Parallelized Protein Purification: Opportunities and Challenges in Early-Stage Biotherapeutics Research & Development

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Abstract

Purification of candidate proteins from a large number of biopharmaceutical samples is a key activity in early-stage discovery programs. A number of automation tools permitting parallelized protein purification, both custom and commercial, that can be applied at a variety of scales, have been developed to meet this challenge. Here we provide a brief overview of state-of-the-art small- and medium-scale parallelized purification approaches that are currently available for various antibody formats as well as other classes of biotherapeutics. Current strengths and limitations in the context of both platform protein purification and process development are discussed.

Introduction

In early-stage biopharmaceutical discovery and development projects, large numbers of constructs are generated from *in silico* or *in vitro* screening programs and must be experimentally evaluated and ranked for their suitability as potential drugs. An understanding of *in vitro* and *in vivo* potency as well as physical and chemical stability is usually required to help rank-order constructs resulting from these screening efforts. Selected lead molecules are produced in mammalian cells (CHO and HEK293) or prokaryotic expression systems (*E. coli* and yeast) and subsequently purified. A variety of analytical assays, cell-based experiments, biophysical characterization tools and early-stage formulation studies are utilized to evaluate these molecules and determine their suitability as drugs as part of nominating a lead molecule.[1,2]

The quantity of soluble protein available for purification often varies and is sometimes limiting for the assay panel planned for the group of constructs under study. Therefore, effective purification capture steps are crucial to maximize recovery. For the various cell-based and *in vivo* studies performed on selected lead molecules, purified products are required having defined levels of endotoxins (pyrogens) and aggregates, and need to be formulated in buffers with excipients at specific protein concentrations. A number of high-throughput methods are now available to perform biophysical and formulation studies[3] requiring streamlined procedures to both buffer exchange

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and to concentrate protein samples in parallel. Once a lead molecule has been identified and a suitable stable cell clone selected, the purification focus shifts from screening to downstream process development. For both platform purification and process development activities, the large number of samples to be purified requires some level of parallelization in order to meet expected product development timelines. A summary of the possible experimental approaches for parallelized protein purification is presented in Table 1, as well as the relationship between sample purification throughput and product quantity using the various methods (Figure 1).

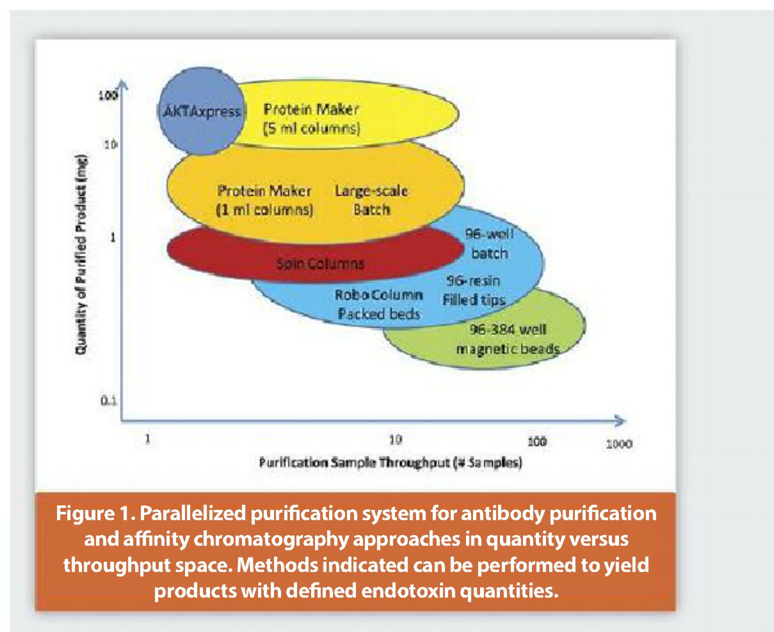

Figure 1. Parallelized purification system for antibody purification and affinity chromatography approaches in quantity versus throughput space. Methods indicated can be performed to yield products with defined endotoxin quantities.

Small Scale Parallelized Purification Approaches

In the early stages of biotherapeutics discovery, design of experiments (DoE) is often applied to identify the optimal protein purification conditions. Depending on the number of factors and responses evaluated, the number of individual experiments can increase exponentially and become difficult to process manually. In addition, while attempting to optimize both target affinity and biophysical properties of proteins, the resulting screening campaigns will generate many samples requiring parallel purification. In general, these early-stage activities require small quantities (less than 1 mg) of purified product to qualify and characterize each protein. Three kinds of purification approaches can be applied: (i) plate based methods, (ii) membrane supports derivatized with ligands and (iii) resin filled pipette tips.

Plate based purification methods

To overcome the challenges associated with small scale parallelized purification, several liquid handler vendors offer high-throughput purification solutions with associated accessories and consumables.[4,5] Vacuum manifolds, positive pressure units and centrifuges, are alternative approaches to achieving an optimal protein purification strategy. All three accessories are compatible with 96 well plates and offer different advantages. Centrifugation is probably the most common approach when performed manually or in a semi-automated fashion,[6] but it is costly to purchase and integrate within a liquid handler. One limitation of the centrifugation approach is the inability to control the flow rate for optimal product binding and elution. In comparison, small scale batch purification (mixing resin with samples or resin filled plates) using vacuum separation can provide comparable product purity to centrifugation-based approaches.[7] Batch purification using vacuum filtration has been applied to the purification of a library of mAbs and bispecifc IgG antibodies.[8,9] Vacuum filtration is affordable and easy to implement on an automated liquid handler. However, due to sample-to-sample variations in viscosity, uneven flow rates can occur across a plate, resulting in differences in protein recovery.

Table 1. Options for Parallelized Protein Purification at Various Scales

Scale	Method(s)	Format(s)	Number of Samples	Affinity Resin Chemistries[1]	Non-affinity Resin Chemistries[1]	Manual Operation	Automated Operation	Reference(s)
µg to low mg	Resin filled pipette tips	5-320 µl resin	Up to 96	Any	Yes	Yes	Yes	5,19-21,42,43
	Membrane plate, modules	SBS plate, modules	96 per plate	Protein A, IMAC	Yes	Yes	Yes	13-17, 54
	Small-scale batch	SBS deep blocks 15 ml or 50 ml tubes	24, 48, or 96 per plate Up to 48 in tubes	Protein A, G	Yes	Yes	Yes	4-10, 12, 18,10, 35-41, 49, 51, 52
	Magnetic beads	SBS plate + magnet	96-384 per plate	Protein A, Protein G, IMAC	No	Yes	Yes	12
	Spin columns	Spin columns	Limited by rotor capacity	Protein A, G, IMAC	No	Yes	With automated centrifuge	
	Robo-Column	Packed beds	Up to 96	Any	Yes	No	Yes	5, 7, 18, 19, 44-52
	Protein Maker	Packed beds	Up to 24	Any	Yes	No	Yes	23,29
multi-mg	Batch	Bottles or flasks	Limited by shaker capacity	Any	No	Yes	No	
	Protein Maker	Packed beds	Up to 24	Any	Yes	No	Yes	23,29
	AKTA Express	Packed beds	Four per unit	Protein A, G, IMAC	Yes	Yes	Yes	24-28

[1]May require custom column packing

To overcome limitations with vacuum, approaches using positive air pressure have been developed for protein purification. Two studies[10,11] describe liquid handler pipetting needles or probes with a cap that can be used to seal the chamber, with application of positive pressure for controlling flow rates. Using this equipment, they have achieved purification of microgram to milligram quantities of protein from various culture volumes. Recently, high-throughput plate based accessories using positive air pressure have become available for various liquid handlers. These units are designed to provide constant and independent air flow to each well of a plate, permitting optimal sample recovery in the event of a clogged well. Both vacuum and positive pressure units, while offering limited control of the flow rate, are readily amenable to high-throughput purification and offer the flexibility of working with any available purification resin.

The use of ligand-immobilized magnetic beads is an alternative strategy, amenable to high-throughput plate based purification and does not require the purchase of costly accessories.[12] Many vendors offer different magnets and a variety of magnetic beads derivatized with a number of small molecules, peptides or proteins for affinity purification. In a screening campaign, it is possible to achieve small-scale automated purification in 96 or 384 well plates, allowing for the recovery of microgram quantities of antibodies for characterization.[12] A drawback of this approach is the cost associated with magnetic beads, which are often intended for single-use applications. Some commercial purification systems are designed to be used specifically with magnetic beads but these systems tend to have limited flexibility as they are not open access and will restrict the user to specific purification kits, type of labware, sample volume or a specific method.

Membrane support based purification strategies

For some time, there has been interest in applying membrane based supports for antibody purification as an alternative to chromatographic resin beads,[13] although it is only recently that commercial products have appeared to permit this in a high-throughput mode.[14] The availability of 96 well plates and spin columns impregnated with Protein A (Takara/CloneTech) within a membrane support permits the purification of multiple samples in parallel. This approach offers clear advantages for high-throughput purification strategies, as the plates are fully compatible with liquid handler platforms and feeds can be applied at high flow rates, as dynamic binding capacity is in principle not limited by volumetric flow rates.[15] In addition, these membrane supports do not present any of the inconveniences associated with, for example, manipulating resin beads and using magnets.

Some of the limitations of packed bed chromatography (pressure, sample diffusion and dilution) can be overcome by using synthetic microporous or macroporous membranes as a chromatography matrix.[16] While this approach can be adapted for many classes of

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chromatographic separations as applied to biotherapeutics, it is mainly utilized with affinity and ion exchange purification methods. Addition of standard functional groups, including diethylaminoethyl (DEAE-), sulfopropyl (S-) and quaternary ammonium (Q-), among other functionalities, permits molecular interactions with the surface of the membrane.[14] Some multimodal membranes (MMM) permit both capture and polishing in a single operation, removing endotoxins and other contaminants. The advantage of this technology is that it allows for fast purification, avoids shifts in pH by utilizing chaotropic salts to effect elution, and minimizes loss and dilution of the product.[17] These purification approaches are good candidates for DoE-driven scouting and screening.

Pipette tip based purification methods

Resin filled tips for chromatography are commercially available from various suppliers, consisting of 1ml resin-packed pipet tips or liquid handler 96 well format microtips containing resin (typically 5-320 µL) having a variety of chemistries and allowing for fast, parallelized purification. The mode of purification can be viewed as either a batch binding technique[18] or as a packed bed method,[19] with the sample being aspirated and dispensed in repeated cycles. The main advantages of this approach are its amenability to automation and the ability to elute the purified product in a very small volume (microliter), resulting in a higher protein concentration. This concentrated purified protein is ready-to-use in various analytical assays, thereby eliminating the tedious process of concentration/ultrafiltration of multiple samples.[20] Potential limitations include entrapment of small amounts of air in the resin, non-specific interactions with some proteins, as well as the typical single-use nature of these products. In spite of this, some protein production facilities have implemented this technology as part of their core purification platform.

Approaches to high-throughput buffer exchange and sample concentration

Buffer exchange (or desalting) is generally performed by either size exclusion chromatography (SEC), or by group separation using Sephadex G25 or other media. In group separation, proteins elute earlier than salts and other buffer components or small contaminant molecules. For high-throughput buffer exchange, a number of 96 well plate products containing appropriate resins are commercially available and can be utilized using either multi-channel pipets or with a liquid handler for sample volumes of 20-130 µl. In most cases, sample recovery is achieved by either gravity or centrifugation, although resin filled tips are also available for use on liquid handlers. For larger volumes, it is possible to adapt gravity desalting columns (3-10 ml bed volume) by positioning these columns in custom liquid handler racks and applying samples with a liquid handler equipped with independent channels.[22,23] Centrifugal 96 well plates with low protein binding ultrafiltration membranes having a molecular weight cut-off of 3 kDa, 10 kDa or 30 kDa are also commercially available. These plates can be used as concentration devices for many protein samples with volumes ranging from 20 to ~500 µL, with larger volumes processed using multiple sample application.

Medium Scale Parallelized Purification Approaches

Along the product development pipeline, the initial large number of candidate molecules is reduced to a smaller number of lead molecules. Early-stage *in vivo* efficacy and toxicology, product characterization studies as well as formulation development of these molecules often requires tens to hundreds of milligrams of purified products. This requirement for larger quantities of purified products in general requires different protein purification approaches than performed for small-scale screening. The available parallelized protein purification strategies at this scale consist of (i) batch methods, (ii) commercially available equipment or (iii) modification of existing equipment to generate a customized solution.

Batch purification

Protein purification in batch mode using bulk chromatography resins is a convenient and inexpensive approach for medium scale parallelized affinity purification. Mass transport between the liquid phase and the stationary (resin) phase is comparable between batch and packed bed chromatography, although this process occurs continuously in columns and discontinuously in batch purification. The number of samples that can be purified in this manner is mainly dependent on production volumes and the available shaker space to facilitate batch binding. Comparable purity and recovery to packed bed purification, as well as low endotoxin requirements, can be readily achieved using this method. The main practical advantage of this method is that it can be easily set up with basic laboratory equipment. However, batch purification becomes a time consuming and laborious method when performed continually for large numbers of productions. Batch purification remains a suitable method, particularly in a research environment.

AKTAxpress

The AKTAxpress™ platform, released in 2003 addresses the challenging issue of how to automate and increase the throughput of medium scale protein purification.[24] This platform is able to address some of the issues with regards to implementing automated and parallelized purification workflows. Up to three groups of instruments, with each group having up to four individual FPLC instruments (up to twelve instruments in total), operated by a single computer, can be utilized simultaneously to purify successive samples via multiple purification steps.[24-27] Each instrument can be utilized for affinity purification of up to four samples successively followed by either buffer exchange or size-exclusion chromatography without user intervention. While this does not represent a true parallel purification system, the increase in sample throughput and the reduction in the number of required trained personnel made this instrument an attractive solution for various users, including a number of structural genomics centers.[25,28]

Protein Maker™

The Protein Maker™, a 24-channel parallel liquid-chromatography workstation has been specifically developed for high-throughput protein purification.[29] Typical chromatography columns with bed volumes of 1 to 5 ml are connected to a column gantry using standard FPLC fittings. The column gantry moves over a 20-position deck, either to dispense liquid to a waste position, or to 24-well plates located on the deck. This system is designed to purify up to 24 samples concurrently, each with an independent flow path, with feed volumes ranging from 10 ml to 1 litre. This number can be increased to 48 samples a day for smaller (~(10 mL) feed volumes.[23] A wide range of chromatographic resins prepacked in 1 ml and 5 ml column formats, identical to those utilized on most FPLC systems, are commercially available.

The instrument is also designed to aspirate from collection plates located on the deck, enabling the creation of automated two-step purification methods for up to 12 samples simultaneously.[29] We have previously described various applications of this instrument for both platform purification as well as purification development of biotherapeutics.[23] Due to the parallelized nature of the purification process, this automated protein purification platform significantly increases the throughput compared to performing equivalent work using a traditional HPLC or FPLC purification system.

Customized solutions

A variety of custom solutions for parallelized protein purification have been developed. For example using a Gilson ASPEC XL4 system, this 4-channel solid phase extraction instrument has been adapted for IMAC purification, yielding purified samples at milligram scale.[11] Up to 60 samples can be purified using this system. A modified 10-channel flash chromatography system is capable of purifying up to 10 samples concurrently by ion-exchange or immobilized metal affinity chromatography (IMAC) as well as buffer exchange.[30] Finally, a modified solid phase extraction system containing loops and switching valves has been successfully utilized to purify up to four samples in parallel, with a capacity for up to 24 samples at 50 mL volume.[31] This system can perform either single step affinity purification, or perform a two-step method involving final sample buffer exchange.

Applications for process development

Quality by design (QbD) has gained industry acceptance as the preferred approach for the development and commercialization of biotherapeutics. QbD involves three primary components: process knowledge that includes a thorough characterization of process inputs and their impact on performance, the relationship between the process and the product's critical quality attributes (CQAs), and the association between CQAs and the products clinical performance.[32,33] However, when working at standard laboratory scale, a systematic exploration of the design space for QbD-based process development requires more extensive experimentation and is therefore more time consuming and requires larger sample quantities. With biotechnology companies operating under ever-increasing pressure towards lowering the cost of manufacturing, High-Throughput Process Development (HTPD) has emerged as a necessary enabler of QbD in a time- and resource-constrained environment.[4] The general methodology used by the industry today combines various HTPD techniques with multivariate statistical analysis such as design of experiments (DoE).[4,34]

High-Throughput Process Development

HTPD is characterized by parallelization, automation (liquid-handling platform) and miniaturization (microscale chromatography formats). These factors allow for reduced experimental time frames, low sample consumption and increased understanding by exploring a large experimental window. For chromatographic applications, there are three high-throughput formats: (i) 96 well plates, (ii) pipette chromatography tips and (iii) miniature packed columns, typically used in an automated manner using a liquid-handling system.

(i) Plate-based purification

Ninety-six well plates have received the majority of focus in high-throughput studies due to simplicity of execution, as well as the small quantity of resin, low sample volume and protein quantity requirements, and the possibility of evaluating up to 96 experimental conditions in parallel allowing for screening a wide range of design spaces. Various mode of chromatography can be adapted to HTS batch binding, including hydrophobic interaction, ion-exchange, mixed-mode, as well as affinity chromatography.[6,35-37] Examples of successful applications of this technique include screens to optimize binding buffers, chromatographic resins and identification of optimal wash or elution buffer conditions.[37-40] Batch binding can also provide information on the thermodynamics of the product–resin interaction, the selectivity between the product and impurities, and binding kinetics.[34-36,41] However, due to the fact that the format and physical arrangement of matrix in the multi-well plate is markedly different from that of a column, quantitative prediction of column performance from HTS batch binding experiments does not always directly translate into column operating conditions due to the lack of resolution of a batch experiment, as well as the absence of dynamic flow.[6,19]

(ii) Pipette-tip based purification

Some studies have explored the use of micro-chromatography tips in the context of HTPD.[5,19] Both cation exchange (CEX) and ceramic hydroxyapatite (CHT) resins have been successfully used in a chromatography tip format with an automated robotic workstation for the development of a purification process for virus-like particles.[42] These tips, operating with a liquid-handler system, have also been used to screen conditions for recovering polyclonal antibodies from ovine sera by mixed-mode cation-exchange chromatography.[43] Both studies[42,43] selected tips over batch binding plates due to the ease of automation, the need of little or no manipulation of plates, as well as the absence of the need for lengthy incubation times required for adsorbent mixing. Resin filled tips have the disadvantage that upon scale-down from regular packed beds to liquid handler-scale, both linear flow velocity and residence time cannot be maintained constant due to the tapered shape of pipette tips. '

A few studies have explored the possibility of applying membrane chromatography in the context of HTPD. A HTPD system using either eight-strip spin ion exchange (IEX) or metal chelate membranes has been developed for evaluation of adsorption capacity, selectivity and for screening binding and elution conditions, using BSA and lysozyme as model proteins.[54] An HTPD platform for membrane chromatography using 96 well IEX membranes plates was successfully implemented for the purification of granulocyte colony stimulating factor (GCSF), yielding results comparable to the traditional laboratory scale.[14]

(iii) Miniature columns

The miniature packed column format is an appropriate high-throughput purification scale-down model for lab scale packed columns, allowing for controlled unidirectional flow, fraction analysis and better separation resolution than other HTPD formats.[44] These columns have been successfully used for optimization of dynamic binding capacity, column wash and elution conditions and impurity clearance,[18] for applying DoE[45] or to optimize non-protein A antibody purification.[46] Moreover, there are multiple examples that demonstrate equivalent product quality and chromatographic behavior for well characterized test proteins using multiple chromatographic media.[18,47-52] By using cation exchange (CEX) miniature columns coupled to a liquid handling system, it has been possible to demonstrate comparability to a larger scale mAb purification platform.[53] A similar conclusion has also reported utilizing a standardized HTPD strategy to optimize the polishing step for a mAb purification.[18] Compared to 96 well batch plates, miniature columns have lower throughput, typically utilized in groups of 8 columns, corresponding to a single column of a SBS-formatted plate. These columns will also consume more sample compared to 96 well plates as resin volume used in plates could be as small as 2 µL while columns smallest bed volume is 50 µL. Even though they are the HTPD format that better simulates purification conditions at larger scales, miniature columns do have limitations as compared to lab scale columns. Concerns reported to impact the predictive capabilities of miniature columns include geometric factors, transport properties according to protein size and resin types, non-linear elution gradients and wall effects.[18,19]

Parallelized purification within the context of downstream or upstream process development requires analytical and characterization assay panels to rank molecules, clones or cell culture processes. A key for making rapid decisions and reducing the required time in the development cycle is to effectively couple process development with parallelized analytics. An example of this is the miniaturized, parallelized purification integrated with high-throughput analytics, including microfluidic capillary electrophoresis and protein quantitation using microfluidic absorbance spectroscopy together, providing a panel of analytical data, including visualization informatics tools.[53] Using small quantities of material and high-throughput data generation and analysis decreases both cost and development cycle time.

The Way Forward

As described in this review, a number of different approaches are available to achieve parallelized protein purification (summarized in Table 1 and Figure 1). There continues to be a shortage of viable commercial equipment to accomplish this task, particularly where multi-milligram quantities of protein are required. While a more fully automated, integrated platform, such as the Protein Expression and Purification Platform (PEPP[55]) is attractive, these solutions require customization on a case-by-case basis, and with the associated costs, scale and complexity are oftentimes outside of the capabilities of many organizations. New chromatographic formats for packed resins continue to be developed, offering improvements in separation resolution, high elution throughput and lower cost[56,57,58] , as well as new varieties of purification membranes.[59,60] Both technologies will ultimately benefit from a means to apply multiple samples in parallel for preparative antibody, antibody fragment and conjugates purification. Beyond chromatographic resins, other protein purification techniques applied to biopharmaceuticals can potentially be parallelized, including aqueous two phase extraction methods.[61] An effort to automate this purification approach has already been performed using a liquid handler.[62] Preparative crystallization as a protein purification technique should be readily amenable for parallelization, as is the case with the preparation of protein crystals for X-ray diffraction studies. This purification approach has been successfully applied to both a Fab[63] as well as a full-length IgG.[64] The use of flocculating polymers that can reduce levels of high-molecular weight species, CHO host-cell proteins and DNA as well as endotoxin can be utilized in a parallelized format.[65] These are all exciting areas for further research and represent further opportunities to enhance the possibilities for applying parallelized protein purification approaches.

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