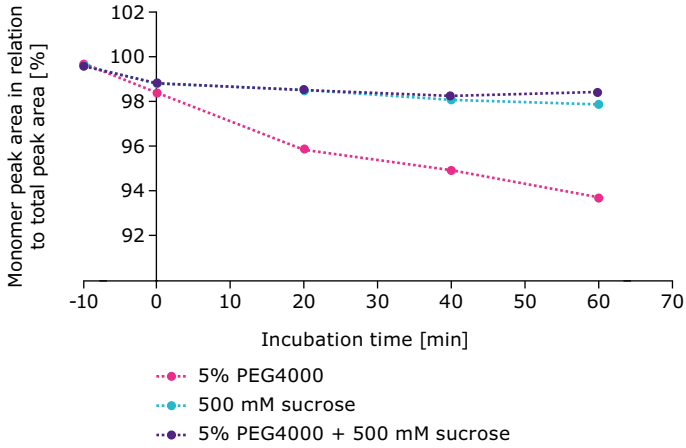

Figure 6.

Formation of aggregates following elution of mAbB from an Eshmuno® A column during low pH hold at pH 2.8 in the presence of a combination of sucrose and PEG4000 compared to sucrose or PEG4000 only. Aggregate content was measured with SE-HPLC directly after elution from Protein A chromatography and after adjusting pH to 2.8 every 20 minutes up to 60 minutes.

Excipient Impact on Cation Exchange Chromatography

To ensure that the applied excipients do not negatively affect subsequent purification steps in downstream processing, their impact on the dynamic binding capacity at a 10% breakthrough level (DBC10) of a cation exchange chromatography polishing step was evaluated.

Figure 7 shows that the measured dynamic binding capacity values agreed with the published data for Eshmuno® CPX columns. At the residence time of five minutes, more than 140 mg protein/mL resin could be loaded onto the column before 10% of the breakthrough UV signal was reached. Comparing results of DBC10 in the absence and presence of excipients, only minor changes can be observed, especially in the presence of sugars and polyols. Nevertheless, the overall results were above 80% of DBC10, which is typically used as a common industry practice for protein load. Therefore, none of the selected excipients showed a negative effect on dynamic binding capacity on cationic exchange chromatography.

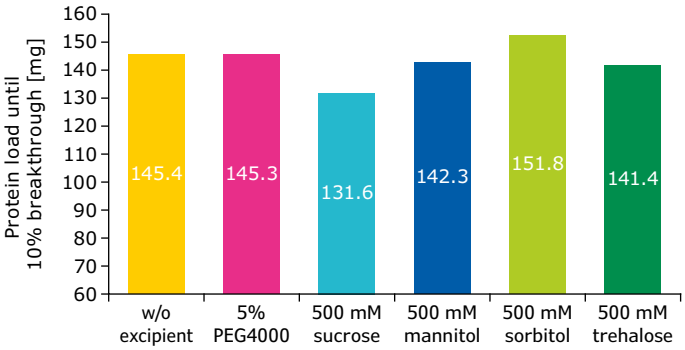

Figure 7.

Dynamic binding capacity (DBC10) values for mAbB with and without excipients tested on an Eshmuno® CPX column. The amount loaded was determined via 10% breakthrough UV signal at 280 nM with the built-in UV sensor.

Conclusion

This study showed that the addition of suitable excipients can have a beneficial effect during the purification of mAbs. During Protein A chromatography with pH gradient elution, addition of PEG4000 led to sharper elution peaks, reduced pool volumes, and enabled a greater reduction of HCPs, which is probably caused by the interaction of PEG4000 with the bound antibody/Protein A ligand complex. The usage of excipients such as PEG4000 results in a higher pool concentration and can lower processing costs in the following unit operation (e.g., smaller tank needed for virus inactivation step) and shorter process times for steps such as sample loading in cation exchange chromatography.

In this study, less stable antibodies were found to be stabilized during the low pH elution or virus inactivation either through the addition of polyol or sugar excipients, such as sucrose, trehalose, mannitol, and sorbitol or the combination of PEG4000 and sucrose. Moreover, positive effects in both Protein A chromatography and virus inactivation steps, such as higher HCP clearance, lower elution pool volume, and stabilization of the antibody can be achieved by the addition of excipient combinations of PEG4000 and sucrose, without performing additional buffer exchange such as diafiltration between the two steps. It has been demonstrated that all selected excipients have no negative impact on virus reduction and binding capacity of cation exchange chromatography. Therefore, there is also no need for removal of excipients during the purification process.

Overall, these studies demonstrated that excipients can improve not only the purification performance in Protein A chromatography but also the protein stability in virus inactivation without harming subsequent chromatographic steps. Therefore, the excipients or combination of those could also be applied for continuous downstream processing.

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