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Application of the Protein Maker as a platform purification system for therapeutic antibody research and development

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ABSTRACT

Within the research and development environment, higher throughput, parallelized protein purification is required for numerous activities, from small scale purification of monoclonal antibodies (mAbs) and antibody fragments for *in vitro* and *in vivo* assays to process development and optimization for manufacturing. Here, we describe specific applications and associated workflows of the Protein Maker liquid handling system utilized in both of these contexts. To meet the requirements for various *in vitro* assays, for the identification and validation of new therapeutic targets, small quantities of large numbers of purified antibodies or antibody fragments are often required. Reducing host cell proteins (HCP) levels following capture with Protein A by evaluating various wash buffers is an example of how parallelized protein purification can be leveraged to improve a process development outcome. Stability testing under various conditions of in-process intermediates, as an example, the mAb product from a clarified harvest, requires parallelized protein purification to generate concurrent samples for downstream assays. We have found that the Protein Maker can be successfully utilized for small-to-mid scale platform purification or for process development applications to generate the necessary purified protein samples. The ability to purify and buffer exchange up to 24 samples in parallel offers a significant reduction in time and cost per sample compared to serial purification using a traditional FPLC system. By combining the Protein Maker purification system with a TECAN Freedom EVO liquid handler for automated buffer exchange we have created a new, integrated platform for a variety of protein purification and process development applications.

1. Introduction

Purification of antibodies and antibody fragments are key activities in the generation of critical reagents for various *in vivo* and *in vitro* assays as part of biotherapeutic lead identification and process development. Often, it is necessary to purify large numbers of antibodies with milligram yield, relatively quickly and at minimal cost. Various strategies are available to achieve such purification outcomes, and can involve to various extents both automated and manual methods [1,2]. While parallelized purification methods yielding sub-milligram quantities of pure proteins based on packed columns, 96-well plates containing small quantities of chromatographic resins or ligands immobilized to the surfaces of membranes have been developed, there are relatively fewer options available for generating purified quantitates of protein in the intermediate (5–100) milligram scale. A few examples of customized solutions to this problem exist, involving integration of existing purification platforms such as the ÄKTA Purifier with a CETAC autosampler [3], ÄKTA Pure [4] or liquid handling robotics [5] have been reported. Other solutions include the design and fabrication of customize robotics platform, including the Protein Expression and Purification Platform [6]. While some commercial instruments for purification of small quantities of protein have been developed, such as the QIAcube for purification of His-tagged proteins [7], there are few examples of commercial instruments that can be utilized for platform purification at milligram scale.

In the context of process development applications, various commercially available scale-down protein purification products have been developed, including Predictor plates (GE) and Robo-columns (GE and Atoll Bio). While very useful for early-stage screening of various chromatographic conditions, the maximum size of the columns possible in these platforms (600 μL bed volume) results in a considerable gap in the scale between screening and further optimization of process

Abbreviations: CHO, Chinese Hamster Ovary; DPBS, Dulbecco's phosphate buffered saline; HC, IgG heavy chain; HCP, host cell protein; IMAC, immobilized metal ion affinity chromatography; LC, IgG light chain; mAb, monoclonal antibody; OAA, one-armed antibody.

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conditions. Some examples of higher throughput, automated solutions to purification process development have been reported [8,9]. While automated, sequential purification of samples is possible using a chromatography system connected to an auto-sampler, this cannot be parallelized using a single instrument, thereby reducing the possible number of samples processed.

A specific instrument which has been designed around accomplishing the task of parallelized, medium scale purification is the Protein Maker system, originated by Emerald BioStructures [10] and subsequently developed and marketed by Protein BioSolutions. The Protein Maker is an automated protein purification platform designed for purification of feed volumes of various sizes, from ~10 mL to 1 L (~1 mg to 100 mg) or more utilizing up to 24 chromatography columns, each with an independent flow path. The main components of the system are (i) the syringe pumps with the associated 9-port valve, mixing syringe and sample lines, which together form the initial portion of the flow path, (ii) the column gantry, columns and associated tubing from the syringe pumps, which form the subsequent portion of the flow path and (iii) the deck, which contains up to 19 positions for SBS format plates and a dedicated waste position.

While purification of a variety of proteins from any number of sources is in principle possible with the instrument, the focus herein are examples of purification of antibodies and their fragments generated from mammalian expression systems. We have utilized the Protein Maker as a key component of a platform purification system that integrates automated buffer exchange implemented on a TECAN Freedom EVO liquid handler. This protein purification platform can be used for both parallelized, small-medium scale purification of antibodies and their fragments, as well in various process development applications.

2. Materials and methods

2.1. Antibody production

Murine IgG samples were produced in hybridoma culture in IMDM supplemented with 10% heat-inactivated FBS and mouse IL-6 by a procedure previously described [11]. For some antibodies, cultures were performed transiently in Chinese Hamster Ovary (CHO) cells as previously described [12]. Productions were harvested by centrifugation or filtration (0.22 μm or 0.45 μm) and IgG containing supernatants stored at 4 °C until purified.

2.2. Purification of mAbs and Fabs

For development of Protein Maker purification methods, protein samples were purified using 1 mL HiTrap columns (GE Life Sciences), including Protein G HP, MabSelect SuRe™ (Protein A) and Ni Sepharose Excel™ mounted on an ÄKTA Purifier 10/100 system. Chromatographic profiles were monitored at 280 nm. Columns were equilibrated in Dulbecco's Phosphate Buffered Saline Solution (DPBS, HyClone Laboratories), the sample applied at the appropriate residence time (1 min for Ni Sepharose Excel or 3 min for MabSelect SuRe or Protein G HP) and a portion of the flow-through fraction collected for subsequent non-reducing SDS-PAGE analysis. Columns were washed with DPBS and bound proteins eluted with two column volumes (CV) of sodium-citrate buffer pH 3.6 (MabSelect SuRe), two CV of 100 mM glycine-HCl pH 2.6 (Protein G HP) or one CV of DPBS with 500 mM imidazole pH 8 (IMAC purification). For proteins eluted from Protein A or Protein G columns, samples were pH adjusted using 1 M solutions of sodium HEPES or Tris–HCl buffer to a final pH of 6–7.

Platform, parallelized purification experiments were performed using the Protein Maker running the Protein Maker v2.0 software (Protein BioSolutions). Purification runs were performed using the 1 mL HiTrap columns and the chromatography conditions (residence time, column washing and sample elution) established using the ÄKTA purification system. Sample and buffer lines were cleaned in place with 0.5 M NaOH and equilibrated in DPBS or appropriate buffer solutions. During purification, a portion of the flow-through fraction was collected for subsequent non-reducing SDS-PAGE analysis. Protein samples were eluted in three steps, consisting of a pre-elution volume, elution volume and post-elution volume. Protein concentration measurements (A_{280} nm) on these fractions were used to establish the final pooled sample.

2.3. Process development for mouse IgG2a purification

The Protein Maker system was used to purify in parallel five murine IgG2a samples from mouse Hybridomas using MabSelect SuRe and Protein G HP 1 mL HiTrap columns. For each mouse IgG2a, 20–22 mL of supernatant (~0.5 to 2 mg of mouse IgG2a, depending on titer) was purified on either column using the purification method described above. For protein G purifications, elution was performed in two steps, first with 100 mM citrate buffer pH 3.6 and then with 100 mM glycine-HCl buffer pH 2.6. Elution fractions were neutralized using 1 M Tris. The quantity of IgG2a contained in elution fractions were determined based on A_{280} nm. For protein G purification, the quantity of IgG2a obtained from the two elution steps was summed for calculating the yield.

2.4. Development of a post-load wash step to improve HCP removal during protein A purification

Data were obtained with three different antibodies expressed in CHO cells. The Protein Maker system was used to perform parallel purifications using MabSelect SuRe 1 mL HiTrap columns. For each antibody, eight wash conditions were tested. For each tested condition, 10 mL of supernatant (16.3 to 17.5 mg of antibodies) were loaded at a residence time of 3 min. HiTrap columns were then washed and antibodies were eluted using 2.5 CV of 100 mM citrate buffer pH 3.0. Elution fractions were neutralized using 1 M HEPES. The quantity of antibody in elution fractions was determined by A_{280} nm. The quantity of HCP in elution fractions was measured using a CHO HCP ELISA kit (Cygnus Technologies).

2.5. Buffer exchange and aseptic filtration

Buffer exchange into DPBS following affinity purification was performed manually either using Zeba-spin columns (Thermo-Fisher Scientific) by centrifugation or using PD-10 desalting columns (GE Healthcare) by gravity according to the manufacturer's instructions. Alternatively, sample buffer exchange using PD Miditrap G-25 columns was automated on a TECAN Freedom EVO150® liquid handler according to gravity protocols from GE Healthcare. The Freedom EVO150® was equipped with a liquid displacement Liquid Handler (LiHa) configured with 8 channels (4 disposable tips and 4 washable tips), a Robotic Manipulator (RoMa), a Tecan Vacuum (TeVac), carriers for 11 microplates, shelf for 4 microplates, one reservoir position for elution buffer, and tip carriers for hanging tips. All tubing and components of the liquid displacement system were cleaned in-place with 0.5 M NaOH for at least 15 min and rinsed with sterile water prior to operations. A script was developed with the flexibility to process from 24 to 96 samples at once. Before starting, the storage solution from PD MidiTrap G25 columns was removed manually, the columns placed in a 24 position custom holder and the rack positioned on the Freedom EVO150® worktable. Purified protein samples from the Protein Maker were stored in 24 deep well microplate (Seahorse Bioscience). The system liquid was replaced with DPBS (HyClone Laboratories) and the script started. First, the RoMa arm brought the column racks onto the TeVac and columns were equilibrated with three bed volumes using the Freedom EVO150® system liquid (DPBS). The equilibration buffer