CHAPTER

9

High-throughput, parallelized and automated protein purification for therapeutic antibody development

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List of abbreviations

ATPS aqueous two phase system
CE-SDS capillary electrophoresis, sodium dodecyl sulfate
CIP clean in place
DBC dynamic binding capacity
DoE Design of Experiments
HCP host cell protein
HIC hydrophobic interaction chromatography
IEX ion-exchange chromatography
LH liquid handler robotics platform
LC-MS liquid chromatography, mass spectrometry
mAb monoclonal antibody
PD process development
PEG polyethylene glycol
SEC size-exclusion chromatography
SPR surface plasmon resonance
UPLC-SEC ultra performance liquid chromatography, size exclusion chromatography

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Introduction

Downstream processing continues to be challenged by the increasing complexity and higher quantities of product resulting from upstream processes, with DSP often described as a bottleneck in the production of therapeutic antibodies. Early stage antibody therapeutic development has different challenges than does the later stages. Initially, there are often many constructs that require production and purification at small to intermediate milligram scale in order to evaluate product attributes and identify manufacturing liabilities via panels of *in vitro* and *in vivo* assays. Various levels of product purity may be required, depending on the kinds of questions to be addressed. Speed, cost and simplicity of operation, where possible, are key factors to be considered in the resulting purification workflows. While parallelization of purification can be readily achieved at microgram and low milligram scale using a variety of liquid handling automation equipment, purification automation and parallelization becomes progressively more challenging as antibody production volumes and product quantity for capture increase. Both commercial equipment and a variety of adapted "in-house" solutions have been described to try to overcome this problem. Here, a survey of chromatographic and non-chromatographic purification approaches are presented in the context of automation, parallelization and their potential for high sample throughput. The survey is by no means exhaustive and the interested reader is referred to previous reviews of this subject area [1–4] for additional perspectives.

Small-scale equipment and strategies

Liquid handling platforms

Scaled-down purification strategies that can handle large numbers of constructs from small volume productions in minimal time are crucial in early-stage discovery projects. The scale-down approach offers the possibility of minimizing volumes for production using transient transfection in mammalian cells, as well as reducing the burden on analytical and characterization studies by optimizing high-throughput analytical methods to analyze many samples in parallel. The ability to parallelize and automate also reduces costs and provides sufficient opportunity to sample the required design space.

A variety of small scale platform and process development related purification methods have been developed using various liquid handling (LH) robotics equipment from a variety of commercial vendors. The most common methods employ the use of resin filled filtration plates that can be utilized in either vacuum filtration, positive pressure or centrifugal mode, magnetic beads to which an affinity resin is coupled to be used with a plate-compatible magnet, small pre-packed columns, OPUS® RoboColumns (Repligen Corp), through which liquids are applied using LH fixed tips, 24 or 96-well filter plates containing membranes immobilized with Protein A, Protein G or ion-exchange functionalities or chromatography resin-filled tips, a common product called PhyTips™ (Phynexus Inc.).

Resin-filled purification tips

Resin filled tips offer the advantage of being readily amenable to a variety of automation platforms. These tips are offered in a wide variety of sizes and different chemistries

depending on the purification application. Aspiration, washing and elution of purified products is achieved simply by controlled liquid movement into and out of the tip. The point has been made that the bidirectional liquid flow in such tips is not as found in conventional packed columns, where liquid flow is usually in one direction, and for this reason they are more similar to a batch purification process [5]. Automation of PhyTips™ in 12 channel format as a platform purification strategy has been described [6]. Using either custom packed 500 μL tips or commercially available PhyTips containing Protein A resin described in Ref [7], successfully developed HT antibody purification platforms capable of purification of up to 2000 antibodies per day from 1 mL cultures.

Magnetic beads

Para-magnetic beads, for example, cross-linked agarose, to which Protein A, Protein G, Protein A/G or Protein L are covalently attached are commercially available from a number of suppliers. Utilizing an appropriate magnetic base integrated with an automated LH platform, magnetic affinity purification resins can offer a fast way to purify small quantities of antibodies from large numbers of small volume samples. Immobilization of the beads by the magnetic base permits LH tips to aspirate liquids for washing and elution of products. An advantage of magnetic beads is the ability to potentially elute purified antibodies in small volumes, resulting in more concentrated samples for buffer exchange or downstream assays. Some considerations with magnetic beads as a potential automated purification option include their cost per unit of purified product, compatibility with standard CIP procedures for recycling of resin as well as the quantity of purified product required. This scale of purification can be particularly attractive for assays that require small quantities of purified protein, including LabChip microfluidic CE-SDS, SPR, UPLC-SEC and LC-MS.

An example of how magnetic protein A based purification has been described for small-scale purification of monoclonal antibodies secreted by yeast cell lines [8]. In these studies, mAbs from 96-well plate cultures were purified using 20 μL per well quantities of BioMag Protein A beads (Qiagen) using a Biomek NX LH platform. The resulting purified samples were subsequently analyzed by HT E-PAGE pre-cast electrophoresis system (Invitrogen). Combined with gel imaging analysis, such experiments can be used to rank-order clones for protein productivity.

New generations of immuno-magnetic beads are being developed from materials other than cross-linked agarose with new properties, allowing for faster antibody purification. Examples include either Protein A or Protein A/G covalently immobilized to magnetic SiO_2 microspheres [9] or Protein A coated and glutaraldhehyde-crosslinked Nickel particles [10]. Improved magnetic bead technologies along with reductions in costs will expand the scope of use for these affinity resins in automated antibody purification.

Plate-based membrane chemistries

A wide variety of membrane-based separations in purification have been described although mainly for larger-scale antibody purification, usually as an intermediate polishing step (reviewed in Ref. [11]). A major advantage of membranes compared to bead-based resins

is that mass-transfer is convection instead of diffusion limited, resulting in much faster binding kinetics at higher flow rates. Another advantage of the membrane-based format is the ability to elute products with a smaller volume of elution buffer, resulting in more concentrated samples prior to desalting or other post-purification treatments. Buffer consumption is also reduced due to the small membrane area. A potential limitation of such an approach is the dynamic binding capacity may be limited as the membrane surface area in a 96-well plate format will be small.

While the options for antibody affinity purification using membranes in plate format are limited, commercially available Protein A and Protein G immobilized in a 24 or 96-well membrane plate format recently have appeared, one product being Capturem™ plates (Takara Bio Inc.). A number of vendors supply 96-well or other plate formats with ion exchange or other membrane chemistries that can alternatively be used for high-throughput antibody purification. Examples include Mustang E and Mustang Q ion exchange chemistries in 96-well plate format (Pall Corp) as well as S- (cation exchange), Q- and Stick PA (anion exchange) and Phenyl (HIC) chemistries in Sartobind 96-well plate format (Sartorius).

Batch purification in plates

Batch purification methods can also be scaled down into different plate formats for execution using liquid handler systems. Clarification options include both centrifugation and vacuum driven filtration. The clarification could be performed off line or integrated as part of the automated purification workflow. Filtration plates can either be obtained commercially, such as Multi-Trap purification plates (GE Healthcare) or made in-house using chromatography resins and appropriate filtration plates. As with clarification, both vacuum-based filtration and centrifugation are options for binding, washing and elution of products. Positive pressure manifolds, used for example in solid-phase extraction, can offer more control of liquid flow rates compared to either centrifugation or vacuum based approaches, and are available for a number of LH systems from different suppliers.

An example of the utilization of resin filled filter plates for HT process mAb purification development has been described in Ref. [5]. In these studies, vacuum filtration was employed in the purification and protein quantitation achieved using 96-well UV plates to order binding capacities for different resins. A similar automated batch purification strategy was employed in Ref. [7] with mAb Select SuRe except that centrifugation was utilized for plate washing and product elution. In this study, the batch binding was performed in separate plates prior to transfer of antibody-loaded resin to filter plates for further processing using the LH.

Robo columns

Opus® Robo-columns (Repligen Corp) are small packed bed columns available in a range of bed volumes from 50 to 600 μL. Unlike resins in plate format, they represent a scale down model for a packed chromatography column. Columns are available either pre-packed or packed with user-defined resins. It is possible to perform step gradients and collect fractions from such columns if appropriate plate-shuttle hardware is available for the LH. These

columns have been demonstrated to run with TECAN EVO LH systems (Tecan Trading AG; [5]) as well as the JANUS liquid handler (Perkin Elmer; [12]). One study [5] utilized 600 μL bed volume Robo Columns POROS HS media for DBC studies and 200 μL bed volumes for product elution studies. In another study [12] work, 100 μL bed volume Robo Columns containing mAb Select SuRe for purification were coupled with a CentriPlate P96 buffer exchange column array (emp Biotech) or HT preparative scale HPLC-SEC. One advantage of HPLC SEC columns in this kind of application is the comparatively higher flow rates that are possible compared to soft gels (cross-linked agarose), resulting in faster cycle times.

Buffer exchange and protein concentration

Workflows for the small scale purification of intact mAbs as well as antibody fragments usually involve at least one step where the sample matrix needs to be modified (buffer exchange) or a sample is required at a higher concentration. It is sometimes difficult to find good options to achieve parallelized buffer exchange and concentration of protein samples, leading to a bottleneck in the workflow that results in additional manual effort, time and cost. Resin filled tips (gel filtration Phytips), OPUS Robo-Columns filled with gel filtration media and 96-well plates such as MultiTrap Sephadex G25 plates, (GE Healthcare) or Zeba spin desalting plates (Thermo Fisher Scientific) in 96-well format are all commercially available options. Selection of the correct product depends on sample application volume, the available LH equipment and how to optimally integrate buffer exchange into the overall purification workflow. While individual centrifugal or gravity based desalting columns are readily available from a number of suppliers, they normally require more manual effort than HT-compatible devices. Some solutions to this have been proposed, for example, integrating gravity desalting columns such as PD-10 or any number of other formats into racks such that an LH can apply samples and the resulting samples collected in blocks [13].

There are fewer options for parallelized concentration of samples prior to purification steps such as SEC. Ultrafiltration plates in 96 well format for small volume sample concentration with different molecular weight have been described, for example, Acroprep Advance filter plates (Pall Corp) or Multiscreen Ultracel-10 96-well plates (Millipore).

Mid-scale purification strategies

Batch methods

Many of the more recently developed parallelized, higher throughput protein purification approaches that utilize commercial equipment and chromatography consumables can be prohibitively expensive for academic and other non-industrial lab environments. There can also be some burden with regards to the availability of trained personnel to develop and execute some of these purification workflows. A relatively simple, if not elegant approach consists of batch based purification, a method which can be readily parallelized with minimal investment in materials. Disadvantages of this approach are that it is labor intensive and can result in variable purification performance, as the level of control in the method is more limited than for pump-based liquid handling.

In batch purification using affinity resins, the product capture step can be either coupled directly to the rest of the work flow or uncoupled. When coupled, clarified supernatant or lysate is applied directly to columns containing defined quantities of purification resin(s), allowed to pass through the resin and samples collected in tubes or blocks. Control of residence time is relatively difficult under gravity flow conditions, unless a multiport pump can be utilized, although it can be better controlled using a vacuum manifold so long as all samples in the manifold flow at a similar rate. A multi column plate adapter, MCPA, has been described and can be utilized for such an application under gravity or vacuum conditions [14].

In an uncoupled batch purification process, resin is first incubated with clarified harvest supernatant for some pre-determined period of time prior to moving the resin/supernatant mixture into columns of appropriate size, which can then be processed by gravity flow to collect the flow-thru. Column washes and product elution are all achieved by adding sequential buffers to the columns, either manually or with a pump. The uncoupled approach is more attractive if larger supernatant volumes are to be used, requiring less operator time, as well as alleviating the issues around residence time, since the product capture step has already occurred. Using appropriate CIP conditions, products having the required endotoxin levels can normally be successfully purified.

Automation options

Mid-scale purification, where purified product requirements are in the tens to hundreds of milligrams, represents one of the most challenging areas for parallelization and automation of protein purification. While custom built, specialized high-throughput and highly automated protein purification equipment, such as that found in the GNF protein expression and purification platform [15] does exist, it is expensive, time-consuming and requires a team with the right kinds of expertize and resources to bring it to life. Purification at this scale also often places more demands on the purification platform in order to deliver products with defined endotoxin levels for cell based and*in vivo* studies, as well with minimal protein aggregates. Ideally, such purification strategies are properly integrated with both upstream clarification technologies as well as analytical assays and sample and IT management in order to create a holistic antibody production platform.

Protein Maker

The Protein Maker (Protein BioSolutions) is a liquid handling platform using syringe pumps, originally developed and sold by Emerald BioSystems for high-throughput, mid-scale protein purification [16]. The current version of the instrument consists of 24 syringe pumps, each having an independent flow path consisting of sample inlet tubing, a nine port switching valve, a mixing syringe at position 5 of the valve (for forming step gradients, if desired) and outlet tubing leading to the column manifold. Buffers and cleaning solutions are delivered from bottles via the syringe pumps and tubing for each flow path to the multiport valve. Chromatography columns of 1 or 5 mL bed volume are most commonly employed, although other columns can be connected singly or in series, depending on the available space and required flow rate. There are a total of 20 deck positions, including one for waste, in which SBS-formatted plates or blocks can be located. Plates having 24, 48 or 96 wells can potentially be utilized for sample collection, depending on how column outlets are organized on the gantry.

Racks located at either the ends or top of the instrument secure samples of various volumes as well as buffer, cleaning and sanitization solutions. Typically, clarified supernatant volumes between 10 or 20 mL and 1 L are applied per flow path. If required, larger volume or higher productivity clarified harvests can be split over multiple columns and eluted products subsequently pooled together. An accessory available for the Protein Maker is a 24-channel, A280 nm absorbance manifold assembly, allowing chromatograms to be recorded and stored for each of the individual flow paths. It is also possible to cool the deck using a circulating cooling bath containing an appropriate solvent system.

In addition to single step column purification, the instrument can also perform automated two-step protein purification of up to 12 samples in parallel by aspirating samples from a plate located on the deck. In this configuration, two sets of 12 columns, for example, 12 Protein A columns and 12 desalting columns, would be connected to gantry A and gantry B, respectively. Products eluted from Protein A into an elution plate would be aspirated and passed over desalting columns located in gantry B, with the final eluted samples collected into a separate receiving plate.

A large variety of therapeutic antibody research and development studies can be executed using the parallelized purification capability of the Protein Maker, with a few of these recently summarized in Ref. [13]. In addition to platform purification of antibodies or antibody fragments at various scales, using a variety of affinity chromatography columns, process development applications, including resin screening studies, Protein A column washing studies to reduce HCPs, or clarified harvest hold stability studies to define optimal hold time and temperature, can be performed. The Protein Maker can also be readily integrated with other platform purification equipment depending on the overall workflow requirements, for example, utilization in combination with a Tecan EVO liquid handler for high-throughput gravity-based buffer exchange of purified samples [13] or utilization of semi-automated size exclusion chromatography using FPLC's. In-process analytical assays including SEC and CE-SDS to analyze product attributes can also be performed.

Modified FPLC and HPLC systems

The general lack in availability, with few exceptions, of commercial higher-throughput, automated protein purification solutions for mid-scale (10−1000 mg) purification has resulted in a number of ingenious adaptations to FPLC and HPLC equipment. In many cases, this involves integration of FPLC's, pumps and switching valves with software and appropriate autosampler equipment. Addition of a standard sample pump, injection valve and sample inlet valve with air sensor can permit automated single-step purification of up to 14 productions of any volume, depending on the instrument and configuration selected. The challenges then become temperature control of supernatant feeds and potentially fractionation capacity for the eluted products.

Commercially available autosamplers, including those from GE/Spark-Holland (ALIAS Bio Cool & Bio-Prep Cool) and Teledyne CETAC (ASX-560) can be readily connected to GE ÄKTA or Bio-Rad NGC systems, as examples, to help improve purification sample throughput. Volumetric dispensers under high pressure, such as the HPD (Spark Holland) offer the possibility of applying larger clarified production volumes to chromatography

columns at flow rates of up to 10 mL/min. Using the HPD system, up to 24 larger volume samples (six samples per unit, utilizing four units) could be automatically loaded onto columns sequentially and purified. As with autosamplers, these and related devices can be integrated with FPLC systems using the appropriate control software and Input/output connections.

For smaller numbers of samples (five or fewer), commercial GE (ÄKTA Pure; [17]) or Bio-Rad (NGC Discover; [18]) instruments can perform automated two or three column purification steps utilizing an additional loop valve and tubing to temporarily store intermediate samples in the purification workflow. A recent strategy utilizing modified ÄKTA PURE or NGC systems to perform an automated sequential Protein A, buffer exchange and SEC workflow on up to seven samples at 500 mg scale has been described [19]. An alternative approach, using legacy equipment such as the ÄKTA Explorer, is to utilize the outlet valve and sample inlet valve position in combination with the sample pump to sequentially reapply eluted samples from one purification step to subsequent columns [20]. Generally, the most common platform purification workflows would include affinity purification using Protein A chromatography followed by buffer exchange or SEC. The introduction of other chromatographic methods, such as HIC, IEX or mixed-mode chromatography would first require development of the appropriate binding, washing and elution conditions suitable for a given product.

The overall approaches possible with newer, off-the-shelf commercial instruments represent improvements to that utilized in the legacy ÄKTAxpress system (GE), where each individual instrument could purify up to 4 samples per day, depending on the type of purification used. Increased throughput is achieved through combining ÄKTAxpress units, with up to 12 modules controlled by one computer. A number of groups have reported successful automated, multi-step purification at multi-milligram scale using these instruments, often for structural studies [21−24,59].

A relatively early effort was made by a group at Amgen to build an integrated HT purification platform utilizing a modified ÄKTA Purifier, and a commercially available CETAC ASX-520 autosampler [25]. This system was named the ÄKTA-AS. A significant challenge at the time was establishing communication between the autosampler and the FPLC system. Modifications to the ÄKTA Purifier were also required, including the addition of valves to direct flow and to be able to move liquid from a waste valve port onto a second column in two-step purification mode. Using this system, samples are served by the autosampler to a 1 mL Protein A affinity column followed by buffer exchange using a 5 mL HiTrap desalting column. Fractionation is achieved in 96-well blocks to facilitate HT analytical experiments. Depending on the sample volumes, the system has a throughput of up to 240 samples (with a maximum volume of 14 mL) or 84 samples (with a maximum volume of 50 mL). Variable sample loading is controlled through integration of an air sensor within the flow path, which is used to stop a pump.

A further modified ÄKTA Purifier or Explorer system has been described, this time the purpose being automated two-step purification of antibodies where the second chromatographic step requires conditioning of the elution from the first step [26]. This second purification step could be one of a variety of chemistries, although in this specific study, cation exchange in bind and elute mode was selected to remove antibody aggregates post Protein A. The elution conditioning for the second purification step is achieved by in-line dilution,

utilizing a static mixer. In this setup, either 5 or 10 mL bed volume Protein A and IEX columns can be integrated to perform two-step purification of up to five samples without manual intervention.

Utilizing Agilent HPLC components an automated, preparative purification system capable of executing consecutive Protein A and SEC chromatography on up to 48 samples at 35 mL production scale has been developed [27]. Using a 10-port valve and two quaternary pumps permitted two SEC columns to run in parallel, with a third quaternary pump dedicated to Protein A purification. As opposed to the normally utilized cross-linked agarose sizing columns, a silica based TSKgel G3000SW column was instead utilized, decreasing the run time approximately 4-fold. Samples were loaded using a CETAC ASX-520 autosampler.

Normally in batch processes, clarification of the harvest is an off-line operation prior to product capture by chromatography. To overcome this challenge, an automated harvesting and two-step purification system using commercially-available components has been described [28]. This system makes use of a separate pump and disposable filtration units to achieve harvest clarification of up to eight, one liter productions. Two ÄKTA Pure 25 systems are connected to the clarification train such that each FPLC instrument can perform both affinity purification, in-line pH adjustment of the eluted product as well as SEC in a continuous manner. The system has been demonstrated to yield products having low endotoxin levels. Using this equipment, the entire process can applied to 8 productions in less than 24 h.

It is also possible to utilize less traditional equipment in order to create an HT purification instrument. An example of this is the development of a positive pressure device for affinity purification by adapting a Gilson ASPEX solid phase extraction system [29]. As configured, this instrument could process up to 60 samples in 18.5 h. While this particular study made use of His-tagged proteins, adaptation to antibody affinity purification would be straightforward. Using a modified Gilson solid phase extraction system with switching valves a 4 channel automated antibody purification instrument, capable of purifying up to 24 samples has been developed [30]. The setup is can also perform two step purification, consisting of Protein A purification and buffer exchange using PD-10 desalting columns in gravity flow mode.

A number of vendors sell Protein A, Protein G, Protein A/G or Protein L spin columns, a convenient format for lab-scale, centrifugation-based antibody purification. Depending on the resin volume of the particular spin column, it is possible to purify several milligrams of product per column. In principle, one could imagine automation of spin columns by building an appropriate column holder, collection reservoir and integration with an automatable centrifuge. There seem to be few examples of commercial solutions to automation of spin columns, one being the QIAcube system (Qiagen), although this appears to be limited to IMAC purification methods [31].

Magnetic resin based separations

Paramagnetic beads to which Protein A or other affinity ligands are covalently attached offer an attractive alternative to traditional resins for small and mid-scale antibody affinity capture and have even been evaluated even at pilot scale [32,33]. A paramagnetic Protein A resin, LOABeads Alkali A, have tolerance to sodium hydroxide for cleaning and are

colored black for easy visibility (Lab on a Bead AB). A particularly attractive feature of these resins is the ability to effectively uncouple the clarification and purification steps, by introducing magnetic beads directly into mammalian culture supernatants to bind secreted products prior to clarification. The cost, however, of these resins can represent a significant investment, especially if used in a single use workflow. Using strong rare earth magnets, it is possible to bind the beads and separate these from cells, permitting rapid isolation of the antibody. Alkali resistant (0.1−0.5 M NaOH) magnetic Protein A (AmMag™) beads have been developed by GenScript, as well as a semi-automated purification system, AmMag®SA. This instrument is able to process up to 12 samples at 5−50 mL production volume concurrently.

A different instrument, the KingFisher system, available in different formats, has also been developed (ThermoFisher Scientific). These instruments can process between 6 and 96 samples to a maximum sample volume of 50 mL, depending on the instrument model and quantity or volume of product to be purified. This instrument contains rods that consecutively transfer and release magnetic affinity resin beads, moving them through binding, washing and elution buffers to effect purification. Protocols that demonstrate applications with either Protein A or Protein G paramagnetic beads have been described.

Membrane-based and monolith devices

As with 96-well membrane plate formats, small membrane capsule devices are commercially available for Protein A affinity purification. Both 1 and 3.5 mL GORE™ protein capture devices for antibody purification are available (W.L. Gore & Associates Inc.). These devices are capable of purification of tens of milligrams of products and are amenable to repeated CIP using 0.1 M NaOH as a cleaning agent. With the lure lock connectors, several units could be utilized with an appropriate column valve and integrated autosampler on an FPLC platform, or alternatively, replace packed beds on an instrument such as a the Protein Maker. A distinct advantage over small packed beds would be the higher flow rates, resulting in comparable quantities of purified products in less time.

Another device is the Sartobind Protein A membrane absorber (Sartorius stedim biotech GmbH) available in a 2 mL format. This device can also be connected via lure lock connectors to FPLC, peristaltic pump or related equipment using flow rates of 5−10 mL/min. As with other Protein A formats binding capacity varies depending on IgG isotype and species. The protein A ligand utilized in these devices is partially stabile under 0.05 M NaOH CIP conditions.

As with 96-well format plates, a number of small (1−5 mL) capsule devices are available in anion exchange, cation exchange and HIC membrane chemistries from various suppliers including Pall and Sartorius, which can be utilized in a similar manner as with small chromatography columns. Depending on the application, these devices can be utilized in either bind-and-elute or flow-thru mode.

Monolith columns having large flow channels share the high mass transfer properties under high flow rates of membrane based devices but with the potential of larger binding capacity. Protein A, Protein G and Protein L devices are available in 1 mL format and larger sizes as well as in a variety of other chemistries (BIA Separations). These devices also circumvent the need for column packing and offer a lower shear environment, due to the large channels, for large molecules. Like other devices, these monoliths can be connected to a variety of

FPLC or related instruments. The application of similar devices for the isolation of immunoglobulins from serum as part of a high-throughput purification workflow has been described [34].

Applications to HT process development

Establishing purification unit operations as part of developing an appropriate downstream purification process remains a significant challenge, facing multiple pressures relating to minimizing time and process costs, achieving required final drug substance/drug product attributes, operate within practical equipment feasibility limits and remain both scalable and technically robust within a cGMP environment. The demands are further exacerbated with complex therapeutic antibody modalities, which may possess inherent design-related liabilities and limitations that have not yet been fully explored nor are well understood until downstream process development is well underway. Inevitably, the time-line for downstream process development shrinks, compacted by continued upstream process development changes on one side and the demands for large quantities of purified product for analytical assay development, product characterization, formulation and pre-clinical*in vivo* studies on the other. High-throughput purification tools combined with statistical methods including Design of Experiment can, in some instances, increase process development efficiency and reduce overall time and effort. Several reviews that cover various aspects of HT downstream process development of therapeutic molecules including mAbs have been published previously [1,35,36,60].

Commercial suppliers related to protein purification products provide a number of HT purification consumables that can be adapted into antibody product process development studies. One of these, OPUS® Robo-columns (Repligen Corp), integrated with an appropriate LH system capable of collecting fractions, have been utilized in a number of studies, including determination of adsorption isotherms [37], column washing and elution studies [38], dynamic binding capacity studies [12] and yield and purity in both bind and elute as well as flow through mode [39]. More detailed descriptions of Robo-column operation on the TECAN EVO [40] or JANUS LH platforms [12,41] are available for the interested reader.

While the miniature column formats do offer benefits, including purification parallelization, reduced requirements for feedstocks and an increased number of automated purification experiments per unit time, there are also limitations. Some of these are related to the nature of the columns themselves. The small bed height as well as diameter mean a reduced number of theoretical plates in the packed bed compared to a longer column, impacting separation resolution, as well as increased wall effects [5]. These would be important considerations in polishing steps. An additional limitation, apt for any small-scale purification method, is the availability of an appropriate set of analytical methods with the corresponding performance characteristics to provide meaningful data for the samples generated [38,41]. Integration of an LH platform capable of multiple modes of HT purification in various formats (columns, tips, plates) with analytical methods including protein quantitation by absorbance and microfluidic electrophoresis (LabChip) has been described as one possible solution to this challenge [41].

An alternative to commercial pre-packed miniature columns is the packing of slightly larger columns (e.g. 2 mL bed volume) of more representative bed height, whose performance may be more predictive to that of lab-scale and larger columns while also allowing for larger product loads [42]. This approach may offer an acceptable compromise between the limitations of miniature packed beds and the increased sample requirements and longer run times of larger lab-scale columns. While these studies were performed using an ÄKTA Explorer 100 system for resin and chromatography condition screening, greater performance could be obtained today utilizing a similar approach with more modern FPLC instrumentation integrated with an auto-sampler and fractionation capability in 96-well plates.

In addition to use of small chromatography columns, batch binding studies in 96-well plate format are often performed as part of antibody purification process development. Commercially available filter plates pre-filled with resin, such as Predictor plates (GE Life Science), Resin seeker plates (TOSOH Bioscience) or Foresight™ pre-packed plates (Bio-Rad Laboratories) are amenable to automation using LH instruments and can be used for chromatographic media scouting, batch uptake studies to determine binding capacity, determine adsorption isotherms under various conditions, understand resin performance with respect to CIP cycles as well as screen resin washing and elution conditions. Filter plates can also be filled in-house with bulk resins from a defined slurry, although there are a number of important considerations when preparing such plates, including the type of filter plate used, agitation conditions for the resin and a quantitative method to dispense resins [43]. Other kinds of studies, such as HCP washing experiments with various buffers [44], and estimation of dynamic binding capacity from uptake studies [45] can also be performed in a similar filter plate format.

Non-chromatographic HT purification approaches

While affinity chromatography, particularly the use of Protein A, has dominated antibody purification for some time, there has been increasing interest in the potential of non-chromatographic approaches, both technically and from a cost of goods perspective. While not yet necessarily adopted, non-chromatographic purification unit operations, especially as part of primary clarification prior to product capture, are applied as required for purification of some products. These steps are often combined with chromatographic steps in designing the overall downstream purification process. Examples of these approaches include large scale crystallization, aqueous two phase extraction, precipitation and polymer-mediated flocculation. Some of these approaches are amenable to high-throughput screening in combination with the appropriate assays to read out on purification performance.

Batch crystallization

Crystallization of antibodies and antibody fragments has been explored as a purification step at both smaller and larger scale as well as a formulation method to increase product stability and even as a subcutaneous delivery format (reviewed in Ref. [46]). While small-scale

crystallization screening experiments can be performed manually, these are in many cases readily amendable to high-throughput robotics, either using traditional liquid handlers or specialized protein crystallization automation equipment. Microbatch under oil screening experiments [47–49] and scaled up (5 mL–1 L scale) stirred tank crystallizers [48,49] can both allow for parallelization of batch crystallization studies.

Precipitation

In addition to using salts such as ammonium sulfate, reversible precipitation of antibodies and related products as a potential purification strategy has been described using ethanol [50], various anionic polyelectrolytes [61], cationic polyelectrolytes [51], polyamines [52] or different molecular weight polyethylene glycols [53–55]. High-throughput experimental methods can be adopted to screen precipitation conditions in plates, including the generation of solubility curves, as well as determine re-solubilization conditions for the precipitated product. Using a TECAN EVO platform a series of polyethylene glycol molecular weights and two pH values were screened with a series of mAbs in filter plates with the protein recovery determined based on A280 nm measurements has ben performed [55]. A related study used a series of linear and branched polyamines at various concentrations to effect precipitation of a CHO produced antibody [52]. In this work, various conditioning of the antibody feed was used in order to evaluate removal of host cell proteins and recovery of product. In these experiments 96-well plates were used for precipitation studies with corresponding filter plates for removing precipitated protein. Utilizing the clarified culture supernatant containing an IgG4 mAb a high-throughput PEG-based precipitation screening system was developed [53]. Using this system, the clarified harvest conditions, including product concentration and pH, as well as optimal PEG molecular weight and concentration leading to the best IgG4 purity and recovery could be readily determined.

Finally, flocculation of CHO produced clarified harvests using polyethylenimine (PEI) or other polymers, such as benzylated poly(allylamine) or SmartPolymer (SmP) can be utilized to reduce host impurities at the clarification step and be combined with filtration to improve subsequent downstream purification processes [56]. As polymer concentration and feed conditions need to be optimized in order to optimize residual clearance and product recovery, high-throughput screening techniques can be brought to bear on the early stages of flocculation clarification development.

Aqueous two-phase systems

Aqueous two-phase processing (ATPS) for protein purification consists of utilizing mixtures of polymer-salt (e.g. phosphate and polyethylene glycol) or polymer-polymer (e.g. dextran and polyethylene glycol) such that the monoclonal antibody product of interest will partition preferentially into one of the aqueous phases, resulting in some degree of purification (reviewed in Ref. [57]). Newer generations of compounds designed for this application, such as self-buffering ionic liquids, hold promise for wider applicability of this method for antibody purification in the future [58].

Using an automated liquid handling system, a high-throughput screening method was developed for ATPS analysis of PEG-salt mixtures as applied to two monoclonal antibodies [62]. Miniaturization of ATPS screening conditions results in reduced requirements for material and the potential for the application of a DoE-based approach to screening as well as appropriate sample replicates for statistical analysis of the data.

Summary and conclusions

An effort has been made here to provide a brief synopsis of the current state of parallelized, automated and high-throughput approaches to antibody purification. Balancing the needs for platform and process development purification applications, as well as appreciating the competition between sample throughput and purified product quantity, does present challenges (Fig. 9.1). While there are no universal solutions, there is often more than one acceptable technical solution at a given purification scale, depending on the specific needs of the user and the equipment available (Table 9.1).

While many off-the-shelf options exist when small quantities of purified protein are required, fewer commercial instruments capable of parallelized purification are available for mid-scale platform purification. More commercial equipment options for mid-scale, parallelized protein purification are required in order to advance the technological capabilities in this area. This deficiency has been tackled by downstream and automation scientists in biotech and biopharma to come up with hybrid solutions using commercial FPLC equipment combined with various components to solve this problem. Integration of FPLCs with auto samplers and other pumping equipment will provide more options for automated, sequential purification at sub-gram purification scale. We can expect to see greater adoption of membrane and monolith chromatography in platform mode, given the advantage of greater mass transfer at higher flow rates and the new products released by vendors. On the process development side, unit operations involving non-chromatographic based separations offer the potential of lower cost, scalable downstream processes that can be integrated with chromatography in a holistic manner. As always, protein purification does not exist in isolation,

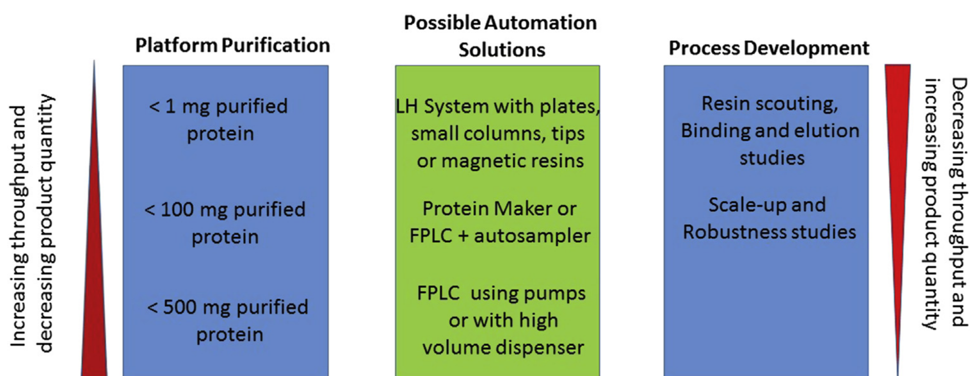

FIG. 9.1 Potential automation solutions for protein purification in both platform and process development modes.

TABLE 9.1 Automation and parallelized antibody purification examples at various scales.

Equipment	Scale	Max Samples/day	Application	Comments
LH + 96 well resin filled plates	μg to low mg	Several hundred	Platform & PD	Vacuum, centrifugation or positive pressure
LH + Robo columns	μg to low mg	96+	Mainly for PD	Selected LH systems. Sample throughput depends on column bed volume and sample volume(s) applied
LH + magnetic resins	μg to low mg	Several hundred	Mainly platform	Difficult to scale due to cost
LH + resin filled tips	μg to low mg	Several hundred	Platform & PD	
Protein Maker	<100 mg	12 to 24 or 48 depending on clarified harvest volume and method used	Platform & PD	Larger supernatant volumes can be divided over additional 5 mL columns. Max number of samples is 12 for two-step purification methods
Batch using columns	<100 mg	~10 per person	Platform	Manual unless peristaltic pump is used. Minimal control of liquid flow rate without a pump
ÄKTAxpress	<100 mg	~4 (2-step method)	Platform	Instrument discontinued by GE, legacy platform
FPLC with sample pump	Low mg to < 1 g	Depends on clarified harvest volume & productivity	Platform & PD	Variable number of samples possible depending on number of sample inlets. In-line air sensor desirable
FPLC with autosampler	<100 mg	Depends on clarified harvest volume, productivity and auto sampler	Platform & PD	Alternative to sample pump for small volume samples, depending on specific auto sampler
Customized FPLC	<500 mg	Depends on clarified harvest volume, productivity and equipment specifics	Mainly platform	Case-by-case setups depending on specific needs and availability of equipment and components

and benefits the most when automation and parallelization are integrated on both the upstream and analytical sides in combination with robust data management and sample tracking capabilities.

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