



SEPARATION OF MONOCLONAL IMMUNOGLOBULIN G AND ITS AGGREGATES USING TOYOPEARL MX-Trp-650M

The importance of proper aggregate removal during polishing of a monoclonal antibody (mAb) for therapeutic use is beyond controversy. Severe anaphylactic reactions have been described in the literature for the application of aggregated proteins as a drug byproduct. Traditionally, ion exchange chromatography or hydrophobic interaction chromatography are utilized to purify a structurally homogeneous product. In case these platforms do not satisfy the requirements for mAb polishing, advanced chromatography resins need to be considered. For instance, mixed-mode stationary phases like TOYOPEARL MX-Trp-650M may pave the way for more challenging polishing applications. This application note intends to give you some insights into how you could start handling mAb polishing with the tryptophan immobilized ligand.

SCREENING FOR THE APPROPRIATE CONDITIONS FOR mAb AGGREGATE REMOVAL

Mixed-mode chromatography is one approach to combine the advantages of hydrophobic interaction chromatography (HIC) and ion exchange chromatography (IEX). The number of potential ligands for mixed-mode chromatography is huge, as potential candidates can be found in various molecular classes. However, the preferred environment for an antibody restricts the ligand choice. Binding and elution with moderate salt and pH conditions, as well as capacities comparable to IEX are in focus of ligand selection. Moreover, the need for an appropriate selectivity sets tight bounds.

To fulfill these expectations, more complex structures are used, if compared to traditional IEX or HIC ligands. In accordance with this, method development becomes more complex, as well. In case no robotic system is at hand, a straight-forward approach how to handle the increased number of parameters affecting the process of binding and elution is described in our first example, the polishing of a humanized, monoclonal IgG. The major factors influencing binding and separation of proteins on TOYOPEARL MX-Trp-650M are the pH and the salt concentration.

ELUTION BY pH GRADIENT

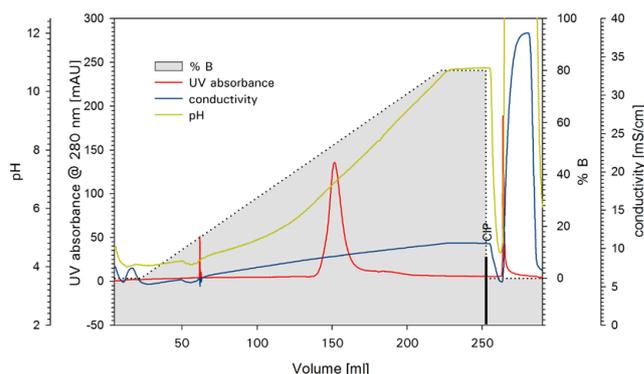


Figure 1b

Column: 6.6 mm ID x 2 cm; Mobile phase A: buffer pH 4.0 + 0.2 mol/L NaCl; Mobile phase B: buffer pH 12.0 + 0.2 mol/L NaCl; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

ELUTION BY SALT GRADIENT

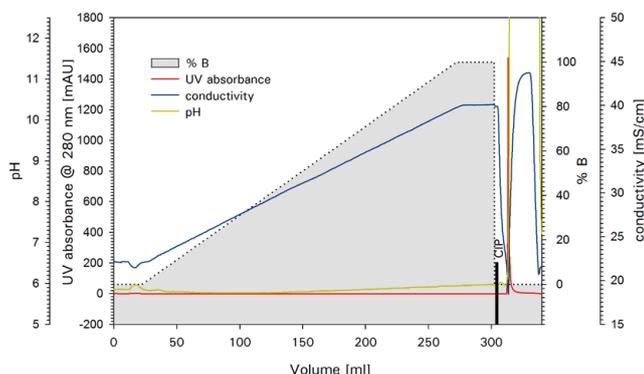


Figure 1a

Column: 6.6 mm ID x 2 cm; Mobile phase A: buffer pH 4.0 + 0.2 mol/L NaCl; Mobile phase B: buffer pH 4.0 + 0.5 mol/L NaCl; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

ELUTION BY COMBINED pH AND SALT GRADIENT

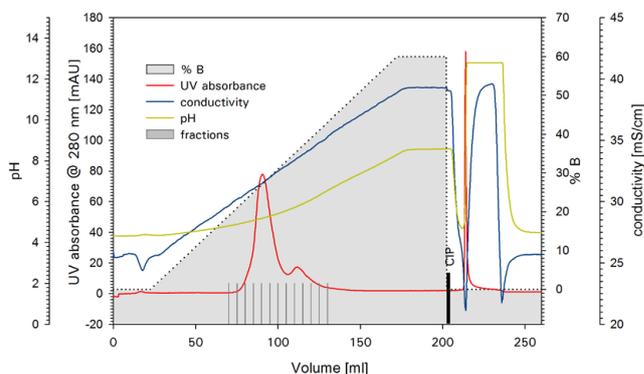


Figure 1c

Column: 6.6 mm ID x 2 cm; Mobile phase A: buffer pH 4.0 + 0.2 mol/L NaCl; Mobile phase B: buffer pH 12 + 0.4 mol/L NaCl; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

For a start, three linear gradient runs will provide hints on the actual working frame for a certain molecule. Figure 1a, b & c show three chromatograms of the mAb sample containing approximately 17% aggregates. The three runs illustrate a salt gradient (constant pH), a pH gradient (constant salt concentration) and a combined salt and pH gradient. The pH span of the applied chromatofocusing buffersystem depends of course on the stability of the sample. These buffer systems are either commercially available as ready to use buffer systems or can be prepared by arranging various (zwitter-) ionic buffer salts with pKs values covering the desired pH span.

While the salt gradient does not allow protein recovery, the pH gradient leads to the elution of one protein peak. In contrast, the combined pH and salt gradient recovers the protein in two peaks, a monomer peak in the front, followed by the aggregates. Quantitative and qualitative analysis of the collected protein peaks was performed by size exclusion chromatography (SEC) using TSKgel G3000SW_{XL}. The corresponding results are presented in figure 2.

mAb AGGREGATE REMOVAL APPLYING AN UP-SCALABLE GRADIENT

The screening gradients are rather time consuming and inefficient in process scale, due to the covered range of pH 4.0 to 12.0. Nevertheless, the retrieved results allow narrowing the pH and salt concentration range to pH 4.0 to 6.0 and 0.2 M NaCl to 0.4 M NaCl, respectively. This frame can be covered by a sodium acetate buffer (Fig. 3). The significantly shortened gradient can be applied for separation, while the aggregate content in the monomer pool is below 1%. SEC chromatograms of the collected fractions are presented in Figure 4.

From these results, we conclude that TOYOPEARL MX-Trp-650M can be utilized as a highly efficient tool for aggregate

SEC CHROMATOGRAMS OF THE COLLECTED FRACTIONS OF COMBINED SALT & pH GRADIENT ON TOYOPEARL MX-TRP-650M (SEE FIG. 1C).

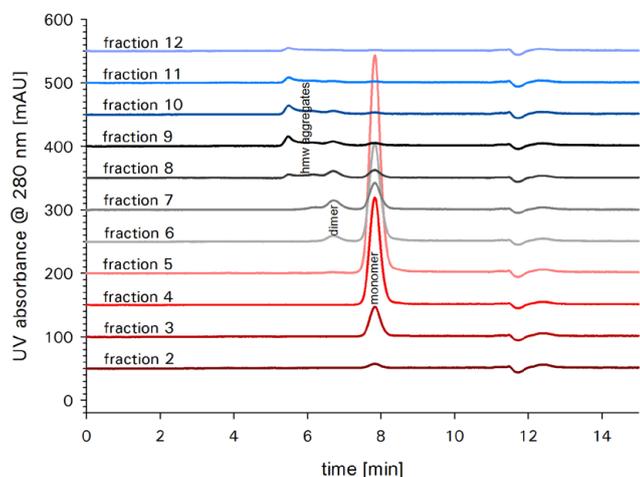


Figure 2

Column: 6.6 mm ID x 2 cm; Mobile phase A: 0.1 mol/L acetate + 0.2 mol/L NaCl, pH 4.3; Mobile phase B: 0.1 mol/L acetate + 0.4 mol/L NaCl, pH 5.6; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

OPTIMIZED pH & SALT GRADIENT ON TOYOPEARL MX-TRP-650M FOR THE SEPARATION OF mAb MONOMERS AND AGGREGATES

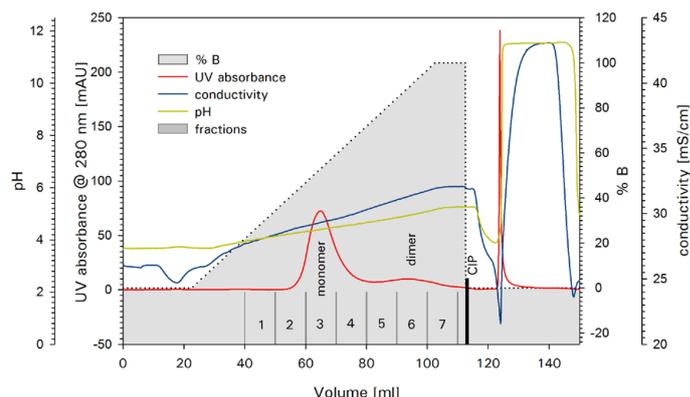


Figure 3

Column: 6.6 mm ID x 2 cm; Mobile phase A: 0.1 mol/L acetate + 0.2 mol/L NaCl, pH 4.3; Mobile phase B: 0.1 mol/L acetate + 0.4 mol/L NaCl, pH 5.6; Linear flow: 150 cm/h; Sample: 10 mg mAb + mAb aggregates (conc. 1 g/L)

removal of mAbs, as it offers capacities comparable to IEX, high recovery and proper selectivity. With the presented screening gradients, it is possible to take advantage of a straight-forward method development approach, which does not necessarily require a robotic system. As a result, method development for mixed mode chromatography with the tryptophan ligand is not more elaborate than for traditional HIC or IEX resin, while it offers outstanding selectivity for mAbs and mAb aggregates. For even more challenging separations, one might consider systematic screening for method development, to play on the modulation opportunities typically owned by mixed mode resins. Enabling both procedures with adequate outcome, traditional method development and advanced systematic robotic screening, characterizes TOYOPEARL MX-Trp-650M as a perfect tool for the polishing of mAbs.

SEC CHROMATOGRAMS OF THE COLLECTED FRACTIONS OF OPTIMIZED GRADIENT ON TOYOPEARL MX-TRP-650M (SEE FIG. 3).

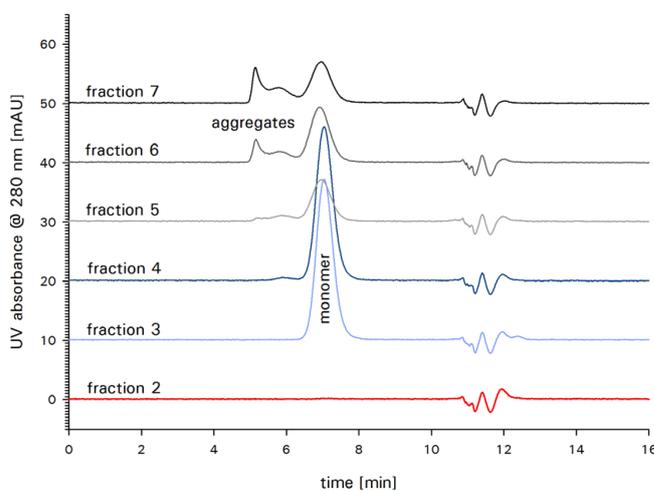


Figure 4

Column: TSKgel G3000SW_{XL} 7.8 mm ID x 30 cm; Mobile phase: 0.1 mol/L sodium phosphate + 0.1 mol/L sodium sulfate, pH 6.7; Flow rate: 1 ml/min; Detection: UV @ 280 nm; Sample: 100 µl of each fraction