Review

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Emerging technologies for the integration and intensification of downstream bioprocesses

Downstream processing is currently the major bottleneck for bioproduct generation. In contrast to the advances in fermentation processes, the tools used for downstream processes have struggled to keep pace in the last 20 years. Purification bottlenecks are quite serious, as these processes can account for up to 80% of the total production cost. Coupled with the emergence of new classes of bioproducts, for example, virus-like particles or plasmidic DNA, this has created a great need for superior alternatives. In this review, improved downstream technologies, including aqueous two-phase systems, expanded bed adsorption chromatography, convective flow systems, and fibre-based adsorbent systems, have been discussed. These adaptive methods are more suited to the burgeoning downstream processing needs of the future, enabling the cost-efficient production of new classes biomaterials with a high degree of purity, and thereby hold the promise to become indispensable tools in the pharmaceutical and food industries.

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The downstream processing (DSP) of biopharmaceuticals is generally more cost-intensive than corresponding upstream (fermentation) processes in the biotech industry and can account for up to 80% of production costs. Moreover, with significant advances in fermentation technologies, which have resulted in titers of over 20 g/l [1], related developments in downstream bioprocessing have failed to keep up in terms of scale and product recovery. An additional challenge that modern downstream practices need to address is the targeting of new classes of bioparticles, such as monoclonal antibodies (mAbs), pDNA, virus-like particles (VLPs) or nanoplexes, as well as whole cells (e.g., stem cells). These different bioproducts and expression systems require a toolbox of robust and scalable techniques to satisfy desired purity and yield in a sustainable and cost-effective manner.

Linear scale-up of traditional DSP methods of harvest clarification, concentration, and primary recovery have reached their limits and are unable to meet current product demand, not only in terms of space requirements, but also in terms of cost, resource consumption and process time. Consequently, technologies that offer process integration, that is, a reduction in the number of unit operations in the initial isolation and purification steps while maintaining high product recovery and purity, would not only increase process economy but also reduce process time. However, chromatographic separations, which require extensive feedstock pre-treatment, have been among the most essential operations in the downstream processing of biopharmaceutical products. Additionally, several chromatographic steps are needed to fulfil the high demands regarding product purity, consistency, and safety [2,3]. Chromatography is a relatively slow process and requires expensive separation media and large volumes of aqueous mobile phases. Therefore, faster production cycles, reduced consumable needs, and/or a reduction of chromato-

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Key Terms

Monoclonal antibodies: Class of biological therapeutics based on antibodies of the exact same structure and properties that have risen to prominence in the last decade for targeted treatment of various diseases.

pDNA: Circular DNA used for gene therapy and vaccine formulations; A novel class of emerging therapeutic bioproducts.

Virus-like particle: Novel class of very large molecule used for vaccination purposes, bearing structural similarity to actual viruses, without the infectious payload, which are difficult to process due to their large size.

Aqueous two-phase separation: Process of selective product isolation from complex mixtures by employing two immiscible aqueous phases.

Expanded bed adsorption chromatography: Chromatographic method employing a fluidized bed for product capture and concentration from complex feedstock in a single step.

Monolith: Macroporous single-block chromatographic material enabling mass transfer solely by convection, which facilitate high flow rates leading to faster process times.

graphic steps within established downstream processes would result in a significant decrease of overall production cost [4,5]. The concept of process intensification refers to any process adaptation or optimization that results in a less resource-intensive biomanufacturing scheme, including, but not limited to, lower water consumption, energy demand and environmental burden.

This review will primarily focus on multifaceted approaches addressing process integration and intensification based on the utilization of non-traditional chromatographic or extractive methods, which include advances in bioprocess material development, hardware design and advantageous modes of operation.

While adsorptive methods, such as chromatography, are widely used in industry as a key purification technology, liquid–liquid extraction technologies, such as aqueous two-phase separation (ATPS) systems, are elegant emerging examples of process integration. In this technique, direct extraction of bioproducts from crude feedstocks can be accomplished by the use of two incompatible polymers or of a polymer and a salt in an aqueous environment. It is a powerful technique that combines moderate to high selectivity, high recovery and high biocompatibility.

A complementary solid–liquid extraction technique, which also allows for the direct sequestration of a targeted bioproduct directly from a crude feed, thereby integrating conventional clarification, concentration, and initial purification into a single unit operation, is expanded bed adsorption (EBA) chromatography **(Figure 1)** [6,7]. Converse to traditional packed-bed chromatography, EBA employs a fluidized adsorbent bed, where fluidization is driven by the flow rate of the mobile phase, which is entering the system from the bottom of the column. Larger biological particles, such as whole cells or cell debris, are allowed to flow unhindered through the interstitial voids within the fluidized column, while the product of interest is allowed to selectively bind to the adsorbent. Additionally, as a result of the relatively high flow rates employed compared with packed-beds, process times can also be drastically reduced.

The successful scale-up of convective flow systems, an example of which are the so-called monoliths [8], have also allowed unprecedented reductions in process times as well as increased recovery rates for the purification of extremely large biomolecules, such as pDNA and VLPs [9–11]. These unique chromatographic media have overcome the limitations of conventional porous bead adsorbents, which were initially designed for much smaller molecules. Convective forces, such as flow rate, the ability to control mass transport in these systems, and larger external pore sizes can consequently accommodate increasingly larger biomolecules [12].

Finally, innovative fibrous adsorbent media that offer faster process times and are easily integrated within current infrastructures can also lead to high-performance disposable systems that meet strict health, safety, and quality requirements. For example, cellulose and other natural polymers have been traditionally used in the recovery and purification of proteins; they have been industrially processed into specific shapes, such as spherical adsorptive microparticles [13] and thin ultra/microfiltration membranes, among others [14]. A novel composite fibre technology known as gPore has been introduced, and offers new kinds of adsorptive supports based on natural fibrous materials, which are packed in various open-structure arrangements. More recently, they have been delivered as non-woven structures as well, which have exhibited reproducible packing properties as well as compatibility with non-clarified feedstock.

Another important trend that has generated an increasing amount of interest lately is the transition from traditional batch-based downstream processing regimens to continuous processes [15,16]. In this respect, methods to transform the technologies discussed in this review into continuous processes exist in the form of simulated moving bed-type systems and their variants [17–20]. Alternatively, promising methods, such as continuous countercurrent tangential chromatography have shown exceptional results [21,22]. Despite the considerable inertia of the biopharmaceutical industry to move away from their established methods, the move towards continuous processing could potentially revolutionize the way bioproducts, especially pharmaceuticals, are produced, in a way similar to how continuous operation modes have done in case of other industries, that is, petrochemical, paper, food or glass. The ability of continuously operated processes to produce higher purity goods at competitive prices will make it imperative to consider the possible application of any new downstream processing methods to be utilized in such a fashion [23].

ATPS

ATPS has been recently revisited for the purification of biomolecules, including biopharmaceuticals such as mAb, growth hormones and growth factors with very impressive results, which in some cases surpassed established chromatographic processes [24].

One of the key advantages of ATPS over traditional techniques is the possibility of handling solid-containing feedstock, and consequently, its application in the early stages of the purification, thereby integrating the clarification and capture of the target biological product in a single unit operation (Figure 1). Another important feature of ATPS is its feasibility of operation in continuous mode, which is particularly relevant when continuous bioreactors are used in the upstream process. This permits immediate clarification and subsequent processing of labile biomolecules.

Aqueous two-phase systems are formed spontaneously upon mixing two solutions of different polymers (e.g., PEG, dextran, ethylene oxide propylene oxide co-polymer [EOPO], polyacrylates), or a polymer and a kosmotropic salt (e.g., phosphate, sulphate, citrate), above a certain concentration. This phenomenon was first observed in 1896, but its potential as a technique for the separation of biological products was only recognized around 1956, with the work of Albertsson [25]. Since then, extraction in aqueous two-phase systems has been successfully used for the fractionation and purification of biological samples containing cells, cell organelles, nucleic acids, proteins, and peptides, among others [26–28]. In fact, ATPS constitutes a gentle technique for the separation of biomolecules, since not only the bulk of both phases contain 70–90% of water, but also because most polymers and salts can have a stabilizing effect on protein structure. Table 1 summarizes some of the biological products that have been purified by aqueous two-phase extraction [29–46,201].

Partitioning of biomolecules in ATPS depends on several parameters, both intrinsic and extrinsic. Intrinsic parameters are governed by the properties of the target biomolecule, including size, electrochemical properties, surface hydrophobicity and conformational characteristics. Extrinsic parameters reflect the characteristics of the two-phase system, including type, molecular weight and concentration of phase forming components; ionic strength, pH, temperature and presence of additives such as affinity ligands, among others [26]. If these extrinsic parameters are properly chosen, highly selective windows can be opened for the purification of the target biomolecules.

The application of ATPS at the process scale has, however, been described only for few biomolecules, including insulin-like growth factor I [34] and human growth factor [47]. This fact can be attributed to the following causes:

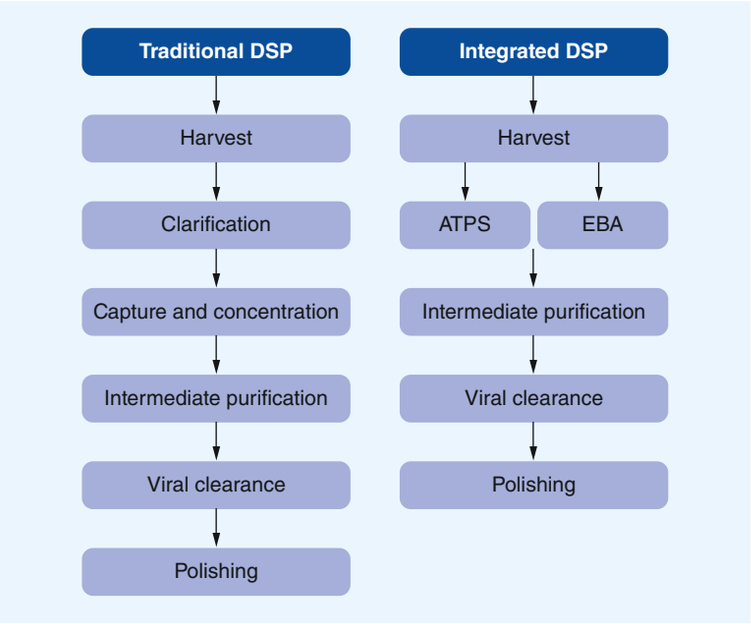

Figure 1. Process flow sheet comparison between a traditional downstream process and an integrated approach, where clarification, concentration and capture can be integrated in a single step.
ATPS: Aqueous two-phase separation; DSP: Downstream processing; EBA: Expanded bed adsorption.

- The mechanisms that govern the partition of biomolecules are not fully understood, although some trends can be established with hydrophobicity being one of the key determinants of protein partitioning [27];
- Method development and optimization is rather empirical and laborious, requiring testing many different parameters at the same time;
- Difficulties may occur during scaling up or scaling down the process;
- Validation of impurity clearance, including the phase-forming components (e.g., polymers) may not be straight forward and may make product approval more difficult by regulatory agencies. In the particular case of mAbs, a questionable platformability of extraction compared with the Protein A capture platform can also be pointed out.

One strategy to make partitioning in ATPS more predictable and selective is to add affinity ligands to one of the phases and thus direct the partition of the target biomolecule towards the phase containing fewer impurities. Different authors have employed this strategy, where both PEG and dextran have been modified with different types of ligands (Table 1). Additional

Table 1. Purification of biological products from distinct sources using aqueous two-phase systems.

Biologicals	Source	System	Ref.
Non-functionalized ATPS			
IgG	Hybridoma CCS	PEG/phosphate/NaCl	[29]
IgG	CHO CCS	PEG/phosphate/NaCl	[30,31]
IgG	CHO CCS	PEG/citrate	[32]
IgG	Hybridoma CCS	PEG/citrate/NaCl	[33]
Insulin-like growth factor	*Escherichia coli* cell debris	PEG/sulphate	[34]
HBsAg	*Saccharomyces cerevisiae*	PEG/sulphate	[201]
Human alpha-1 antitrypsin	Natural milk whey	PEG/citrate	[35]
Rotavirus-like particles	Insect cell culture homogenate	PEG/phosphate	[36]
B19 virus-like particles	Sf9 Insect cell culture paste	PEG/ammonium sulphate	[37]
pDNA	*E. coli* lysates	PEG/sodium citrate/ ammonium sulphate	[38]
pDNA	*E. coli* lysates	PEG/ammonium sulphate	[39]
Functionalized ATPS			
IgG	CHO CCS	PEG-glutaric acid/dextran	[40]
IgG	CHO CCS	PEG/dextran/TEG-COOH	[41]
IgG	Hybridoma cell culture	PEG-mimetic green/ dextran	[42,43]
pDNA	*E. coli* lysates	PEG/dextran/PEG-IDA-Cu(II)/DNA binding fusion protein (LacI-His_6-GFP)	[44]
pDNA	*E. coli* lysates	PEG/dextran/PEGylated PEI	[39]
Red blood cells	Sheep and human RBC mixture	PEG-antibody/dextran	[45]
Hematopoietic stem cells	Umbilical cord blood	PEG/dextran/anti-CD34 antibody	[46]

ATPS: Aqueous two-phase separation; CCS: Cell culture supernatant; CHO: Chinese hamster ovary; HBsAg: Hepatitis B surface antigen.

sophistications of these methods by utilization of affinity micro- or nano-particles are known, as well [48]. The use of soluble ligands, however, adds an extra impurity to be removed in downstream unit operations.

Another interesting approach to ease process development is by using design of experiments to speed up process optimization and, at the same time, facilitating a more thorough evaluation of the process variables. Many published studies make use of a central composite design to optimize the ATPS composition, in terms of polymer type and molecular weight, ionic strength, pH and tie-line length. Another possibility to improve process development is to use high throughput screening techniques such as robotic pipetting stations [49,50] or microfluidic devices [51]. Both tools have the potential to effectively accelerate bioprocess design and optimization in a cost effective manner.

Two significant aspects that could further foster the use of ATPS at large scale are that:

- Scale-up has been described as easy and reliable [52];
- The systems can be processed in continuous mode using typical liquid–liquid extractors such as mixer-settlers or extraction columns [53–55].

Recently, a continuous ATPS process incorporating three different steps (extraction, back-extraction, and washing) was set up and validated in a pump mixer-settler battery (Figure 2). This ATPS process allowed the recovery of IgG from a CHO cell supernatant with a global recovery yield of 80%, a final total purity of 97% and protein purity of more than 99%. A PER.C6 cell supernatant was also processed with a total recovered yield of 100%, a final total purity of 97% and an HCP purity of 95% [54]. The economic viability of this ATPS process has been evaluated and compared with the currently established platform based on Protein A chromatography [24]. The ATPS process was shown to be considerably advantageous in terms of process economy and operation, especially when high titre cell culture supernatants were processed (> 2.5 g/l). It is able to continuously purify the same level of mAbs, reducing the annual variable operating costs by at least 39%.

One of the key features of ATPS is to be amenable to process integration and intensification. ATPS can be used to purify biomolecules directly from a crude feedstock, that is, from unclarified cell cultures/broths, with or without additives such as antifoam. In this case the cell harvest is directly integrated with the capture step without any clarification step in between (Figure 1). Indeed, different ATPS systems have been effectively used as primary recovery steps in the purification of biomolecules, produced in either prokaryotic or eukaryotic hosts. Proteins produced in *Escherichia coli* generally form inclusion bodies or are secreted into the periplasmic space and need to be clarified to remove cell debris and solubilized to yield the active protein.

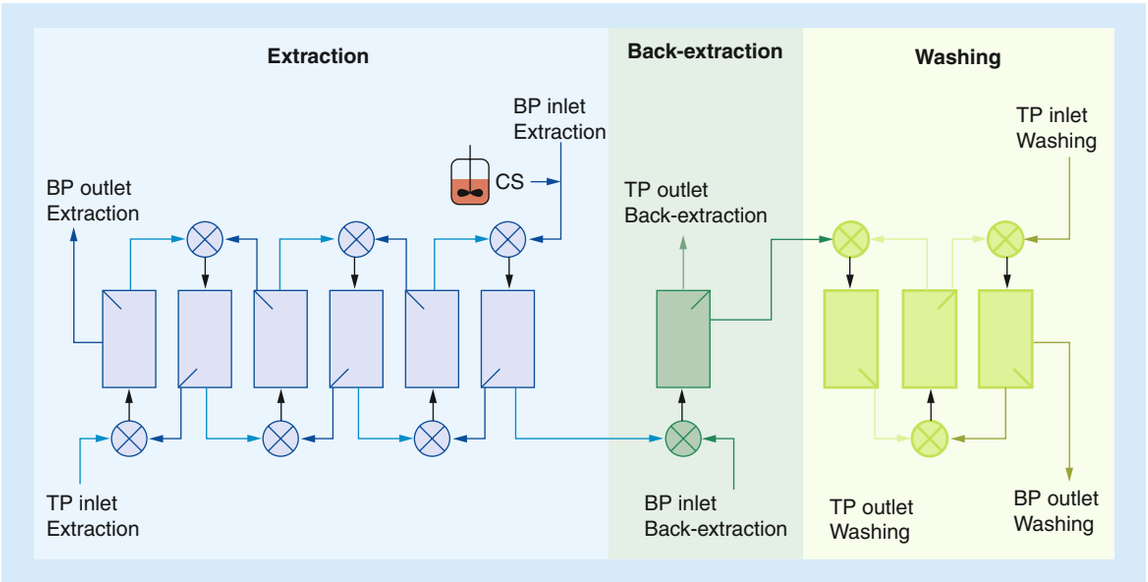

Figure 2. Continuous aqueous two-phase separation-based process flow diagram, designed for the purification of human antibodies from a cell culture supernatant. This includes an extraction step performed in six stages where most high-molecular-weight impurities are removed, followed by a single-stage back-extraction step and ending with a washing step performed in three stages, which allows removal of low-molecular-weight compounds. Numbered rectangles represent the different stages of the extraction step; circles with crosses represent mixing pumps.
BP: Bottom phosphate-rich phase; CS: Cell supernatant; TP: Top PEG-rich phase.

Typically, these processes suffer from low efficiency due to the high viscosity and density of the bulk solutions, caused by the presence of high concentrations of chaotropic agents (e.g., 4 M urea), which can make subsequent solid–liquid separations (e.g., centrifugation) difficult. ATPS can be successfully used in these cases either after (when inclusion bodies are formed) or simultaneously with cell disruption (when soluble proteins are formed), allowing not only the removal of cell debris and host cell proteins and DNA, but also the preservation of protein activity. These integrated processes have been described and successfully applied in the purification of insulin-like growth factor [34] and human growth hormone [47]. It must be noted that system composition can be affected by the presence of a fermentation broth. Hence, researchers should expect a change in composition when passing from preliminary studies performed with pure proteins in diluted buffers to studies with real feedstocks. Interestingly, phase separation typically occurs for lower concentration of polymers when real feedstocks are used. Nevertheless, these strategies will definitely contribute to a dramatic cut on the manufacturing costs of bioproducts and to an increase in product integrity and stability, which, when combined, will ultimately lead to the production of more affordable products.

Further advances in ATPS can arrive from the use of non-conventional phase-forming components, such as ionic-liquids, surfactants and stimuli-responsive polymers, including temperature-, pH- and photo-responsive polymers. These 'smart polymers' (see **Table 2**) offer a rather convenient way to recycle the polymer by simply changing environmental conditions such as temperature, pH, ionic strength or light [56–66]. Most partitioning studies have been performed using the thermo-responsive EOPO copolymer, which can be easily recycled through thermoseparation. Increasing the temperature above the polymer's cloud point induces the formation of a two-phase system, where the concentration of EOPO polymer in the bottom phase is usually 40–60%, while the top phase contains almost 100% water [57]. Typical phase-forming components such as PEG or dextran are more difficult to recycle and commonly require specifically designed unit operations. These could include:

- Diafiltration or dialysis with an ultrafiltration membrane;
- Organic solvent extraction followed by evaporation of the solvent;
- Crystallization for salt components;
- Specific adsorption techniques such as lectin or boronate chromatography for dextran or other carbohydrate-based polymers. Nevertheless, the cost involved in developing and validating these unit operations may not justify this option.

Table 2. Smart polymers used for aqueous two-phase extraction.

Copolymer (trade name)	Composition	M_r (Da)	Characteristics	Ref.
$EO_{50}PO_{50}$ (UCON 50-HB-5100; UCON 50-HB-3520; UCON 50-HB-2000)	Co-polymer of EO and PO composed of 50% EO and 50% PO	5100; 3520; 2000	Temperature responsive (CPT 1% solution = 50–53°C)	[56]
$EO_{50}PO_{50}$ (Breox PAG 50A 1000)	Co-polymer of 50% EO and 50% PO	3900	Temperature responsive (CPT 10% solution = 50°C)	[57]
$EO_{30}PO_{70}$	Co-polymer of 30% EO and 70% PO	5000	Temperature responsive (CPT 10% solution = 40°C)	[57]
HM-EOPO	C14H29-group coupled to the end of the polymer EOPO composed of 66 EO and 14 PO groups	8000	Temperature responsive (CPT 3% solution = 14°C)	[58]
Poloxamers (Pluronic)	Triblock co-polymercs of EO-PO-EO (%EO 10–80%)	1100–8400	Temperature responsive (CPT = 5–65°C)	[59]
pAEDS	Polymer composed by diallyaminoethanoatedimethyl sulfoxide	35,000	pH responsive (precipitates with the addition of 0.95 eq. HCl)	[60]
pDAB	Copolymer of 2-(dimethylamino) ethyl methacrylate, acrylic acid, and butyl methacrylate (5.8:17.7:1)	37,000	pH responsive (precipitates at pH = pI = 4.1)	[61]
pNNC	Copolymer of N-isopropylacrylamide (NIPA), N-vinyl-2-pyrrolidone (NVP), chlorophyllin sodium copper salt (177:9:2)	7100	Light responsive (precipitates by irradiation with visible light at 488 nm)	[62]

CPT: Cloud point temperature; EO: Ethylene oxide; PO: Propylene oxide.

Second generation expanded bed adsorption chromatography

EBA chromatography is based upon the utilization of a bed of fluidized and classified adsorbent beads; the expansion of which is controlled by the upward flow rate of the mobile phase at the bottom of the column. The method relies on a population of adsorbent beads with defined particle size and density distribution that causes a restricted local mobility of the individual adsorbent particles along the height of the column. The downward gravitational force experienced by the particles is balanced by upward viscous forces exerted by the mobile phase following Stoke's law. Crude feedstock containing the product of interest can be directly loaded into a fluidized column containing beads of appropriate functionality, and can be effectively washed to eliminate trace non-binding contaminants (Figure 3). Elution can then be carried out either in expanded mode or packed-bed mode.

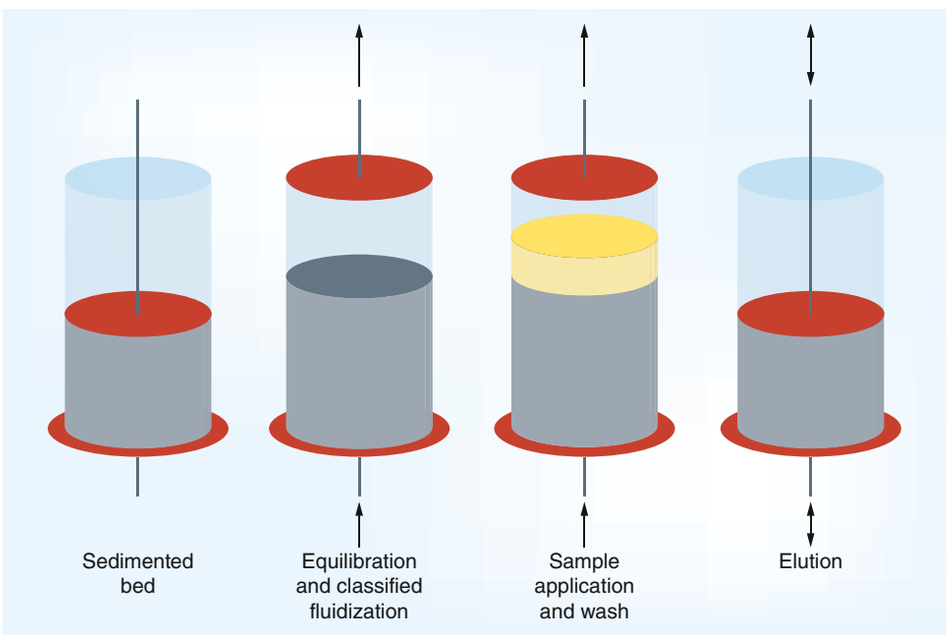

Figure 3. Principle of expanded bed adsorption chromatography.

This new concept for the isolation of bioproducts employing fluidized adsorbent beads was introduced by Draeger and Chase more than two decades ago [67]. This early work was exclusively performed with common Sepharose beads [67,68]. Soon after, particles weighted by a quartz-core were introduced [69]. The pharmaceutical industry previously employed fluidized beds for the recovery of antibiotics. This included batch systems for streptomycin and semi-continuous methods for novobiocin [70,71]. Since its introduction, EBA chromatography has been employed for protein purification in a wide array of expression systems, for example, mammalian, bacterial, yeast, as well as algal systems along with their homogenates [72–74]. EBA also allows for immediate processing of

sensitive products, for example, proteins that are subject to protease attack. Process times are decreased by more than 50% [75]; corresponding product recovery and purity are comparable to conventional multi-step methods [76].

After EBA had been commercially introduced in the early 1990s, shortcomings of the technology soon became apparent in day-to-day operations. The first generation of EBA adsorbents, which consisted of slightly heavier agarose-based beads, (e.g., possessing a core of quartz, zirconia, metals or alloys) promoted unfavorable biomass–adsorbent interactions (Figure 4), which in turn provoked the failure of many proposed industrial processes [77–79]. These interactions introduced a variety of detrimental effects, such as particle agglomeration, improper bed fluidization leading to channelling and stagnant zones, reduced product binding capacity, and elutriation of the adsorbent (due to reduction in its specific weight), among others. In some cases, for certain combination of feedstock type and adsorbent mode, a complete collapse of the fluidized bed was observed. The adverse effects of different biomass types on adsorbents of different composition are found extensively in literature [80]. Furthermore, the low density of these adsorbent beads limited the maximum flow rate that could be employed, leading to longer process times. However, the development of second-generation EBA adsorbents (e.g., DSM Rhobust™) has led to significant improvements in EBA performance [81]. These high-density tungsten carbide-cored beads coated with cross-linked agarose are available in several types of functionality (IEX, HIC, mixed-mode, or affinity) and possess sufficient densities (~2.8–3.2 g/ml) to offer much higher operational flow rates, in the range of 300–600 cm/h [76]. The higher drag forces associated with faster flow velocities also contribute to biomass detachment from the bead leading to lower buffer consumption [82]. Coupled with an effective and uniform flow distribution system, such as the rotating fluid distributor, this technology is now scalable up to 60 cm (internal diameter) columns (Rhobust™ Flex system) [76]. Additionally, ideal bed expansion factors for optimal product capture have been determined to be within 1.8–2.2-times the sedimented bed height [83], and while changes in mobile phase viscosity (e.g., between buffer and crude feedstock) might lead to bed instability, sensitive ultrasonic detectors have been adapted to the expanded bed systems, which automatically adjust flow rates to maintain ideal bed expansion via a feedback loop [84]. Numerous examples clearly highlight the huge potential contained in the EBA methodology [85–87]. Excluding the cost of adsorbents, an economic advantage of up to 50–70% has been reported by Walter and Feuser [88]. These results are directly linked to the reduction of necessary unit operations from three to four in a traditional purification scheme to just one for EBA [88]. To generate a well-fluidized and efficient processing system based on EBA, a key element is controlling the deposition of biomass, which will be discussed below.

On one hand, experimental techniques have been developed, which allow for performance evaluation via key indicators, such as finite bath cell adsorption, the cell transmission index via pulse experiments, and residence time distribution analysis, which indicates proper fluidization and operation of the expanded

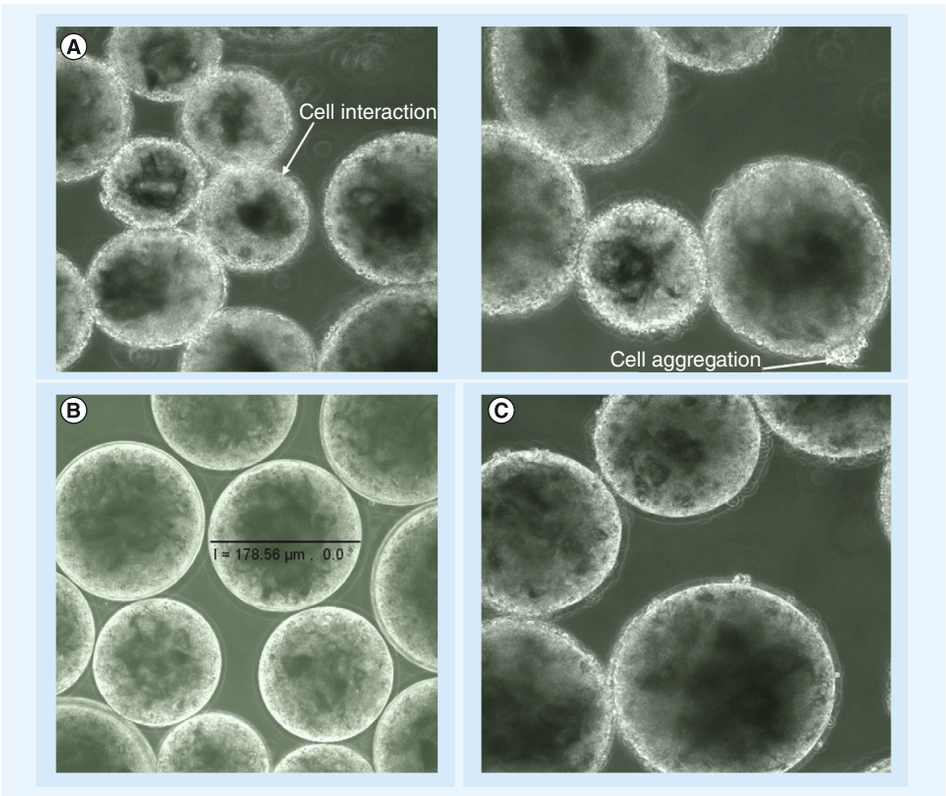

Figure 4. Binding of *Saccharomyces cerevisiae* yeast cells onto Streamline DEAE (average size 200 µm). (A) The interaction and aggregation of yeast cells onto the Streamline DEAE. **(B)** No cell interaction onto Streamline SP (a negative control for no cell interaction). **(C)** Low interaction of yeast cell onto PVP-coated Streamline DEAE.
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bed under real process conditions [85]. On the other hand, further process optimizations of EBA towards reducing biomass–adsorbent interactions have recently emerged in the form of theoretical investigations into the physico-chemical properties of the beads, biomass, as well as the mobile phase [89,90]. These involve the use of refined colloid theory methods (extended DLVO theory) that take into account hydrophobic, polar, and electrostatic interactions between colloidal entities [91]. When Coulomb-type interactions are predominant, it has been shown that assessing the surface charges of cells and adsorbents (via zeta potential measurements) suffices as a quick screening method to evaluate biomass–adsorbent interactions [85].

Several strategies, guided by theoretical calculations described earlier, can be applied in order to optimize process conditions and minimize biomass–adsorbent interactions [85]. For example, increasing the ionic strength of the mobile phase eliminates most electrostatic interactions. Additionally, adjusting the pH of the system results in altered zeta potentials [92]. These adjustments in conditions can be coupled to appropriate adsorbent design, for example, by shielding the bead surface with cell repellent polymers **(Figure 4)** [93], yielding an integrated strategy for EBA process development [85]. Together with the significant recent technological innovations mentioned earlier, second generation EBA has finally matured into a competitive downstream technology that will prove disruptive to current practices in the biotechnology industry.

Convective flow systems

In conventional chromatographic separations, the product of interest is carried along with all the soluble impurities through the solid stationary phase, for example a chromatographic resin packed in the column, with the flow of a liquid mobile phase. The components present in the mixture are separated according to the differences in migration rates that arise from the differences in the physical properties (size-exclusion chromatography), interaction strengths (ion-exchange and hydrophobic interaction chromatography) or affinity properties (affinity chromatography). The movement of the solutes between the two phases and through the column, however, can be driven by either diffusion or convection. Diffusion mass transport is a random thermal movement that is driven by concentration differences of the component in space, and is inversely proportional to the size of the component. Therefore, diffusion is a process that always takes time and can be accelerated only to a certain extent. On the other hand, convective mass transport is induced by an external force, such as a pressure difference, where the mass transfer rate is controlled by an external flow rate, usually induced by gravity or a pump, and is practically independent of molecular size.

Conventional chromatographic matrices for biochromatography were first developed for the purification of small protein molecules and were beaded particles ranging in size from 50 to 500 µm, having pores with diameters of up to 30 nm [12,94] to gain enough surface area to reach high binding capacities for small proteins. Mass transfer through the column packed with beads is based on convection (the mass transfer between the particles) and diffusion (the mass transfer into the pores of the particle). For a very small species (e.g., ions or small organic molecules) the diffusion coefficients are high and the time needed for the molecule to enter the pore is relatively short. The increase in the flow rate does not significantly influence the efficiency of the column. On the other hand, large molecules (e.g., proteins, pDNA and VLPs) for which the diffusion coefficients are at least two orders of magnitude lower and the transfer of the molecule into the pore is additionally hindered due to the small pore diameter/molecule diameter ratios ($d_P/d_M \leq 10$ [12]), more time is needed for the molecule to diffuse into the pore. Consequently, column characteristics, such as resolution and dynamic binding capacity, decrease considerably when the flow rate increases. Regarding the purification of very large compounds, the accessibility of the active surface in conventional beads is an issue; since the size of large antibodies (IgM) is in the range of 20 nm and the size of pDNA, VLPs and viruses is much larger (above 100 nm), these compounds cannot enter small pores. Subsequently, the available surface for binding decreases dramatically, since most of the macromolecules bind to the outer shell of the beads, and as a consequence, the capacity drops from more than 130 mg/ml for small proteins to 1.5 mg/ml or lower for, for example, pDNA [95].

To avoid diffusion and capacity limitations, new chromatographic media were developed, such as non-porous and perfusion particles, chromatographic monoliths, and membranes **(Figure 5)**. Non-porous particles completely eliminated diffusion limitations, leading to significantly enhanced mass transfer. However, as a result of low active surface area, being only on the outer surface of the particle, capacities are also low. This can be overcome by using smaller particles, but columns packed with these particles exhibit extremely high back pressures [4].

Perfusion particles have additional flow through pores of larger diameter (0.6–0.8 µm) within the particle, where the molecules are additionally transported by convection. They still contain smaller closed pores to gain the sufficient surface area to bind

small proteins as conventional porous matrices, but those pores are still too small for pDNA or viruses to enter, which permits the binding of these molecules, once again, solely at the surface of macropores [95]. Moreover, since flowthrough pores in the particle are much smaller than pores in the inter-particle space, most of the mobile phase flows around the particles rather than through the perfusion pores due to the lower back pressure, thereby diminishing the convective effect [96]. Membranes are another type of convective-based support that have excellent hydrodynamic properties. However, because of the low surface area and high dead volume that enhance extra column effects they lack the capacity and resolution (selectivity) [10].

To overcome most of the problems listed above for different types of chromatographic supports, chromatographic monoliths were introduced. They consist of a single block of material that contains highly connected flow through macropores that enable the transport of the solute to the surface solely by convection. Silica monoliths, prepared by sol–gel synthesis, also contain mesopores and are developed for the separation of small molecules. While the polymethacrylate monoliths, having only macropores, are the most widely used monoliths for protein, pDNA, and virus separation, purification and concentration, their physical structure ensures that their entire surface area is accessible for large molecules to bind, which results in very high capacities for large solutes. For example, the capacity of polymethacrylate monoliths ranges from 8 up to 14 mg/ml for pDNA [97–99] and from 10^9 pfu/ml to more than 10^{12} pfu/ml for viruses [100–102], depending on the virus and impurity concentration present. As a consequence of the convective mass transport, they exhibit flow-independent resolution and binding capacity for all sizes of biopharmaceuticals. Before the year 2000, chromatographic monoliths were tested for only laboratory and small preparative purposes. In that year, however, Podgornik and co-workers published the principle of constructing large monolithic columns that were based on a radial geometry column design [103]. Based on this, the scale-up of monolithic columns was successfully solved [8] and the polymethacrylate monolithic columns, also known as Convective Interaction Media (CIM) monoliths, have become very interesting for industrial applications. It is notable, however, that the size of these media is currently limited to a maximum of 8 L columns, which will need to be amplified in the future for larger scale applications. Although the obvious trade-off for CIM media is the relatively lower quantitative binding capacities as compared with higher surface area beaded adsorbents (see **Table 3**), CIM trumps these beaded adsorbents by offering a far superior method to purify new classes of enormous bioparticulate entities such as pDNA and viral particles.

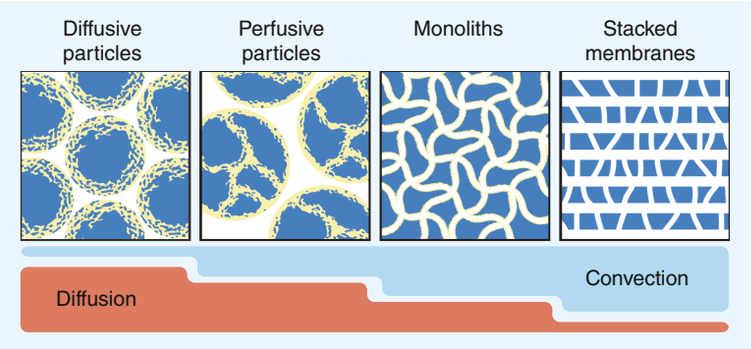

Figure 5. Mass transport in contemporary chromatography formats [10]. Areas of diffusive transport are indicated in yellow, convective transport in white, and support matrix in blue.

One of the first industrial applications of CIM chromatographic monoliths was purification of pDNA by Boehringer Ingelheim [104]. Before the most appropriate chromatographic support was chosen, a thorough comparison study was performed [99,105], which showed the best binding capacity for plasmids and practically flow-independent properties for monolithic columns **(Figure 6)**. The productivity of the process was also calculated and it was shown that by using monolithic columns a 15-times higher productivity could be achieved. At that time no HIC monolith was commercially available, therefore, the first purification step (using HIC) was still performed on the conventional bead matrices. However, because of various advantages using CIM monolithic columns in the downstream processing of pDNA, progress has been made and new purification processes have been developed using solely monolithic chromatographic columns which have, consequently, achieved much higher process productivity [11].

The increased vaccine production and the need for large quantities of purified viral vectors for a promising new field of gene therapy emerging at the end of the previous century led to an intensive search for new tools to substitute the conventional purification techniques such as density gradient ultracentrifugation, precipitation and two-phase extraction. Namely, these methods were unable to deliver fast, efficient, and scalable downstream processes for these biopharmaceuticals. Chromatography was soon recognised as the most promising tool for these purposes but the size of viral particles presented a huge challenge for conventional particle packed columns, while polymethacrylate monoliths with previously described characteristics were ideally suited for the task. Since then, numerous

Table 3. Comparison between various types of adsorbent systems.

	Ligand chemistry	Shape	Flow rates (cm/h)	Back pressure (bar)	Capacity (mg/g)	Biomass compatibility (% wet weight)	Mass transfer limitations	Productivity	Material costs	Geometry	Chemical stability (0.1 N NaOH)
gPore	Any[†]	Micro-fibre network	40–600[‡]	~0.2[§]	100–200[¶]	0–20[#]	Moderate	Requires complete process analysis on an individual basis	Moderate	Variable[††]	Yes
Monoliths	Any	Porous Solid	~500	~0.1	~10	0	Low	Requires complete process analysis on an individual basis	Expensive	Column	Yes
Membranes	Any	Porous Solid	250–450	~1	~55	0	Low	Requires complete process analysis on an individual basis	Expensive	Shallow bed	Yes
Packed beds	Any	Porous Solid	20–300	~1.5	~80	0	High	Requires complete process analysis on an individual basis	Expensive	Column	Yes
Expanded bed adsorption	Any	Porous Solid	100–300	~0.5	~80	≤8–10	High	Requires complete process analysis on an individual basis	Expensive	Column	Yes

[†]SP, DEAE, epoxy, dyes, IDA, Protein A/G under development.
[‡]Tested experimentally. Potential for improvement.
[§]Standard processing pressures.
[¶]Dynamic binding capacities for hen egg-white lysozyme and total human IgG onto cation-exchanger material.
[#]Depending on fibrous network density.
[††]Possible configurations include axial or radial column, spiral cartridge and disposable units.

plant, human and bacterial viruses (tomato mosaic virus, bacteriophage T4, influenza, adenovirus and lentivirus, to name just a few) were successfully purified using CIM monolithic columns [106–109]. Increased productivity of monoliths compared with conventional virus purification methods was demonstrated with different viruses and bacteriophages [9,107]. A very illustrative demonstration as to how the introduction of CIM monolithic chromatography into the DSP of viruses can shorten the process was shown with tomato mosaic virus, where DSP was shortened from 5 days to only 2 h **(Figure 7)**.

Fibre-based adsorbent systems

Cellulose is one of the most abundant polymers in nature. It is found in the cell walls of green plants as a structural component. Made up of 200 to tens of thousands of β-linked D-glucose units, its stability is mainly due to the hydrogen bonds formed between the hydroxyl groups of the juxtaposed chains. This arrangement leads to compact, water-insoluble micofibrils, despite the hydrophilic nature of its building blocks.

The cellulosic microfibrils are arranged into crystalline domains, with a high degree of organization, and amorphous domains. In the case of cotton cellulose fibres, a 65–70% degree of crystallinity is observed, which stems from the hydrogen bridges formed between the polymer chains. However, some hydroxyl groups remain available for hydrogen bonding interactions with water in this phase. In contrast, all hydroxyl groups, even the ones in the interior, are accessible in the amorphous phase and are available for further direct chemical modification [110], which is advantageous for increasing binding capacities of fibre-based adsorbents. However, the utilization of functionalized cellulose fibres creates a pellicular type of adsorbent, which in turn increases the resolving power of the system.

For chemical or biochemical applications, cellulose is most commonly employed in the granular or beaded form as a stationary phase in partition chromatography, for example in thin layer chromatography or as beads packed in a column, thereafter used for the separation of a multitude of molecules, ranging from organic compounds to macromolecules and biopolymers. Cellulose and its major derivatives, nitro- and acetate cellulose, are also employed in DSP, mainly in the filtration, clarification and ultrafiltration processes of cell suspensions and protein-rich solutions. It is also used as an adjuvant, to facilitate the removal of cells and cellular debris, colloids and insoluble material. More recently, electrospun cellulose nanofibers, with high internal surface area, have been studied as a novel protein adsorbent material [111]. This material shows

similar binding capacity and permeability as compared with commercial adsorptive membranes, and offers similar process advantages as the *gPore* materials discussed herein.

Cotton fibres contain more than 95% cellulose, with varying length and fibre diameters between species [112]. Egyptian cotton *(Gossypium barbadense)* is particularly appropriate for the development of adsorptive fibrous material, due to its extra-long and thin fibres (extra-long-staple). Native cotton fibre has a highly ordered and low water-absorbing structure. Alkali-treatment, such as mercerization, is traditionally used to partially loosen this ordered structure, leading to a swelling of the cotton linters. Additionally, alkali treatments reduce crystallinity and increase the accessibility of cellulose hydroxyl groups to the solvent [113]. However, further chemical modifications are necessary for a permanent stabilization of the swollen polymer.

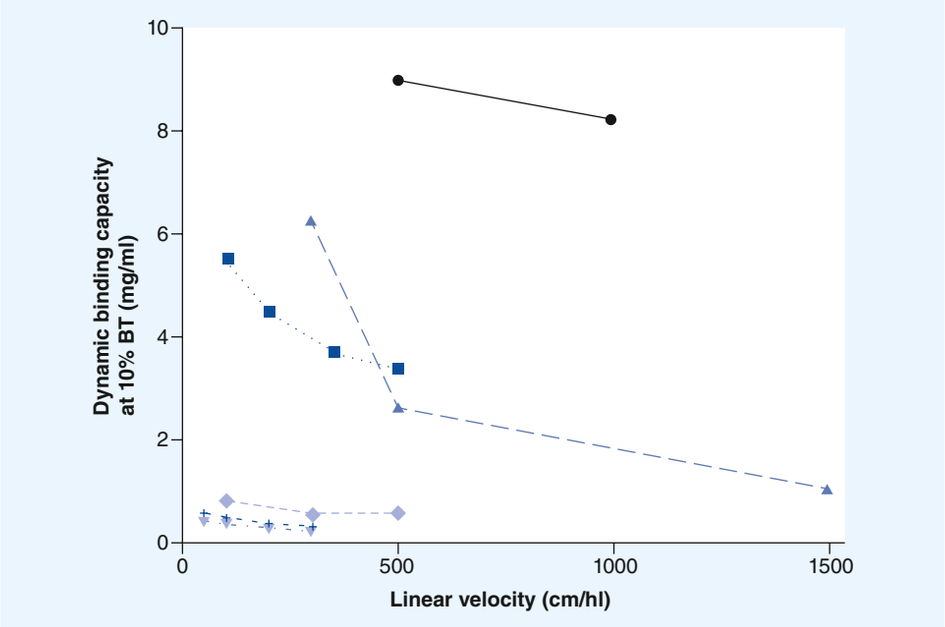

Figure 6. Comparison of different supports (1–2 ml, 0.34 ml disk) regarding dynamic binding capacity (10% breakthrough) for pDNA. Feed stock: purified pDNA solution. Source 30Q, Toyopearl DEAE 650M, DEAE Sepahrose FF represent a class providing low capacity. Q Ceramic HyperD 20 and Fractogel EMD DEAE (S) showed a higher dynamic binding capacity for pDNA at the lower linear velocities. CIM DEAE was the only support, which provided a high capacity (>8 mg/ml) at high flow rates (500–1000 cm/h).
BT: Breakthrough.
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Recently, fibrous materials have become an interesting subject for further research, due to their facile functionalization and beneficial physiochemical properties. They are rapidly becoming a viable alternative to beaded adsorbents (in packed bed mode), due to their competitive cost and excellent process factors, such as yield and purity. The most prominent feature is the ability to apply a wide variety of different functionalities to the fibre-matrix, making these materials adaptable and versatile. The functionalization of the fibres can be facilitated with various chemical and physical methods.

Ionizing radiation was applied to induce graft polymerization of the swelled cellulose with polymethacrylates to add selective adsorptive properties [114,202]. The *gPore* composite material contains more than 60% cellulose, preserving the 3D fibre structure. Water uptake experiments, as well as microscopy images, revealed that the grafting modification does not disturb the physical integrity of the material. This is due to the highly penetrative nature of gamma rays, permitting a homogenous and efficient modification of the material [202].

Confocal microscopy demonstrates the protein adsorption capability in the bulk fraction of *gPore* fibres with IgG labelled with Cy3 fluorescent dye electrostatically adsorbed to the fibre wall, which is composed of a sulfopropyl poly-methacrylate/cellulose composite. The protein diffusion path in the fibre wall is shorter than 10 µm, compared with a more than five-times longer path in the beaded materials [115].

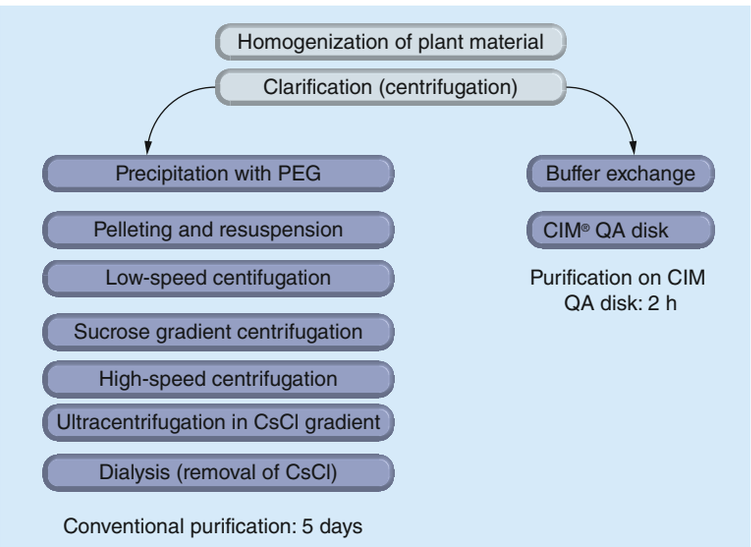

Figure 7. Comparison of conventional tomato mosaic virus purification and tomato mosaic virus purification using CIM QA monolithic support.
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Hen egg white lysozyme was employed as a model protein so as to evaluate the dynamic binding capacity of a fibrous cation exchanger material produced according to the *gPore* technology (SP-*gPore*). The packed fibrous adsorbent allowed for operational flow rates between 40 and 350 cm/h. Under such experimental conditions, capacity values were found in the range of 160 to 280 mg protein/g adsorbent.

To evaluate the resolution power of the packed fibrous adsorbent (SP-*gPore*), chromatography cycles were run utilizing an increased gradient development (20 CV) and employing a mixture of three commercially available proteins. As shown in **Figure 8**, with three different flow rates the SP-*gPore* material showed a very good resolution, which could be maintained even at high flow rates, that is, of up to 900 cm/h. Therefore, flow rates can be increased by a factor of six while maintaining a similar chromatographic behaviour in terms of resolution power. It can be observed that resolution at high flow rate has been better than commonly observed for a beaded material (SP Sepharose) [115]. The combination of both factors allows for the reduction of buffer consumption by gradient shrinking during the process development and leads to a decrease in working time, improving overall chromatographic productivity.

Another advantage provided by the fibrous adsorbent systems is the low-pressure drop observed during chromatography, especially at high flow rates. The permeability coefficient calculated according to Darcy's Law at a low Reynolds number yielded a value of 1×10^{-7} cm^2 for a fibre-packed column [115]. As a comparison, this value is two times higher than the values of columns packed with ion-exchange beads [116,117]. Although many factors can influence permeability, fibre compactness is one of the most important factors determining the observed column back pressure. Considering that the permeability value reported above was measured with a very compact configuration of the fibrous web, more open fibre configurations will most likely lead to even better back pressure performance. Moreover, adsorptive fibres can be easily arranged in an open structure that allows biomass tolerance while retaining its hydrodynamic features. This characteristic becomes an advantage, as it results in simplifying the recovery of bioproducts directly from a cell-containing feedstock.

In regard to the structure of the packing, different arrangements have been studied in the form of stacked discs, simple rolls, or in parallel or twirled arrangements, either in packed or open forms in randomly and non-randomly packed beds. A column with SP-*gPore* fibres in a non-randomly packed open structure double

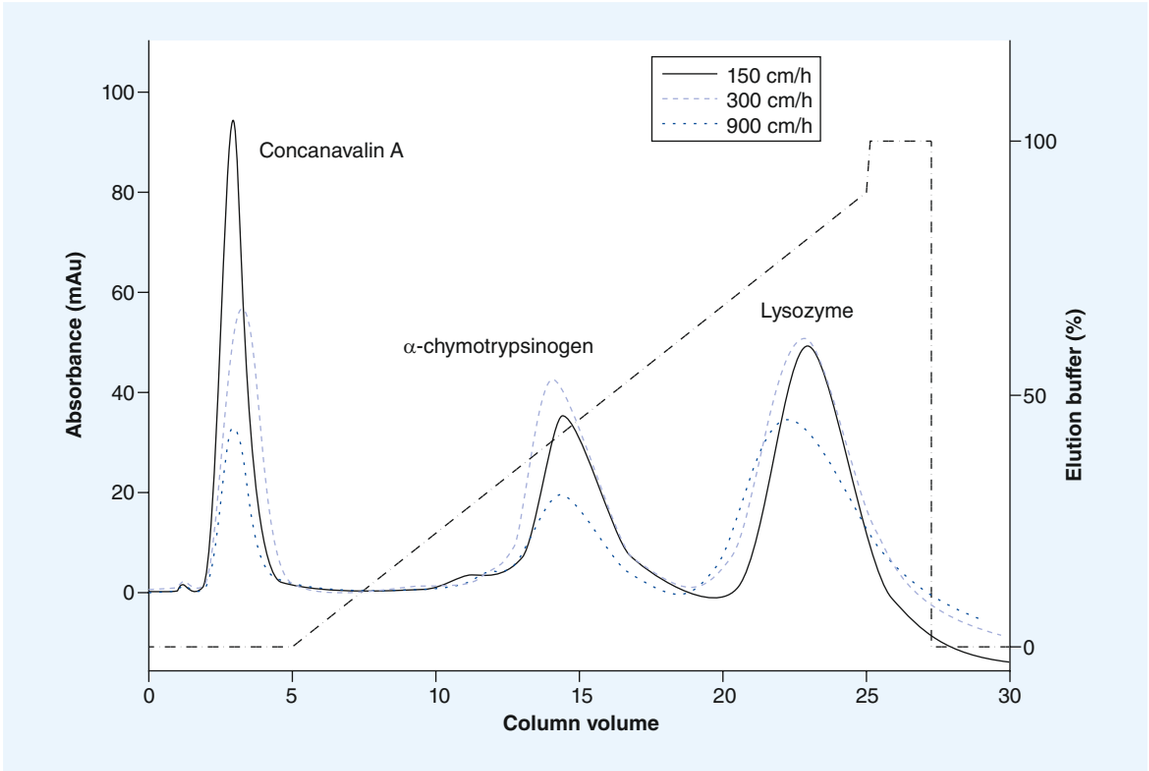

Figure 8. Resolution behavior of SP gPore at an absorbance of 280 nm at three different flow rates.
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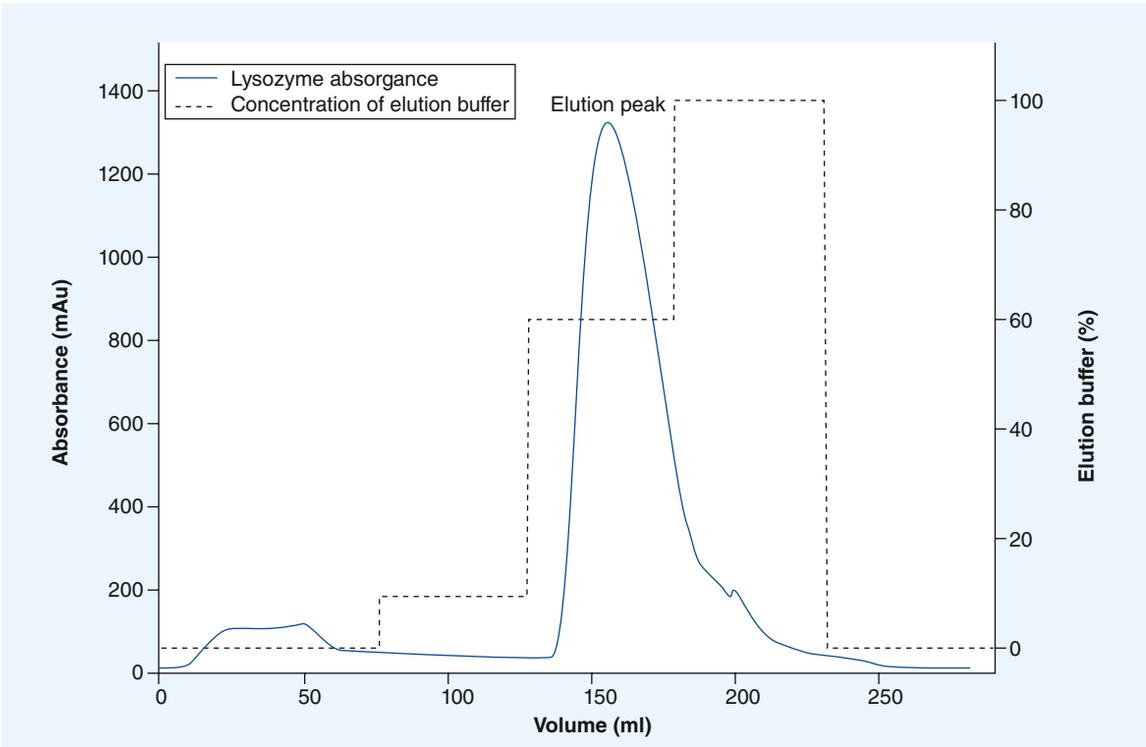

Figure 9. Experiment for biomass tolerance of SP gPore under simulated process conditions. Lysozyme was mixed with yeast cells and applied to the column with SP gPore. Subsequently, the column was washed and the lysozyme eluted (peak at 150 ml).
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roll was tested to demonstrate the ability to process a cell containing feed stream (Figure 9).

An artificial mixture composed of 5 g/l lysozyme (as a model protein for purification) and 1.5% disrupted yeast cells was employed to realistically simulate process conditions generally encountered during the direct purification of macromolecules from a particle containing feedstock. The whole fragmented cell suspension was applied to a cartridge containing the fibrous adsorbent, employing a system based on peristaltic pumps with a linear flow velocity of 150 cm/h. After washing with the loading mobile phase, the elution of the target protein lysozyme was carried out by increasing the conductivity of the mobile phase; the resulting chromatogram elution profile is shown in Figure 9 [115]. Lysozyme is selectively bound to *gPore* and recovered as pure protein from either biomass-filtered or biomass-unfiltered feedstock. The material shows almost no back pressure and no clumps, indicating high biomass compatibility. More recently, these adsorptive fibres were arranged in an optimized non-woven fabric as a single-use device, in order to standardize an adsorptive matrix for an industrial production process. Furthermore, *gPore* has made significant strides in overcoming some of the drawbacks of packed fibres that have prevented high throughput and binding capacities, including low porosities, irregular packing, and most importantly, low biomass compatibility [118,119].

At present, the adsorptive separation of bioproducts can be attained utilizing beaded supports, perfusive monoliths, or membrane adsorbents, all of which have found industrial applications in various bioprocessing niches (see Table 3 for a general comparison). The recent availability of packed fibrous adsorbents, which are amenable for highly productive and cost-effective bio-separations, will add a powerful tool to the current downstream processing portfolio.

Future perspective

The biopharmaceutical industry is growing at an unprecedented rate, especially with regard to complex therapeutic molecules, such as mAb [120]. While it will remain a cost-intensive endeavour to develop and bring such products to the market, significant cost-reduction potential can be realized by incorporating innovative methodologies in downstream bioprocessing early on into the development process. These methodologies should be able to satisfy the ever-increasing global consumer demand for high quality bioproducts, which will play a much more prominent role in the future.

Process integration and intensification should be a key feature in the design of new bioprocesses. In this regard,

techniques such as aqueous two-phase extraction can be a valuable alternative to conventional solid–liquid separation techniques and high-resolution techniques, although EBA chromatography has the potential to surpass traditional technologies in this field as well. They allow integrating the clarification, concentration and capture of biomolecules from crude feedstocks in a single unit operation, with high selectivity and recovery. The challenges posed by new classes of bioproducts require a paradigm shift in approaches for their capture and purification. Together with ATPS and EBA, convective flow systems have also been shown to possess distinctive advantages in meeting these challenges. In terms of enhancements to traditional chromatography methods, disposable, cost-effective, and high performance adsorbent materials, which also allow for shorter process times and lower consumption, have matured in the form of fibre-based adsorbents, such as *gPore*. In the coming years, as new classes of biotherapeutics are developed, continuous-mode adaptions and combinations of these technologies will be needed in order to usher them to the market.

It is noteworthy that the technologies discussed in this review are not mutually exclusive, and should be viewed as complementary and versatile tools in the arsenal of modern industrial bioprocess technology.

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Executive summary

Background

- The cost intensive nature of pharmaceutical production have pushed downstream processing needs and requirements into the focus of research, especially for new molecule classes, that is, pDNA or virus-like particles
- Integration and intensification are the biggest challenges faced by downstream technologies.
- Transformation from batch-based to more economical continuous processing is an important recent development, attracting a lot of attention.

Aqueous two-phase separation

- Aqueous two-phase separation technology is especially suited to early purification, due to its ability to handle solid containing feedstock, integrating clarification and capture.
- Possible to be operated in a continuous mode.

Second generation expanded bed adsorption chromatography

- Expanded bed adsorption is based upon a fluidized bed, on which crude feedstock can be directly loaded, thereby integrating clarification, capture, and concentration in a single step.
- Novel bead materials have been developed, which have tackled several problems previously plaguing this methodology, and have allowed the full potential of expanded bed adsorption to be realized.

Convective flow systems

- Mass transfer in methacrylate monolithic columns, which are easily scalable, is based on convection leading to flow-independent resolution and binding capacity for all sizes of biopharmaceuticals leading to shorter separation time and higher productivity.
- The decrease in contact time of the target molecule with the chromatographic support is important for labile molecules as this reduces the possibility of conformational changes in their structure.
- The structure of methacrylate monoliths with highly connected flow through pores (with the pore diameter of up to 6 mm) enables purification of very large molecular entities such as viruses, virus-like particles, pDNA and others with high process yield.

Fibre-based adsorbent systems

- Disposable purification technologies can be based on functionalized naturally occurring fibres, for example cotton, which is a cheap and renewable starting material.
- The benevolent physico-chemical characteristics and ability to be functionalized with a wide variety of different moieties makes for a versatile and flexible system.

Future perspective

- The new technologies allow considerable savings to be realized, leading to cheaper medication and other classes of bioproducts.
- The emergence of new classes of biopharmaceuticals has evoked a paradigm shift in the industry from established downstream processes.

References

1 Zouwenga R, D'Avino A, Zijlstra G. Improving productivity in bioreactors. *Genetic Engineering and Biotechnology News* 1 April (2010).

2 Jungbauer A, Hahn R. Polymethacrylate monoliths for preparative and industrial separation of biomolecular assemblies. *J. Chromatogr. A* 1184(1–2), 62–79 (2008).

3 Asenjo JA, Andrews BA. Protein purification using chromatography: selection of type, modelling and optimization of operating conditions. *J. Mol. Recognit.* 22(2), 65–76 (2009).

4 Štrancar A, Podgornik A, Barut M, Necina R. Short monolithic columns as stationary phases for biochromatography. In: *Modern Advances in Chromatography.* Freitag R (Ed.). Springer Berlin Heidelberg, NY, USA, 49–85 (2002).

5 Schügerl K, Hubbuch J. Integrated bioprocesses. *Curr. Opin. Microbiol.* 8(3), 294–300 (2005).

6 Hjorth R, Leijon P, Frej A-KB, Jägersten C. Expanded bed adsorption chromatography. In: *Bioseparation and Bioprocessing.* Wiley-VCH Verlag GmbH, Weinheim, Germany, 199–226 (2008).

7 Thömmes J. Fluidized bed adsorption as a primary recovery step in protein purification. In: *New Enzymes for Organic Synthesis.* Springer Berlin Heidelberg, Berlin, Germany, 185–230 (1997).

8 Podgornik A, Jančar J, Merhar M *et al.* Large-scale methacrylate monolithic columns: design and properties. *J. Biochem. Bioph. Methods* 60(3), 179–189 (2004).

9 Adriaenssens EM, Lehman SM, Vandersteegen K *et al.* CIM® monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles. *Virology* 434(2), 265–270 (2012).

10 Gagnon P. The emerging generation of chromatography tools for virus purification. *BioProcess Int.* 6(S6), 24–30 (2008).

11 Smrekar F, Podgornik A, Ciringer M *et al.* Preparation of pharmaceutical-grade plasmid DNA using methacrylate monolithic columns. *Vaccine* 28(8), 2039–2045 (2010).

12 Jungbauer A. Chromatographic media for bioseparation. *J. Chromatogr. A* 1065(1), 3–12 (2005).

13 Gemeiner P, Beneš MJ, Štamberg J. bead cellulose and its use in biochemistry and biotechnology. *Chem Pap-Chem Zvesti* 43(6), 805–848 (1989).

14 Gemeiner P, Polakovič M, Mislovičová D, Štefuca V. Cellulose as a (bio)affinity carrier: properties, design and applications. *J. Chromatogr. B* 715(1), 245–271 (1998).

15 Plumb K. Continuous processing in the pharmaceutical industry: changing the mind set. *Chem. Eng. Res. Des.* 83(6), 730–738 (2005).

16 Poechlauer P, Manley J, Broxterman R, Gregertsen B, Ridemark M. Continuous processing in the manufacture of active pharmaceutical ingredients and finished dosage forms: an industry perspective. *Org. Proc. Res. Devel.* 16(10), 1586–1590 (2012).

17 Ludemann-Hombourger O, Nicoud RM, Bailly M. The 'VARICOL' process: a new multicolumn continuous chromatographic process. *Sep. Sci. Technol.* 35(12), 1829–1862 (2000).

18 Andersson J, Mattiasson B. Simulated moving bed technology with a simplified approach for protein purification – separation of lactoperoxidase and lactoferrin from whey protein concentrate. *J. Chromatogr. A* 1107(1–2), 88–95 (2006).

19 Mazumder J, Zhu JX, Ray AK. Optimal design of liquid-solid circulating fluidized bed for continuous protein recovery. *Powder Technol.* 199(1), 32–47 (2010).

20 Müller-Späth T, Krättli M, Aumann L, Ströhlein G, Morbidelli M. Increasing the activity of monoclonal antibody therapeutics by continuous chromatography (MCSGP). *Biotechnol. Bioeng.* 107(4), 652–662 (2010).

21 Shinkazh O, Kanani D, Barth M, Long M, Hussain D, Zydney AL. Countercurrent tangential chromatography for large-scale protein purification. *Biotechnol. Bioeng.* 108(3), 582–591 (2011).

22 Napadensky B, Shinkazh O, Teella A, Zydney AL. Continuous countercurrent tangential chromatography for monoclonal antibody purification. *Sep. Sci. Technol.* 48(9), 1289–1297 (2013).

23 Jungbauer A, Peng J. Continous bioprocessing: an interview with Konstantin Konstantinov from Genzyme. *Biotechnology J.* 6(12), 1431–1433 (2011).

24 Rosa PAJ, Azevedo AM, Sommerfeld S, Baecker W, Aires-Barros MR. Aqueous two-phase extraction as a platform in the biomanufacturing industry: economical and environmental sustainability. *Biotechnol. Adv.* 29(6), 559–567 (2011).

25 Albertsson P-A. Chromatography and partition of cells and cell fragments. *Nature* 177(4513), 771–774 (1956).

26 Albertsson P-A. *Partition of Cell Particles and Macromolecules.* Wiley, NY, USA (1986).

27 Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: a perspective. *J. Chromatogr. A* 1218(49), 8826–8835 (2011).

28 Kula MR. Trends and future prospects of aqueous two-phase extraction. *Bioseparation* 1(3–4), 181–189 (1990).

29 Andrews BA, Nielsen S, Asenjo JA. Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. *Bioseparation* 6(5), 303–313 (1996).

30 Azevedo AM, Rosa PAJ, Ferreira IF, Aires-Barros MR. Optimisation of aqueous two-phase extraction of human antibodies. *J. Biotechnol.* 132, 209–217 (2007).

31 Rosa PAJ, Azevedo AM, Sommerfeld S, Mutter M, Aires-Barros MR, Baecker W. Application of aqueous two-phase systems to antibody purification: a multi-stage approach. *J. Biotechnol.* 139(4), 306–313 (2009).

32 Azevedo AM, Rosa PAJ, Ferreira IF, Aires-Barros MR. Integrated process for the purification of antibodies combining aqueous two-phase extraction, hydrophobic interaction chromatography and size-exclusion chromatography. *J. Chromatogr. A* 1213(2), 154–161 (2008).

33 Azevedo AM, Gomes AG, Rosa PAJ, Ferreira IF, Pisco AMMO, Aires-Barros MR. Partitioning of human antibodies in polyethylene glycol – sodium citrate aqueous two-phase systems. *Sep. Pur. Technol.* 65, 14–21 (2009).

34 Hart RA, Lester PM, Reifsnyder DH, Ogez JR, Builder SE. Large scale, *in situ* isolation of periplasmic IGF-I from *E. coli*. *Nat. Biotechnol.* 12(11), 1113–1117 (1994).

35 Boaglio A, Bassani G, Picó G, Nerli B. Features of the milk whey protein partitioning in polyethyleneglycol-sodium citrate aqueous two-phase systems with the goal of isolating human alpha-1 antitrypsin expressed in bovine milk. *J. Chromatogr. B* 837(1–2), 18–23 (2006).

36 Benavides J, Mena JA, Cisneros-Ruiz M, Ramírez OT, Palomares LA, Rito-Palomares M. Rotavirus-like particles primary recovery from insect cells in aqueous two-phase systems. *J. Chromatogr. B* 842(1), 48–57 (2006).

37 Luechau F, Ling TC, Lyddiatt A. Recovery of B19 virus-like particles by aqueous two-phase systems. *Food Bioprod. Process.* 89(4), 322–327 (2011).

38 Gomes GA, Azevedo AM, Aires-Barros MR, Prazeres DMF. Purification of plasmid DNA with aqueous two phase systems of PEG 600 and sodium citrate/ammonium sulfate. *Sep. Purif. Technol.* 65(1), 22–30 (2009).

39 Duarte SP, Fortes AG, Prazeres DMF, Marcos JC. Preparation of plasmid DNA polyplexes from alkaline lysates by a two-step aqueous two-phase extraction process. *J. Chromatogr. A* 1164, 105–112 (2007).

40 Azevedo AM, Rosa PAJ, Ferreira IF *et al.* Affinity-enhanced purification of human antibodies by aqueous two-phase extraction. *Sep. Purif. Technol.* 65(1), 31–39 (2009).

41 Rosa PAJ, Azevedo AM, Aires-Barros MR. Affinity partition of human antibodies in aqueous two-phase systems. *J. Chromatogr. A* 1162, 103–113 (2007).

42 Zijlstra GM, Michielsen MJF, de Gooijer CD, van der Pol LA, Tramper J. IgG and hybridoma partitioning in aqueous two-phase systems containing a dye-ligand. *Bioseparation* 7(2), 117–126 (1998).

43 Zijlstra GM, Michielsen MJF, de Gooijer CD, van der Pol LA. Separation of hybridoma cells from their IgG product using aqueous two-phase systems. *Bioseparation* 6(4), 201–210 (1996).

44 Barbosa HSC, Hine AV, Brocchini S, Slater NKH, Marcos JC. Dual affinity method for plasmid DNA purification in aqueous two-phase systems. *J. Chromatogr. A* 1217(9), 1429–1436 (2010).

45 Karr LJ, Shafer SG, Harris JM, Van Alstine JM, Snyder RS. Immunoaffinity partition of cells in aqueous polymer 2-phase systems. *J. Chromatogr.* 354, 269–282 (1986).

46 Sousa AF, Andrade PZ, Pirzgalska RM *et al.* A novel method for human hematopoietic stem/progenitor cell isolation from umbilical cord blood based on immunoaffinity aqueous two-phase partitioning. *Biotechnol. Lett.* 33(12), 2373–2377 (2011).

47 Persson J, Andersen DC, Lester PM. Evaluation of different primary recovery methods for *E. coli*-derived recombinant human growth hormone and compatibility with further down-stream purification. *Biotechnol. Bioeng.* 90(4), 442–451 (2005).

48 Miranda MV, Fernández-Lahore HM, Dobrecky J, Cascone O. The extractive purification of peroxidase from plant raw materials in aqueous two-phase systems. *Acta Biotechnol.* 18(3), 179–188 (1998).

49 Bensch M, Selbach B, Hubbuch J. High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. *Chem. Eng. Sci.* 62(7), 2011–2021 (2007).

50 Oelmeier SA, Dismer F, Hubbuch J. Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation. *Biotechnol. Bioeng.* 108(1), 69–81 (2011).

51 Silva DFC, Azevedo AM, Fernandes P, Chu V, Conde JP, Aires-Barros MR. Design of a microfluidic platform for monoclonal antibody extraction using an aqueous two-phase system. *J. Chromatogr. B* 1249, 1–7 (2012).

52 Rosa PAJ, Azevedo AM, Aires-Barros MR. Application of central composite design to the optimisation of aqueous two-phase extraction of human antibodies. *J. Chromatogr. A* 1141(1), 50–60 (2007).

53 Rosa PAJ, Azevedo AM, Sommerfeld S, Baecker W, Aires-Barros MR. Continuous aqueous two-phase extraction of human antibodies using a packed column. *J. Chromatogr. B* 880, 148–156 (2012).

54 Rosa PAJ, Azevedo AM, Sommerfeld S, Mutter M, Baecker W, Aires-Barros MR. Continuous purification of antibodies from cell culture supernatant with aqueous two-phase systems: from concept to process. *Biotechnol. J.* 8(3), 352–362 (2013).

55 Rosa PAJ, Ferreira IF, Azevedo AM, Aires-Barros MR. Aqueous two-phase systems: a viable platform in the manufacturing of biopharmaceuticals. *J. Chromatogr. A* 1217(16), 2296–2305 (2011).

56 Ferreira IF, Azevedo AM, Rosa PAJ, Aires-Barros MR. Purification of human immunoglobulin G by thermoseparating aqueous two-phase systems. *J. Chromatogr. A* 1195(1–2), 94–100 (2008).

57 Persson J, Nystrom L, Ageland H, Tjerneld F. Purification of recombinant proteins using thermoseparating aqueous two-phase system and polymer recycling. *J. Chem. Technol. Biotechnol.* 74(3), 238–243 (1999).

58 Persson J, Johansson HO, Tjerneld F. Purification of protein and recycling of polymers in a new aqueous two-phase system using two thermoseparating polymers. *J. Chromatogr. A* 864(1), 31–48 (1999).

59 Rabelo APB, Tambourgi EB, Pessoa A. Bromelain partitioning in two-phase aqueous systems containing PEO-PPO-PEO block copolymers. *J. Chromatogr. B* 807(1), 61–68 (2004).

60 Waziri SM, Abu-Sharkh BF, Ali SA. Protein partitioning in aqueous two-phase systems composed of a pH-responsive copolymer and poly(ethylene glycol). *Biotechnol. Prog.* 20(2), 526–532 (2004).

61 Biao N, Junfen W, Xuejun C. Preparation and recycling of aqueous two-phase systems with pH-sensitive amphiphilic

terpolymer PADB. *Biotechnol. Prog.* 25(3), 820–824 (2009).

62 Wang W, Wan JF, Ning B, Xia JN, Cao XJ. Preparation of a novel light-sensitive copolymer and its application in recycling aqueous two-phase systems. *J. Chromatogr. A* 1205(1–2), 171–176 (2008).

63 Freire MG, Claudio AFM, Araujo JMM *et al.* Aqueous biphasic systems: a boost brought about by using ionic liquids. *Chem. Soc. Rev.* 41(14), 4966–4995 (2012).

64 Li Z, Pei Y, Wang H, Fan J, Wang J. Ionic liquid-based aqueous two-phase systems and their applications in green separation processes. *Trends Anal. Chem.* 29(11), 1336–1346 (2010).

65 Oppermann S, Stein F, Kragl U. Ionic liquids for two-phase systems and their application for purification, extraction and biocatalysis. *Appl. Microbiol. Biotechnol.* 89(3), 493–499 (2011).

66 Qu F, Lue F, Zhang H. Smart polymer based aqueous two-phase systems applied in bio-molecule separation and purification. *Prog. Chem.* 22(1), 125–132 (2010).

67 Draeger NM, Chase HA. Protein adsorption in liquid fluidized-beds. In: *Advances in Separation Processes.* Davies, K (Ed.). Taylor and Francis Ltd, London, UK, 161–172 (1990).

68 Chase HA. Purification of proteins by adsorption chromatography in expanded beds. *Trends Biotechnol.* 12(8), 296–303 (1994).

69 Hjorth R, Kämpe S, Carlsson M. Analysis of some operating parameters of novel adsorbents for recovery of proteins in expanded beds. *Bioseparation* 5(4), 217–223 (1995).

70 Bartels CR, Kleimann G, Korzun JN, Irish DB. A novel ion-exchange method for the isolation of streptomycin. *Chem. Eng. Progr.* 54, 49–51 (1958).

71 Belter PA, Cunningh.Fl, Chen JW. Development of a recovery process for novobiocin. *Biotechnol. Bioeng.* 15(3), 533–549 (1973).

72 Anspach FB, Curbelo D, Hartmann R, Garke G, Deckwer W-D. Expanded-bed chromatography in primary protein purification. *J. Chromatogr. A* 865(1–2), 129–144 (1999).

73 Gagnon P. Technology trends in antibody purification. *J. Chromatogr. A* 1221, 57–70 (2012).

74 Niu HF, Wang GC, Lin XZ, Zhou BC. Large-scale recovery of C-phycocyanin from *Spirulina platensis* using expanded bed adsorption chromatography. *J Chromatogr B*, 850(1–2), 267–276 (2007).

75 May T, Pohlmeyer K. improving process economy with expanded-bed adsorption technology. *BioProcess Int.* 9(1), 32–36 (2011).

76 Doeven M, den Boer P. EBA technology: enabling the direct capture of pharma proteins. *Innov. Pharm. Technol.* 36, 66–68 (2011).

77 Fernández-Lahore HM, Kleef R, Kula MR, Thommes J. The influence of complex biological feedstock on the fluidization and bed stability in expanded bed adsorption. *Biotechnol. Bioeng.* 64(4), 484–496 (1999).

78 Fernández-Lahore HM, Geilenkirchen S, Boldt K, Nagel A, Kula MR, Thommes J. The influence of cell adsorbent interactions on protein adsorption in expanded beds. *J. Chromatogr. A* 873(2), 195–208 (2000).

79 Lin DQ, Thömmes J, Kula M-R, Hubbuch JJ. The influence of biomass on the hydrodynamic behavior and stability of expanded beds. *Biotechnol. Bioeng.* 87(3), 337–346 (2004).

80 Feuser J, Walter J, Kula MR, Thömmes J. Cell/adsorbent interactions in expanded bed adsorption of proteins. *Bioseparation* 8(1–5), 99–109 (1999).

81 Stevenson RL. Second-generation expanded bed adsorption scales from lab to plant. *Am. Lab.* 45(2), 36–39 (2013).

82 Vennapusa RR, Aguilar O, Mintong JMB, Helms G, Fritz J, Lahore MF. Biomass-adsorbent adhesion forces as an useful indicator of performance in expanded beds. *Sep. Sci. Technol.* 45(15), 2335–2344 (2010).

83 Jahanshahi M, Ghoreishi AA, Vasheghani-F E, Khavarpour M, Abedijaber A. Comparative study of hydrodynamic behavior of liquid expanded bed adsorption: mathematical and short-cut methods. *Braz. J. Chem. Eng.* 26(2), 299–306 (2009).

84 Vatanakul M, Zheng Y, Couturier M. Flow characterization of a three-phase circulating fluidized bed using an ultrasonic technique. *Can. J. Chem. Eng.* 81(6), 1121–1129 (2003).

85 Hubbuch J, Thömmes J, Kula M-R. Biochemical engineering aspects of expanded bed adsorption. In: *Technology Transfer in Biotechnology.* Kragl U (Ed.). Springer Berlin Heidelberg, Berlin, Germany, 101–123 (2005).

86 Kaleas KA, Schmelzer CH, Pizarro SA. Industrial case study: evaluation of a mixed-mode resin for selective capture of a human growth factor recombinantly expressed in *E. coli*. *J. Chromatogr. A* 1217(2), 235–242 (2010).

87 Ng MYT, Tan WS, Abdullah N, Ling TC, Tey BT. Effect of different operating modes and biomass concentrations on the recovery of recombinant hepatitis B core antigen from thermal-treated unclarified *Escherichia coli* feedstock. *J. Biotechnol.* 138(3–4), 74–79 (2008).

88 Walter JK, Feuser J. Novel approach and technology in expanded bed adsorption techniques for primary recovery of proteins at large technical scale. In: *Extended reports from the 4th International Conference on Expanded Bed Adsorption*. FL, USA, 8–11 September 2002.

89 Vennapusa R, Hunegnaw SM, Cabrera RB, Fernández-Lahore M. Assessing adsorbent-biomass interactions during expanded bed adsorption onto ion exchangers utilizing surface energetics. *J. Chromatogr. A* 1181(1–2), 9–20 (2008).

90 Vennapusa RR, Binner S, Cabrera R, Fernández-Lahore M. Surface energetics to assess microbial adhesion onto fluidized chromatography adsorbents. *Eng. Life Sci.* 8(5), 530–539 (2008).

91 van Oss CJ. *Interfacial Forces in Aqueous Media.* Taylor & Francis, Boca Raton, FL, USA (2006).

92 Lin DQ, Zhong LN, Yao SJ. Zeta potential as a diagnostic tool to evaluate the biomass electrostatic adhesion during ion-exchange expanded bed application. *Biotechnol. Bioeng.* 95(1), 185–191 (2006).

93 Vennapusa RR, Fernandez-Lahore M. Effect of chemical additives on biomass deposition onto beaded adsorbents. *J. Biosci. Bioeng.* 110(5), 564–571 (2010).

94 Tiainen P, Per-Erik G, Ljunglof A, Larsson PO. Superporous agarose anion exchangers for plasmid isolation. *J. Chromatogr. A* 1138(1–2), 84–94 (2007).

95 França dos Prazeres DM. Chromatographic separation of plasmid dna using macroporous beads. In: *Macroporous Polymers: Production Properties and Biotechnological/ Biomedical Applications.* Mattiasson, B, Kumar, A, Galaev, IY (Eds). CRC Press, FL, USA 335–361 (2009).

96 Rodrigues AE, Lopes JC, Lu ZP, Loureiro JM, Dias MM. Importance of intraparticle convection in the performance of chromatographic processes. *J. Chromatogr.*, 590(1), 93–100 (1992).

97 Benčina M, Podgornik A, Štrancar A. Characterization of methacrylate monoliths for purification of DNA molecules. *J. Sep. Sci.* 27(10–11), 801–810 (2004).

98 Krajnc NL, Smrekar F, Černe J *et al.* Purification of large plasmids with methacrylate monolithic columns. *J. Sep. Sci.* 32(15–16), 2682–2690 (2009).

99 Zöchling A, Hahn R, Ahrer K, Urthaler J, Jungbauer A. Mass transfer characteristics of plasmids in monoliths. *J. Sep. Sci.* 27(10–11), 819–827 (2004).

100 Gerster P, Kopecky E-M, Hammerschmidt N *et al.* Purification of infective baculoviruses by monoliths. *J. Chromatogr. A* 1290, 36–45 (2013).

101 Kramberger P, Honour RC, Herman RE, Smrekar F, Peterka M. Purification of the *Staphylococcus aureus* bacteriophages VDX-10 on methacrylate monoliths. *J. Virol. Methods* 166(1–2), 60–64 (2010).

102 Smrekar F, Ciringer M, Štrancar A, Podgornik A. Characterisation of methacrylate monoliths for bacteriophage purification. *J. Chromatogr. A* 1218(17), 2438–2444 (2011).

103 Podgornik A, Barut M, Štrancar A, Josić D, Koloini T. Construction of large-volume monolithic columns. *Anal. Chem.* 72(22), 5693–5699 (2000).

104 Urthaler J, Buchinger W, Necina R. Industrial scale cGMP purification of pharmaceutical grade plasmid-DNA. *Chem Eng. Technol.* 28(11), 1408–1420 (2005).

105 Urthaler J, Schlegl R, Podgornik A, Štrancar A, Jungbauer A, Necina R. Application of monoliths for plasmid DNA purification development and transfer to production. *J. Chromatogr. A* 1065(1), 93–106 (2005).

106 Bandeira V, Peixoto C, Rodrigues AF *et al.* Downstream processing of lentiviral vectors: releasing bottlenecks. *Hum. Gene Ther. Method* 23(4), 255–263 (2012).

107 Kramberger P, Peterka M, Boben J, Ravnikar M, Štrancar A. Short monolithic columns – a breakthrough in purification and fast quantification of tomato mosaic virus. *J. Chromatogr. A* 1144(1), 143–149 (2007).

108 Smrekar F, Ciringer M, Peterka M, Podgornik A, Štrancar A. Purification and concentration of bacteriophage T4 using monolithic chromatographic supports. *J. Chromatogr. B* 861(2), 177–180 (2008).

109 Urbas L, Jarc BL, Barut M *et al.* Purification of recombinant adenovirus type 3 dodecahedric virus-like particles for biomedical applications using short monolithic columns. *J. Chromatogr. A* 1218(17), 2451–2459 (2011).

110 Kalia S, Kaith BS, Kaur I (eds.) *Cellulose Fibers: Bio- and Nano-Polymer Composites.* Springer-Verlag Berlin Heidelberg (2011).

111 Hardick O, Dods S, Stevens B, Bracewell DG. Nanofiber adsorbents for high productivity downstream processing. *Biotechnol. Bioeng.* 110(4), 1119–1128 (2013).

112 Lewin M. *Handbook of Fiber Chemistry.* CRC Press, FL, USA (2006).

113 Wakelyn PJ, Bertoniere NR, French AD *et al. Cotton Fiber Chemistry and Technology.* CRC Press, FL, USA (2006).

114 Borsa J, Tóth T, Takács E, Hargittai P. Radiation modification of swollen and chemically modified cellulose. *Radiat. Phys. Chem.* 67(3–4), 509–512 (2003).

115 Gavara PR, Cabrera R, Vennapusa RR, Grasselli M, Fernandez-Lahore M. Preparation, characterization, and process performance of composite fibrous adsorbents as cation exchangers for high throughput and high capacity bioseparations. *J. Chromatogr. B* 903, 14–22 (2012).

116 Du KF, Yan M, Wang QY, Song H. Preparation and characterization of novel macroporous cellulose beads regenerated from ionic liquid for fast chromatography. *J. Chromatogr. A* 1217(8), 1298–1304 (2010).

117 Keener RN, Fernandez EJ, Maneval JE, Hart RA. Advancement in the modeling of pressure-flow for the guidance of development and scale-up of commercial-scale biopharmaceutical chromatography. *J. Chromatogr. A* 1190(1–2), 127–140 (2008).

118 King JK, Pinto NG. Short fibrous supports for preparative chromatographic separations of biomolecules. *J. Chromatogr.* 609(1–2), 61–68 (1992).

119 Singh A, Pinto NG. Polymeric short-fiber chromatographic supports for downstream processing of biomolecules. *React. Polym.* 24(3), 229–242 (1995).

120 Gura T. Magic bullets hit the target. *Nature* 417(6889), 584–586 (2002).

» Patents

201 Development Center For Biotechnology: US5462863A (1995).

202 M. Fernandez-Lahore and M. Grasselli: WO2008107196 (2008).