Automated Two-Column Purification of Trastuzumab on the NGC Chromatography System with Modifications for Inline Neutralization

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Abstract

NGC Chromatography Systems are versatile instruments for protein purification. Here we describe the optimal NGC System configuration for automated tandem immunoglobulin G (IgG) antibody purification with inline pH neutralization and size exclusion chromatography (SEC). This rapid two-column purification technique minimizes the exposure of antibodies to low pH, preventing aggregation. The modifications described here allow for a continuous, automated antibody purification workflow from complex sample to pure antibody at the tens-of-milligrams scale.

Introduction

The workflow for the purification of antibodies most commonly starts with an affinity capture step using Protein A or G resins. Recovery of the bound antibody from the affinity matrix requires an acidic elution step. This sudden drop in the solution pH can lead to aggregation of the antibody. In manufacturing, holding the affinity elution at acidic conditions is a useful strategy for viral inactivation. However, the resulting antibody aggregates must be removed prior to final therapeutic formulations. While advantageous at the industry level, where production scales are large, at the R&D level, where antibody quantities may be limited, the need to have a rapid purification protocol that can minimize aggregation and isolate the prominent monomer fraction from aggregates is more important. Here we describe the modification of an NGC Chromatography System to perform a two-column tandem purification of the biosimilar trastuzumab, commercially known as Herceptin. The workflow first involves an antibody affinity capture and elution purification step, followed by rapid inline neutralization of the eluate and a subsequent polishing step to separate the antibody aggregates from monomers by SEC (Figure 1).

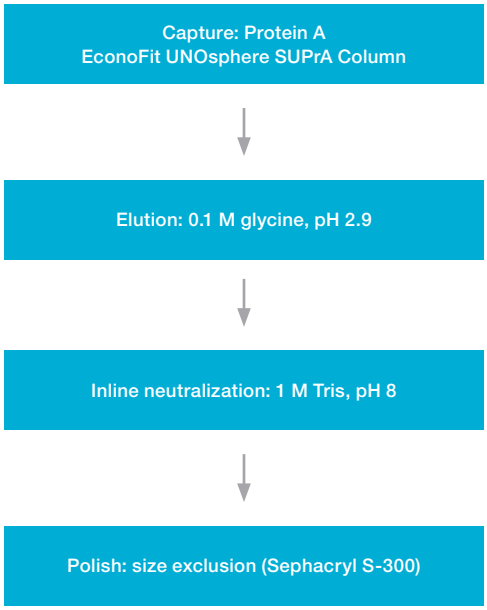

Fig. 1. Workflow for the automated tandem purification of trastuzumab with inline neutralization.

Materials and Methods

Biosimilar

Human embryonic kidney (HEK) 293 cell culture supernatant containing the biosimilar trastuzumab was obtained from Absolute Antibody (catalog #Ab00103). The supernatant was filtered through 0.22 µm polyethersulfone (PES) membrane vacuum filtration units (Corning Incorporated, #431098) prior to performing chromatography. Since the pH and conductivity of the cell culture were similar to phosphate buffered saline (PBS), no buffer exchange was needed before loading the sample on the Protein A column.

Chromatography

All purification steps were performed at room temperature on an NGC Quest 100 Plus Chromatography System (Bio-Rad™ Laboratories, Inc., #7880004) equipped with additional modules (sample pump, two column switching valves, two buffer inlet valves, sample inlet valve, pH valve, and outlet valve) (Figure 2). Detailed configuration instructions for this workflow are described in bulletin 3360. Antibody capture was performed on a 5 ml UNOsphere SUPrA Protein A Column (Bio-Rad, available prepacked as EconoFit Column, #12009323) operating at 2.5–3.0 ml/min for all sample loading and column wash steps. UNOsphere SUPrA elution and neutralization were done at 0.5–0.75 ml/min. The subsequent final polishing step was done on a Hi-Prep 16/60 Sephacryl S-300 HR Column (Cytiva, #17116701) operated at 0.5–0.75 ml/min. The buffer used was PBS (Bio-Rad, #31098). Protein was eluted from the UNOsphere SUPrA Column using 0.1 M glycine-HCl, pH 2.9, and inline neutralization was performed using 1.0 M Tris-HCl, pH 8.0, at a mixing ratio of 4:1 (volume eluate:volume 1 M Tris-HCl) (Figure 3).

The NGC System tubing was configured to allow simultaneous elution from the UNOsphere SUPrA Column and subsequent acid neutralization prior to loading onto the size exclusion column (Figure 2). System pump A was connected to column valve #1 (UNOsphere SUPrA Column). The outlet port of this column valve was connected to a static Y-mixer chamber (14 µl volume, IDEX Health & Science LLC, #P-514). System pump B was connected directly to the Y-mixer. The UNOsphere SUPrA eluate (0.1 M glycine, pH 2.9) and 1 M Tris-HCl fluid streams mixed in the chamber and were directed to the SEC column installed on column valve #2. A 5 µm filter cartridge (Bio-Rad, #7500703) was placed in between the Y-mixer and the SEC column to remove any large insoluble material from contaminating the column. Fractionation steps utilized either an outlet valve for flow-through material or the NGC Fraction Collector with Racks (Bio-Rad, #17002070) for the main SEC elution peak.

Electrophoresis

Purification was analyzed by SDS-PAGE. Ten microliters of each fraction were mixed with an equal volume of 2x Laemmli Sample Buffer (Bio-Rad, #1610737) and heated to 95°C for 5 min. Samples and Precision Plus Protein Unstained Protein Standards (Bio-Rad, #1610363) were then loaded onto a 4–20% Criterion TGX Stain-Free Protein Gel (Bio-Rad, #5678092). Gels were stained with Bio-Safe Coomassie Stain (Bio-Rad, #1610786) and imaged on a ChemiDoc MP Imaging System (Bio-Rad, #12003154).

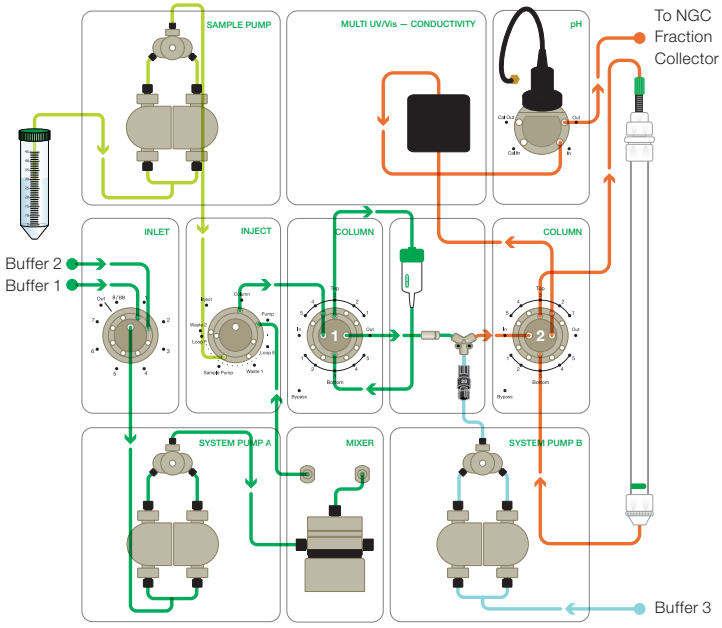

Fig. 2. NGC Chromatography System configured for tandem purification of antibodies using inline neutralization. The inlet valve is primed with two buffers: PBS, pH 7.8 (buffer 1), and 0.1 M glycine, pH 3.0 (buffer 2). System pump B delivers 1 M Tris, pH 8.0 (buffer 3), for inline pH neutralization. Arrows denote the direction of flow within each path. Sample pump flow path (■); system pump A flow path (■); system pump B flow path (■); neutralization flow path (■). PBS, phosphate buffered saline; UV, ultraviolet; Vis, visible (light).

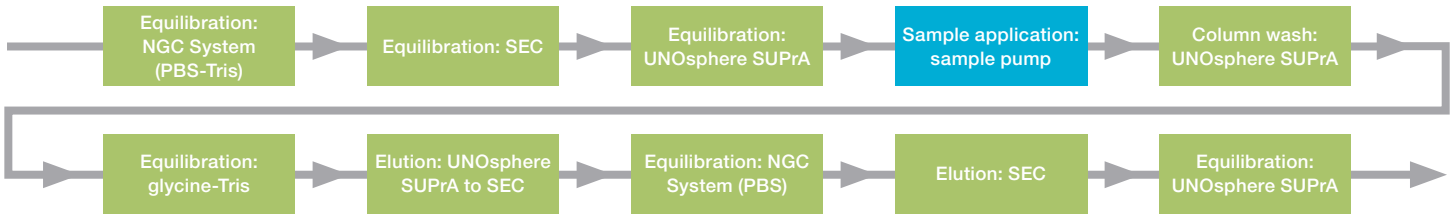

Fig. 3. ChromLab Software method outline for the tandem purification of trastuzumab.

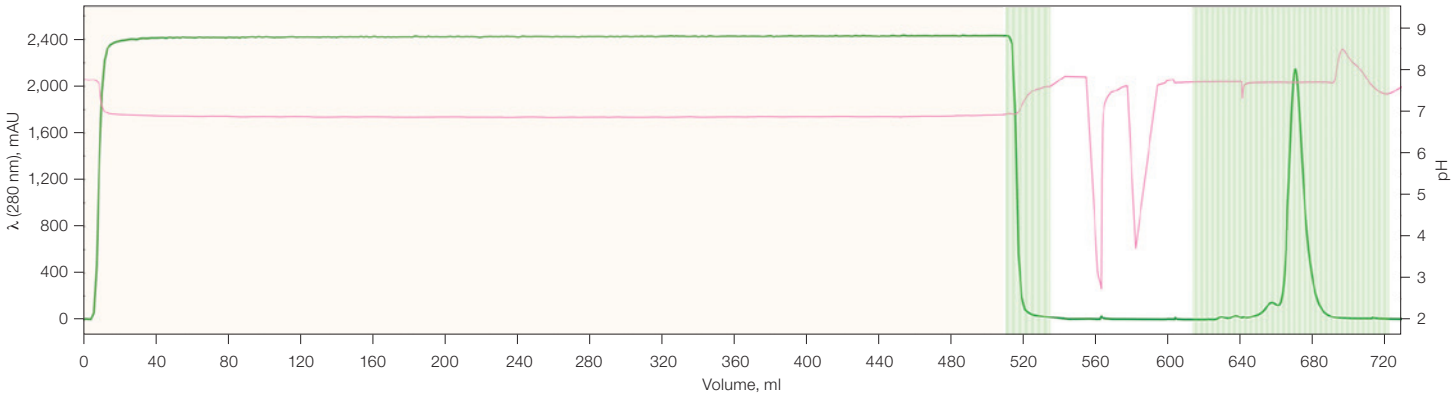

Fig. 4. Chromatogram of the purification of trastuzumab by tandem affinity and size exclusion chromatography. HEK 293 supernatant (500 ml) containing trastuzumab was injected onto a 5 ml UNOsphere SUPrA Column followed by subsequent elution, inline neutralization, and injection onto a 120 ml size exclusion column. UNOsphere SUPrA flow-through fractions were collected as one fraction via the outlet valve and 1.5 ml exclusion fractions were collected into a 2.0 ml deep well microplate on the NGC Fraction Collector. The absorbance (280 nm, —) and pH trace (—) are shown as well as the fractions (■).

Results and Discussion

The NGC System tubing was modified to incorporate an inline neutralization step using a low mixing volume Y-mixer. The acid-eluted material from the UNOsphere SUPrA Protein A Column was immediately neutralized and filtered prior to loading onto the SEC column. Incorporating a sample pump and sample inlet valve allows for the potential to incorporate multisample automation to increase throughput. Elution from the UNOsphere SUPrA Column occurred in reverse flow to minimize the elution volume that would be injected onto the SEC column. From a 500 ml sample loading volume, 30 mg of purified trastuzumab was obtained (Figure 4). The ultraviolet (UV) chromatogram of the SEC elution clearly shows a main peak and a shoulder. Gel analysis of the peak fractions shows that both the main peak and shoulder are composed of the biosimilar; however, the shoulder peak is larger in size and is approximately the size of an antibody dimer (Figure 5).

The flow-through material from the UNOsphere SUPrA Column was processed a second time through this entire protocol and no antibody was found in the final size exclusion elution, indicating a 100% yield in the purified biosimilar. Proof-of-concept experiments using purified bovine γ-globulin showed that around 50 mg of monomer material could be purified when saturating the UNOsphere SUPrA Column. The potential upper limit of antibody yield can be found based on the dynamic binding capacity of UNOsphere SUPrA Resin, which for a 5 ml column would theoretically be 150 mg based on human IgG. The maximum loading volume for the 120 ml SEC column should be less than 5 ml, so elution of the UNOsphere SUPrA Column in reverse flow aids in minimizing the elution volume. This entire two-column tandem purification workflow can be completed in less than 5 hr (depending on sample loading volume and whether the size exclusion column is pre-equilibrated) and yields pure monomeric antibodies that can be used at the research level. The total time for the experiment shown in Figure 4 was 11 hr (Table 1).

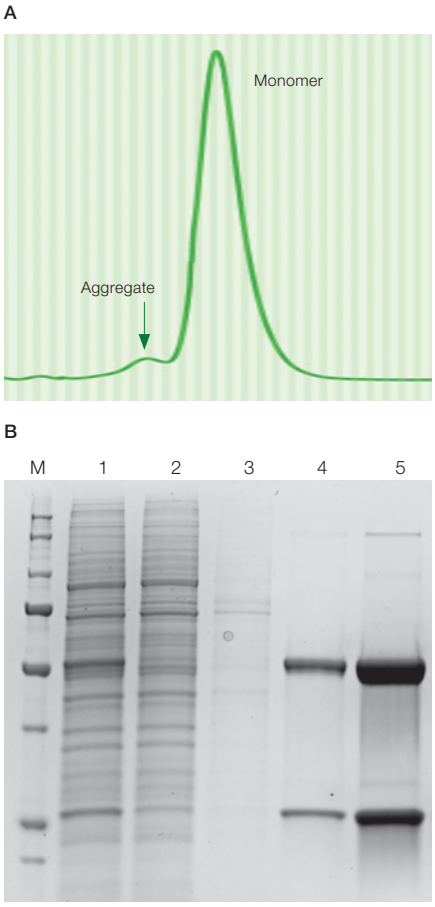

Fig. 5. A, chromatogram and B, SDS-PAGE electrophoresis gel summarizing the tandem purification of trastuzumab. UV trace represents the size exclusion elution peak in Figure 4. M, Precision Plus Protein Unstained Standards; 1, sample load; 2, UNOsphere SUPrA Column flowthrough; 3, UNOsphere SUPrA Column wash; 4, aggregate peak (approximately 1.0 µg); 5, monomer peak (approximately 7 µg). The aggregate peak includes 5% of the elution peak area. The gel was stained with Bio-Safe Coomassie Stain.

Table 1. Process timetable for the tandem purification of trastuzumab shown in Figure 4.

Phase	Flow Rate, ml/min	Time, min	Comment
System equilibration	20	2	
SEC column equilibration	0.6	200	Variable, 0.1–1 column volume of SEC
UNOsphere SUPrA Column equilibration	3	8	
Sample application (500 ml)	2.5	213	Variable, dependent on sample volume
UNOsphere SUPrA Column wash	3	8	
System equilibration (UNOsphere SUPrA Column elution/neutralization)	0.6	5	
UNOsphere SUPrA Column elution	0.6	15	
NGC System equilibration (SEC)	0.6	10	
SEC elution	0.6	200	
UNOsphere SUPrA Column/NGC System equilibration	3	10	
Total time		**671**	

High-throughput automated purification of antibodies can be achieved in this workflow by using either a sample pump combined with sample inlet valves for a total of 15 input samples of virtually infinite volume or with a third-party autosampler for up to 84 50-ml sample injections (bulletin 6896). Additionally, sample collection can be scaled to meet the throughput demands by incorporating sample outlet valves and up to two NGC Fraction Collectors.

Conclusions

The NGC Chromatography System is a versatile and customizable platform for the purification of biomolecules. Here we have demonstrated the modification of the NGC System for the purification of the biosimilar trastuzumab. The modifications described here allow for a continuous, automated antibody purification workflow from complex sample to pure antibody. The methodology outlined here is amenable to research laboratories developing antibody therapeutics and desiring to produce pure antibodies at tens-of-milligrams scale for further study and development. High-throughput purification is possible by using a sample pump or third-party autosampler for sample injections. Finally, addition of a third NGC Column Switching Valve would allow a second polishing step, such as ion exchange or mixed-mode chromatography (either before or after the SEC column), to be added if any contamination by host cell proteins or DNA is observed.

Bibliography

Mazzer AR et al. (2015). Protein A chromatography increases monoclonal antibody aggregation rate during subsequent low pH virus inactivation hold. J Chromatog A 1415, 83–90.

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