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A fully automated three-step protein purification procedure for up to five samples using the NGC chromatography system

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ABSTRACT

The drug discovery process in the biopharmaceutical industry usually starts with the generation of plasmids coding for certain proteins. Due to advances in cloning techniques the generation of thousands of different plasmids is not a limiting factor anymore. The next step is the expression and evaluation of the proteins. In recent years significant progress has been made in the miniaturization of protein expression and purification. These processes have been adapted to robotic platforms and hundreds of proteins can be expressed and purified in parallel. As a consequence of miniaturization, the protein purification is restricted to a one-step process. In addition the amount of purified protein is usually in the µg-range. This might be suitable if a sensitive initial screening assay is available. However, when larger amounts of proteins are required robotic platforms are no longer appropriate. In addition, a one-step purification procedure is often not sufficient to obtain pure protein preparations. To address this topic we have used the NGC chromatography system for automated purification of up to five samples using a three-step purification procedure. The first chromatographic step is the capture step followed by a desalting step. The final purification was done using size exclusion chromatography. This set-up reduces the overall-time needed for protein production, needs minimal operator invention, is easy to handle and thus increases the throughput.

1. Introduction

Biotherapeutics are one of the fastest growing class of therapeutics in the pharmaceutical industry. More than 200 biopharmaceuticals, e.g. antibodies, hormones, cytokines, and enzymes have been approved for market authorization [1,2]. The prerequisite for recombinant protein expression is the recombinant DNA coding for the desired protein. Techniques for high throughput generation of large libraries containing protein expression vectors have advanced considerably within the last years [3]. In order to keep pace with the increased speed of expression construct generation, protein expression has been miniaturized and automated with microbes as well as with mammalian cells [4–6]. Accordingly, protein purification has been minimized and adapted to robotic platforms [7,8]. This is suitable for protein construct screening where usually only small amounts of protein, typically in the µg range, are required in the initial screening assays. Particularly this is the case for most antibodies, where the initial screening assay is typically a binding assay to the antigen [9]. Besides antibodies, fusion of peptides or proteins to the constant fragment (Fc-domain) of an immunoglobulin is a widely used concept for generation of biopharmaceuticals. The addition of the Fc-domain has several desirable properties; it often stabilizes the protein, enhances its expression and simplifies the purification since the fusion protein can be purified by Protein A chromatography [11]. In terms of pharmacokinetic properties fusion of a protein to the Fc-domain leads to an extended serum half-life since the Fc-fusion protein can be recycled by the neonatal Fc receptor (FcRn) [12].

If more material is required for the initial assays or expression levels are low the supply of protein might become a bottleneck in the discovery workstream. Accordingly larger expression cultures are required and purification becomes more demanding. An automated purification platform dedicated for purification of such proteins is the ProteinMaker [10]. Computer controlled chromatography systems, such as the Aekta systems, are popular in the industry and have also been adapted for automated protein purification. Most of the automated multi-sample, medium scale, multi-step protein purification systems have incorporated devices from a third party vendor or used custom made equipment [12–16].

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Here we have used the NGC-chromatography system to set up an automated three-step purification of for up to five Fc-fusion proteins. Neither parts from a third party vendor nor custom made equipment were required for the purification procedure. All devices (valves, pumps, detectors, collectors) were directly available from the vendor and could be easily configured on site. The protein purification procedure starts with clarified culture supernatants from mammalian cell cultures. There is no upper limit concerning the sample size. Target proteins are captured on individual Protein A columns and are subsequently desalted and finally passed over a size exclusion chromatography column. Cleaning steps and re-equilibration steps are also included. The time for purifying Fc-tagged proteins from five 1-L culture supernatant is about 35 h.

2. Materials and methods

2.1. Automated instrumentation

A NGC Discover 100 Pro system equipped with two additional column switching valves, one additional inlet valve module and air detectors was used. The buffer blending valve was not incorporated. The system contains two buffer pumps capable of flowrates of up to 100 mL/min and a sample pump. The flowpath was designed to allow the samples to pass the UV-detector twice. The first time after the capture column, which allows detection of the flowthrough, and after elution from the size exclusion chromatography column (Fig. 1). The chromatography system was controlled by the Chromlab software (Biorad) version 5.0.2.11.

2.2. Protein expression and purification

The proteins were cloned into an expression vector under a CMV promotor. Protein expression was done by transient transfection of FreeStyle HEK293-F cells (Thermo Fisher Scientific). Cells were grown in non-baffled shake flasks (Corning) at 110 rpm, 37 °C and 8% CO_2. Cells used for transfection were grown to a cell density of 1.2×10^6 cells/mL. For transfection, DNA was mixed with linear Polyethyleneimine (PEI) at a ratio of 1: 3 in Optimem I- Medium (Thermo Fisher Scientific). After 20 min incubation at room temperature the transfection mixture was added to the cell culture. Cell cultivation was done using Freestyle F17 medium supplemented with 6 mM glutamine. After 6 days cells were separated from the culture broth by centrifugation (30 min at 4.500 g and 4 °C). Cell pellets were discarded and the supernatant was cleared by 0.22 µm sterile filtration. The cleared culture supernatants were used for protein purification.

Up to five supernatants containing Fc-tagged proteins could be processed at the same time. Proteins were captured on two connected 5 mL HiTrap Protein A columns (GE Healthcare) for each protein. The columns were equilibrated with phosphate buffered saline (PBS, Gibco). After sample loading, columns were washed first with PBS and then with 0.1 M citrate buffer, pH 6.0. Proteins were eluted with 0.1 M citrate, pH 3.0 and immediately passed over a HiPrep Desalting 26/10 column (GE Healthcare) equilibrated in PBS. The peak fractions eluting from the desalting column were stored in a 20 mL sample loop and the desalting column was washed and re-equilibrated with PBS. Five storage loops are connected to the system and the purification procedure can hence be repeated up to four times. Further purification was done by size exclusion chromatography using a Superdex 200 20/60 pg column (GE Healthcare) equilibrated in PBS. Proteins were subsequently eluted from the storage loops and directly loaded onto the size exclusion chromatography column. After elution of each protein, the column was washed with PBS. The peak fractions were collected and purity was checked by non-reducing 4–12% SDS-PAGE with MOPS-buffer as running buffer (Thermo-Fisher Life Technologies). 15 µL from each fraction was mixed with 5 µL LDS-sample buffer (Thermo-Fisher Life Technologies) before loading of the gel. Gels were stained with InstantBlue (Expedeon, Cambridgeshire, UK). Endotoxin measurements were done using the PTS Endosafe-device (Charles River).

3. Results

A picture of the system and the sample flow path is shown is shown in Fig. 1. The culture supernatants (samples) were connected to an inlet valve followed by an air sensor. The usage of an air detector allows loading samples of nearly any volume. Sample loading was stopped by air detection. Buffers used for equilibration (PBS), Protein A column washing (0.1 M citrate pH 6.0) and Protein A elution (0.1 M citrate, pH 3.0) were connected to a second inlet valve. Up to eight different buffers can be connected to an inlet valve. 0.5 M NaOH was used for column regeneration and connected as buffer B to the system. The samples were loaded onto the Protein A column and upon air detection in the sample flowpath the corresponding Protein A column was washed with 6 CV of PBS and 3 CV of 0.1 M citrate, pH 6.0. Elution from the Protein A column was done with 0.1 M citrate, pH 3.0. The wash buffers and the first elution fractions (one CV) were directed directly into the waste. Then, column valve 2 switched to include the desalting column in the flowpath and the protein eluted from the Protein A column was directly desalted by passing over the desalting column. As soon as the UV280 nm signal reached 200 mAU, the outlet valve switched and the protein was pumped via the injection valve and the column valve (or loop valve) into the storage loop. Setting the detecting level of the UV280 nm signal to 200 mAU proved to be optimal in our case. For other proteins different settings might be more adequate, but this needs to be checked case by case. Alternatively, automated peak-detection can be used. Collection into the loop was done during the next 1.5 CV. The immediate desalting step after the acidic elution from the Protein A column keeps the exposure of the protein in the acidic buffer as short as possible. Next, the desalting column was washed with 15 CV (note: the CV-value used in the software refers to the CV of the Protein A column) with PBS. Alternatively, the desalting column can also be washed with an additional 0.5 M NaOH wash step, but since we had not yet detected any cross-contamination we directly re-equilibrated the desalting column with PBS for the sake of convenience. This procedure was repeated up to four times, depending on the amount of samples. Further purification of the proteins was done using size exclusion chromatography. The samples were pumped from the storage loop directly on the size exclusion chromatography column, now bypassing column valve 1. The corresponding flowchart as displayed in the Chromlab software is shown in Fig. 2. For our Fc-fusion proteins the first 100 mL eluting from the size exclusion chromatography column were directed into the waste and fractions were collected for the next 150 mL. Again, this might be adjusted for other proteins or peak detection can be used for fraction collection. After elution, the size exclusion chromatography column was re-equilibrated with PBS. Again, an additional washing step with 0.5 M NaOH could be included at this point. In our daily used procedure we have included a final cleaning-in-place process after purification of all samples was finished. The general protocol for an automated purification and cleaning-in-place procedure is depicted in Fig. 3.

3.1. Purification of recombinant Fc-tagged proteins

An automated purification cycle for five samples (each 1 L) including column equilibration and cleaning took approximately 35 h. During the purification the chromatography system was completely left unattended. In addition to monitoring the UV280 nm absorbance, the pH-value was monitored. The piping system was designed in a way that each elution step from a column could be monitored via the UV und pH detectors. This allowed an improved control of the process. A chromatogram for a purification of five different Fc-tagged proteins is shown in Fig. 4. A close-up view of the elution from the first size exclusion chromatography and a corresponding SDS-PAGE is depicted in