Perspective

Anything but Conventional Chromatography Approaches in Bioseparation

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Abstract

While packed bed chromatography, known as conventional chromatography, has been serving the biopharmaceutical industry for decades as the bioseparation method of choice, alternative approaches are likely to take an increasing leading role in the next few years. The high number of new biological drugs under development, and the need to make biopharmaceuticals widely accessible, has been driving the academia and industry in the quest of anything but conventional chromatography approaches. In this perspective paper, these alternative approaches are discussed in view of current and future challenges in the downstream processing field.

1. Introduction

Conventional packed-bed chromatography using bead-based stationary phases is ubiquitous at both analytical and preparative scales. It has been the workhorse in the purification of recombinant and engineered proteins such as monoclonal antibodies (mAbs), forming the basis of downstream processing trains in the biopharmaceutical industry [1-3]. Packed-bed chromatography is normally associated with high separation efficiencies promoting the recovery of biomolecules with high yield and purity. Despite its indisputable benefits in providing biological drugs for the society, conventional chromatography is far from perfect. In fact, it is a time-consuming batch process, often difficult to scale-up especially at large scales. In addition, chromatographic adsorbents are highly priced (e.g. protein A resins cost $5,000 – 15,000/L) and are associated with high water consumption during operation (e.g. 1500 to 3500 L per kg mAb produced assuming an initial mAb titres of 2 to 5 g/L), contributing to the high cost and environmental footprint of the downstream processing stage [4-6].

As we continue to improve our understanding of the complexity, diversity and broad base of human diseases, the nature of biopharmaceutical drug candidates being developed for commercial applications expands. In response to the significant improvements recently seen in upstream productivity (titre), a diverse range of downstream technologies, alternative to conventional chromatography, are either under development or already implemented in industrial settings to address the emerging bottleneck [7]. Originally developed by NASA [8], the Technology Readiness Level index (TRL: 1-9) can be used to assess and track the development

of innovative technologies from concept to industrial implementation. This concept is adapted to the technologies explored in this review correlated against their resolution potential relative to conventional chromatography (Figure 1).

The key drivers for successful implementation of innovative technologies in an industrial setting focus around technologies that offer: **1)** a disruptive approach to cell or protein purification which presents a distinct technical advantage to biopharmaceutical process development and/or production over conventional unit operations; **2)** greater throughput and process intensification to ultimate reduce the cost of biodrug production; **3)** the appropriate regulatory approval to ensure patient safety.

Many alternatives to conventional chromatography offer greater productivity and production facility throughput, thus reducing the overall cost of goods of a medicine, namely monoliths and membrane-based bioseparations [9]. Industry consensus predicts integrated and/or continuous process technologies (TRL: 8) will be the next step-change in biomanufacturing strategy [10]. The opportunity to intensify the chromatography resin utilisation (through high cycling or counter-current chromatography), combined with a reduction in the overall facility footprint, hold great potential to drive cost of goods down. Technologies such as membrane chromatography and monoliths (TRL: 9) are already applied in commercial drug production and are also well suited to adhere to this high intensity (and continuous) vision of biomanufacturing in the future.

In addition, there are a number of novel technologies that offer technical or process economic advantages over conventional chromatography. Protein crystallization (TRL: 9) has long been applied to many industrial biomanufacturing processes in

batch-mode as a cost-effective method to achieve high purity drug product [11], often applied to recombinant insulin production [12] and many other oral dose-based therapies [13]. Continuous crystallization (TRL: 3) may offer a further evolution of this technique [14]. Recent developments in automated High throughput Process Development (HTPD) equipment, combined with Design of Experiments (DoE) approaches to process development, enabled the wider application of Aqueous Two- and Three-Phase Extraction (TRL: 9) as a scalable and cost-effective approach to biomanufacturing of products such as interleukin, human growth hormone, insulin-like growth factor and monoclonal antibodies at an industrial scale [15].

2. Non-conventional chromatography with alternative adsorbents

The physical phenomena that determine the rate of biomolecules adsorption onto a chromatographic adsorbent include both mass transfer processes and adsorption kinetics. However, with the exception of some affinity adsorbents, mass transfer processes control the overall rate at which biomolecules are adsorbed onto chromatographic stationary phases. The transport of biomolecules through a packed-bed chromatographic column can be divided into 3 parts [16]: i) convective flow through the interparticle space associated with the flow rate imposed; ii) film diffusion, responsible for the transport of biomolecules from the bulk of the solution into the boundary layer surrounding the stationary phase; and iii) pore diffusion that accounts for the transport of biomolecules within the pores, which is considered the rate limiting step. This paradigm is however changing due to the

recent improvements in stationary phases. Modern beads are more rigid and exhibit high dynamic binding capacities even for very short residence times.

Chromatography on convective flow devices such as monoliths, nanofibers and membrane adsorbers has been emerging as a rapid and feasible alternative to conventional chromatography on bead-based supports, especially for large biomolecules such as DNA, virus and virus-like particles [17]. A distinct advantage of convective flow devices lies on the independence of the binding capacity on the flow rate. Monoliths and membranes have large through-pores that enable convective flow. As a consequence, mass transfer is not limited by pore diffusion as in conventional column chromatography. With pore diffusion absent, and considering film diffusion much faster than pore diffusion [16], mass transport limitations are drastically reduced and separation occurs solely based on differences in the binding kinetics of each molecule present, i.e., based on the affinity between the biomolecules and the stationary phase. The presence of large through-pores, decreases drastically the surface area per unit of volume available for binding when compared to a packed-bed column [18]. This decrease in the specific surface area does not affect the binding of very large molecules, such as nucleic acids, virus or even cells. The majority of the binding sites are located in open channel structures, becoming much more accessible to large biomolecules than traditional inner pore surfaces, which may even translate an increase in the dynamic binding capacity, specially at high flow rates. For most proteins, however, this decrease in the specific surface area is an important drawback in terms of binding capacity which can be 4-10 times lower than traditional packed-beds [19]. For monoclonal antibodies, a maximum binding capacity of 7.2 mg/mL has been reported for CIM-

protein A monolith [20], while MabSelect SuRe LX™ and Toyopearl® AF-rProtein A exhibit static binding capacities over 100 mg/mL [21]. This is probably the reason why monoliths and membranes have found their niche in polishing steps operated in flow-through mode when the impurity levels have already been considerably reduced by previous capture steps.

Conventional pressure-driven membrane technology has been extensively used so far as primary purification stage for biomolecules due to the high separation efficiency, low operation cost, and the facile up- and downscaling [22]. Membrane adsorbers, on the other hand, are well implemented at industrial scale, mostly to capture contaminants in the polishing stage [23]. Despite the extensive use of membranes for bioseparations, membrane fouling remains a key issue [24]. Although future improvements in membrane anti-fouling properties [25] are expected to facilitate the applications of membrane technology for bioprocessing even further, the development of single use membranes brings the enhanced extraordinary advantages towards closed processing and usage of lower clean room classes. As a perspective, with the increased product titres that can be achieved using optimized media and cell lines, a number of membrane technologies could become attractive as principal purification steps in alternative to chromatography. These include innovative membrane-assisted crystallization/precipitation [26], both of which can be operated in a continuous mode by using high selective membranes as the product titer increases, working in the logic of process intensification strategy.

Monoliths combine the advantages of uniform flow distribution typical of packed-beds and the convective mass transport efficiency of membrane adsorbers. These advantages translate into consistent capacity and high-resolution separation regardless of flow rate, solute size and mobile phase viscosity [27]. There are however some limitations regarding monoliths scale-up [18]. There are also concerns regarding the reproducibility of the well-defined, inter-connected network of channels within the monolithic structure at large scale [28]. To overcome these limitations, alternative fabrication technologies have been proposed such as additive manufacturing using 3D-printing, where monoliths would be simply printed rather than cast [29]. Another strategy has been the incorporation of different types of nanoparticles – from neutral, to charged, gold and magnetic nanoparticles - within the monolith structure to enhance separation and capacity [30, 31].

Other adsorbents, including magnetic beads, have been considered extremely versatile separation processes due to the possibility to purify cells, viruses, proteins and nucleic acids directly from crude samples, using fast and gentle processes [32-35]. In addition, magnetic adsorbents can be easily integrated with other non-chromatographic approaches, including aqueous two-phase systems, with improved performance in comparison to the isolated processes [36]. Although the implementation of magnetic based purifications in large-scale has not been fully achieved yet, there are several advances confirm the potential of this methodology, namely in GMP validation of processes and magnetic separation equipments [37, 38].

3. Continuous chromatographic processes

The move towards continuous integrated biomanufacturing lead to increasing research in the field of continuous chromatography. Continuous chromatography works as a counter current chromatography, known as simulated moving bed chromatography (SMB), that allows the separation of binary mixtures in a very efficient manner. In biopharma and biotechnology binary mixtures are rarely present, as mainly multicomponent mixtures must be separated.

An option in continuous chromatography is annular chromatography, where the stationary phase is packed into a rotating annulus. The separation mixture and solvents are fed at the top, and the separated compounds are continuously collected at the bottom [39]. Although this is the most elegant solution of continuous chromatography, it suffers from mechanical problems. It is very difficult to seal the rotating parts over long periods of time and therefore the manufacture of such systems has ceased several years ago [40]. Currently, and most probably in the future, counter current loading will be the most popular (pseudo)-continuous chromatographic method for capture of proteins and other biopharmaceuticals from a clarified culture supernatant. Counter current loading, and respective skids, are offered by many companies and different trade names such as capture SMB or BioSMB [41, 42]. For example, the polishing methods in an integrated continuous process are preferably made in the so-called flow through mode, where the product is in the flowthrough and the impurities are bound [43]. This leads to high productivity and reduction of column size and buffer consumption.

Apart from packed chromatography, membrane separations also show great potential in continuous processes. The easy integration of membrane separation stages in large-scale continuous production systems will provide economic feasibility and less or no environmental issues. In addition to cost, continuous membrane processing has also the potential to provide smaller overall facility footprints, more flexible production, and significant improvements in product quality through enhanced control and uniformity of the microenvironment within the manufacturing process provided by advanced, high-selective, membrane separations.

With the state of art, continuous chromatography systems and stationary phase materials for continuous integrated biomanufacturing systems can be readily established. Still, several challenges remain. It is necessary to work in a functional closed system to avoid microbial contamination, a problem that is not fully solved yet [44]. In addition, packing density will change over time and therefore the switch time must be adapted. This requires a control system and appropriate monitoring with real sensors or so-called soft sensor using a statistical model that can be trained by several runs [45]. A challenge will be also the development of the control space, virus clearance studies and the development of continuous processes at small scale, for which equipment and development concepts are not yet available.

4. Aqueous two-phase systems

Aqueous two-phase systems (ATPSs) are a type of liquid-liquid separation based on the formation of two immiscible water-rich phases, which are formed above certain critical concentrations of two mutually incompatible solutes. The most explored

systems are the polymer-salt and polymer-polymer, together with alcohol-salt, ionic-liquid based systems, either as phase forming components or adjuvants, and responsive-polymer systems [46].

The concept of using ATPSs to separate and purify biological compounds from fermentation broths has been under investigation for three decades since it was introduced by Albertsson [47]. Several types of biological molecules and particles, including small organic compounds such as metabolites and antibiotics [48, 49], different size proteins, plasmids [50], large macromolecular complexes such as virus-like particles [51] and also animal cells [52] have been partitioned in ATPSs.

ATPS provides a simple yet integrated approach for feed clarification, biomolecule concentration and partial purification. The purification of monoclonal antibodies from CHO and hybridoma cell supernatants, is an example, where >70% host-cell proteins removal and >75% HPLC purities were achieved with yields above 95% in a single step [53]. ATPS based separations also show some promise for industrial scale in continuous purification processes [54-56], although the requirement of large amounts of expensive polymers being one of the major impediments to the use of ATPSs in industrial applications. A multi-stage separation at lower salt concentrations allowed a high yield and purity of IgG using 33% less salt [57].

Partitioning of biomolecules in ATPSs can be shifted between the top or bottom phase depending on the type of system chosen, polymer concentration and molecular weight, ionic strength and pH. These large number of variables also serves to highlight the challenge of empirically optimizing a given ATPS for a particular target, thus justifying a demand for good modelling and high-throughput screening approaches [58].

ATPS-based partition can be considered an alternative to the chromatographic, filtration or other solid phase-based dominating modern bioprocesses. Such alternative is of renewed interest in regard to cost effectiveness for large scale bioprocessing. Traditional strengths of liquid-liquid systems in terms of ease of scaling, and ability to handle complex feeds at high target titres, have become much more valuable as bioprocessing moves to larger and denser, fermentation feeds.

5. Crystallization

Crystallization has been used for the purification of almost all small-molecule pharmaceuticals and in a wide range of conventional chemical industries. With increasing titre concentrations, crystallization becomes a viable option for the recovery of biopharmaceuticals, as already shown for insulin [13], enabling potentially cost-effective separation and efficient manufacturing over conventional downstream separation approaches. Additionally, crystalline proteins offer the advantage in products of higher purity and stability which can benefit formulation, storage, and drug delivery options [59].

One of the key challenges of this process is that proteins are notoriously difficult to crystallize. The increase in flexibility and number of groups that are able to hydrogen-bond impact on the ability to nucleate into a condensed crystalline form. It is well known that nucleation is a conception stage and governs the entire crystallization process. Major efforts to enhance the protein crystallization ranging from the use of electric field [60], magnetic fields [61, 62], ultrasound [63], microgravity [64], surfaces [65], porous substrates [66, 67] tailored membranes [68] to soft templates such as DNA origami [69] have been undertaken - to facilitate

their nucleation, primarily for structural determination.

For crystallization as a purification process for biopharmaceuticals, the separation of the target protein has to be achieved often from a complex multicomponent multiphase suspension. Additionally, process scale-up from the 10's nL to 100's mL and larger is necessary. These objectives may differ significantly to those for structural determination. Over the last decade, much progress has been achieved to demonstrate the feasibility of crystallization. Batch crystallization of proteins at scales of 100's mL has been achieved for simple model proteins including fragments [70] and full-length antibodies [20], and up to 1 mL for a therapeutic monoclonal antibody (anti-CD20) [71]. Selective crystallization from protein mixtures has been demonstrated with the use of well-defined narrow pore size distributions nanotemplates [72]. This selectivity is on the basis of a relationship between the nucleant and solute surface properties such as dimension and surface property [73], in good agreement with modelling [74, 75].

Current efforts in protein crystallization process development have focused on a range of continuous flow crystallization platforms including circulatory and oscillatory flows [76, 77], tubular plug flow [78], and oscillatory flow reactors (OFR) (eg meso-OFR) [79]. Progress has been made in understanding the influence of frequency and amplitude of oscillation [80], flow and mixing [81] to the development of a workflow for continuous crystallization [14]. The scientific community has been active in developing various control strategies ranging from electric field assisted crystallization [82], seeding [83], real-time monitoring, including the use of process modeling tools [84].

Despite much progress, barriers to industrial adoption of continuous crystallization

remain. Digital design approaches based on molecular modelling for protein variability, process modelling for design and control can act as the catalyst and provide the impetus to accelerate the technology readiness level of crystallization. Additionally, crystallization may also open up new purification routes for more complex and next generation product therapeutics.

5. High Throughput Process Development

A biopharmaceutical production process involves many processing steps that all have to be sized and operated correctly to achieve to a product that can meet strict criteria as put forward by regulatory agencies. Designing such a process is finding an optimum in a multi-dimensional parameters space. Traditionally, such processes were developed with a combination of heuristics and lab scale experimentation, leading to long development times, considerable use of material, and ultimately suboptimal processes [85].

Nowadays in industry, lab scale experimentation has been replaced by High Throughput Screening (HTS), or High Throughput Experimentation (HTE) approaches using robotics, reducing consumables and development time considerably [86, 87] (Figure 2). Miniaturized experimentation for screening and property determination can be done using these robotic systems, for chromatographic resin selection, isotherm determination, protein solubility determination [88, 89], extraction [90], among others. The use of HTS/HTE for Process Development (PD) is coined High Throughput Process Development (HTPD) and has evolved to include both upstream cell culture, downstream capture

and purification and product formulation. Nowadays all large biopharmaceutical companies use HTS in their PD approaches.

The evolution of HTPD includes, alongside miniaturization and automation in experimentation, the emergence of *mathematical modeling and process simulation* [91]. Further miniaturization attempts towards 384 wells [92] as well as *chip based* isotherm determination to be used in a first principles mechanistic model based process design approach [93, 94] were undertaken. The benefits are obvious: less sample volume is used which is critical at the early stages of process development where material is scarce, and normally used for toxicological studies and clinical trials. However, using smaller volumes brings issues with evaporation of sample volume and mixing. At such small scale, diffusive mixing takes over from vigorous turbulent mixing, which might complicate the experimental work and data interpretation. Miniaturization efforts resulted also in chromatography on a chip [95-97] and Aqueous Two Phase Systems on chip [51, 98, 99] for screening purposes. Examples where model based hybrid HTPD is applied can now be found in literature, and key examples included HTPD for typical downstream bio separation operations [100] where the next level includes the use of neural networks trained by mechanics models to design biopharmaceutical purification sequences [101].

In the years to come, with the increase computer power, we will see a further coupling of HTS with modeling, be it mechanistic modeling or machine learning [101]. Standardization of chip based PD is foreseen as well as the use of Big Data and the emergence of property databases to be used for *In-silico* PD. Considering the large and rapidly expanding interest in developing portable analytical tools for

several fields of application, ranging from environmental to medical analysis, and the emerging features of ATPS within the analytical field, microfluidic applications using ATPS are expected to be a trending field of research in the years to come [58]. The microbead-based microfluidic platform provides the possibility of a high degree of integration and scalability, in an automated and simple manner, with potential for the development in miniaturized biosensing and biotechnology assays, as well as in the optimization of separation conditions for biopharmaceuticals and other biomolecules. As bead-based microfluidic bioassays and processing become more established, modeling and simulation of the system will be required [102].

6. Conclusions

The need for life-saving biopharmaceuticals, easy accessible to all, will continue to grow. There is a clear need to develop purification processes with higher productivity and versatility, in order to embrace the diversity of biological products under development and at clinical stages. Non-conventional chromatography adsorbents will be more prominent in the purification of biological products, including viruses, virus-like particles and cells, which are larger, more complex and labile than proteins. The commercial-scale manufacture of high value biological drug products is currently performed using batch processes. Although batch operations facilitate the design and optimization of the single unit operations and easily allows off-line measurements of key product quality attributes between processing steps, the adoption of continuous bioprocessing is expected to alleviate the several economic [103] and regulatory challenges that are being currently faced by biopharmaceutical industry [104].

In a highly regulated industry with scarce resources for bioprocess development in many companies, significant barriers must be overcome to implement radical new technologies in bioseparation [105]. All new and existing technologies applied to the licensing and sale of biomanufacturing products must conform to strict regulatory guidelines recommended by agencies such as the Food and Drug Administration (US) and the European Medicines Agency (Europe). These guidelines are referred to as current Good Manufacturing Practice (cGMP) and ensure patient safety, while also encompassing fundamental environmental and sustainability considerations. A key area of focus for new technology components or consumables are leachable and extractable profiles that may contaminate the drug substance. The selection of appropriate materials of construction, and associated technology vendor assurance, are pre-requisites for successful technology implementation in an industrial setting. Patient safety should be considered early in the new product design process to assure rapid progress through the Technology Readiness Levels and industry acceptance.

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Conflict of interest

The authors declare no financial or commercial conflict of interest

Figure legends

Figure 1. Positioning anything-but conventional chromatography approaches in Bioseparation with respect to the Technology Readiness Level (TRL). TRL 1 – basic principles observed; TRL 2 – technology concept formulated; TRL 3 – experimental proof of concept; TRL 4 – technology validated in lab; TRL 5 – technology validated in relevant environment (industrially relevant environment in the case of key enabling technologies); TRL 6 – technology demonstrated in relevant environment (industrially relevant environment in the case of key enabling technologies); TRL 7 – system prototype demonstration in operational environment; TRL 8 – system complete and qualified; TRL 9 – actual system proven in operational environment.

Figure 2. Relationship between sample volume and information density in the context of High Throughput Process Development.

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