Review

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Perspectives on adsorption technology as an effective strategy for continuous downstream bioprocessing

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Abstract

The biological industry, but also biorefining platforms in the context of the circular economy, have been fertile terrains for the development of a palette of novel products of increasing structural diversity and fields of application. To mention only a few of the more complex bioproducts, we can consider monoclonal antibodies, plasmid DNA, virus-like particles and exosomes. Of course, some products of industrial relevance are much simpler in nature. Irrespective of the product class and field of application, a shift from batch processing to continuous manufacturing is currently happening. This is due to the fact that operating in the continuous mode can result in process intensification, and therefore increased productivity with lower environmental impact can be realized. Some biological production schemes, e.g. those based on perfusion cultures, are well known in the pharmaceutical industry. Likewise, continuous fermentation is now utilized to produce ethanol from biomass. In many cases, continuous downstream operations are desired but difficult to implement; there is a pressing need to better understand the available options for product recovery and purification. Some traditional methods require re-evaluation in modern contexts and some others, of experimental nature, require further improvement before actual implementation. In this article, we present an overview of the most promising methods that would allow for robust downstream bioprocessing options in the near future – with a focus on adsorption technologies.

Keywords: adsorption; chromatography; wool-based fibres; membranes; aqueous two-phase systems

ABBREVIATIONS

AEX	anion-exchange column
ATPS	aqueous two-phase systems
CCTC	counter-current tangential chromatography
DBC	dynamic binding capacity
DX	dextran
EBA	expanded bed adsorption
HCP	host cell protein
LC	liquid chromatography
LSCFB	liquid–solid circulating fluidized bed
mAb	monoclonal antibody
MCSGP	multicolumn counter-current solvent gradient purification
MSFB	magnetically stabilized fluidized bed
MW	molecular weight
MWCO	molecular weight cut-off
PCC	periodic counter-current
PDVF	polyvinylidene fluoride
PEG	polyethylene glycol
PES	polyethersulfone
PPG	polypropylene glycol
PS	polysaccharide
SBC	static binding capacity
SC	sodium citrate
SMB	simulated moving bed
SPTFF	single-pass tangential flow filtration
TF	tangential flow
UF	ultrafiltration
VLP	virus-like particle capacity

INTRODUCTION

Downstream processing, defined as the dovetailed unit operations required for the product recovery and purification from biological resources, has always been a bottleneck during biomanufacturing. Challenges were encountered in the mid-1940s, when penicillin was produced by fungal fermentation on an industrial scale, in the early 1980s, when the modern

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biopharmaceutical industry was established, and at the turn of the century, with massive amounts of monoclonal antibodies being produced in mammalian cell cultures. This challenge persists today, with a variety of products and feedstocks to be processed in the continuous mode of operation. Nowadays, large amounts of bioproducts must be purified without affecting biological structure and activity.

An analysis of commonly deployed methods for downstream bioprocessing will indicate that a few robust operations are preferred, depending on the nature of the product under consideration. Operations like precipitation and crystallization, which are useful with organics, are only exceptionally employed with labile macromolecules – although some notable exceptions exist. Proteins and enzymes are preferentially processed also by the partition of adsorption, including preparative chromatography when high resolution is needed. These methods can also be applied to pDNA or mRNA, a type of product of more recent addition to the therapeutic portfolio. Large entities, such as virus-like particles (VLPs) and nanoplexes, can also be recovered via adsorption and partition, but materials and systems of exceptional characteristics are required to that end.

In this review, we have considered methods that are suitable for continuous downstream bioprocessing operations. In doing so, we have concentrated our vision on the development of resilient adsorption-based approaches. Some adsorption-based systems, to operate in a continuous or semi-continuous manner, will require the integration into hybrid schemes, where other methods, like ultrafiltration, can play an important role. Therefore, operations that are ancillary to adsorptive bioproduct recovery or that are competing options are also briefly presented. Some products, such as polysaccharides, are rarely amenable to recovery via adsorption owing to their characteristic very high molecular weight and complex rheological behaviour, thus typically necessitating tangential-flow operations for satisfactory processing.

Porous adsorbent beads have been traditionally employed in the form of packed beads in process chromatography. The prototypical structural and functional features of such beaded adsorbents are briefly reviewed below. However, as will also be presented to the reader, there is now a trend to depart from this picture -into adsorbents that are fibrous in nature or that are fluidized rather than packed. These changes are helping practitioners to adapt adsorptive processes with enhanced possibilities, including the ability to work in continuous mode with systems that are biomass tolerant (integration) and sustainable (intensification).

The concept of (downstream) process integration, as already proposed by Prof. Maria-Regina Kula in Düsseldorf (Germany) in the 1980s, entails the development and implementation of novel unit operations that can replace the use of several conventional ones, thereby creating enhanced performance and cost advantage. The key to integration is the recovery of the product in the presence of biomass, as demonstrated in the case of aqueous two-phase extraction or fluidized/classified (expanded) bed chromatography.

On the other hand, (downstream) process integration refers to designs that allow for processing operations to take place with equipment of reduced dimensions while keeping mass/time productivity. In this case, the feedstock is continuously refined. This concept was proposed in the 1990s by Luuk van der Wielen in Delft (The Netherlands) and, since then, has gained acceptance in the UK and the continent. This technological approach is exemplified by operations such as simulating bed chromatography, counter-current adsorption or rotating annular chromatography, to mention a few.

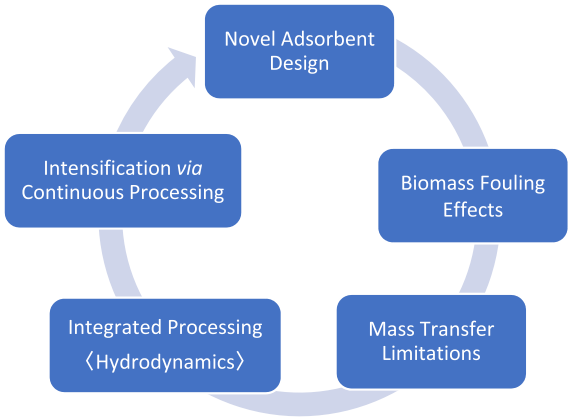

Figure 1. Design principles for biomass-tolerant continuous adsorption systems.

Figure 1 conceptually depicts our approach to the synthesis of advanced adsorptive processes that would allow for both process integration and intensification. Novel adsorbents can be designed based on traditional beads with new properties (e.g. higher material density, biomass fouling tolerance, ligand-less affinity) or with new materials (composite fibres, adsorptive belts). There is a definitive link between material development and process performance, including synergy between engineered adsorbents and well-designed processing schemes.

Summarizing, this paper presents to the reader an overview of the current alternatives for the recovery and purification of valuable bioproducts. Methods and techniques that are apt for continuous processing are highlighted with a preference in adsorptive processing. This paper is an integral part of an issue devoted to the topic of continuous biomanufacturing where relevant topics that are of scientific and practical interest are covered.

ADSORPTION AND CHROMATOGRAPHY

Characteristics of beaded adsorbents

Spherical particles manufactured from cross-linked polymers such as agarose, methacrylate, acrylamide or polystyrene–divinylbenzene can be applied to purify various bioproducts by employing ion-exchange, affinity or hydrophobic interaction chromatography.[1] Protein adsorption (Fig. 2) in chromatography is mainly governed by convection or diffusion; in certain cases, external forces such as gravitation and electrophoresis can play a role. In a second phase diffusion specific forces, which are of electrostatic origin, govern the transfer of particles through the adsorption boundary layer. Finally, a physicochemical interaction arises between the adsorbed molecule and the interface or previously adsorbed molecules. In addition to electrostatic and Van der Waals forces, hydrogen bonding could also be relevant.[2]

During the chromatographic separation of large species (e.g. macromolecules), transport resistance at the pore level is the dominant contribution to the total mass transfer resistance; porosity is highly dependent on the structural characteristics of the adsorbent beads. Pore connectivity (tortuosity) defined as the number of bonds (pores) connected to a single node is an essential parameter in the characterization and construction of porous particles.[3]

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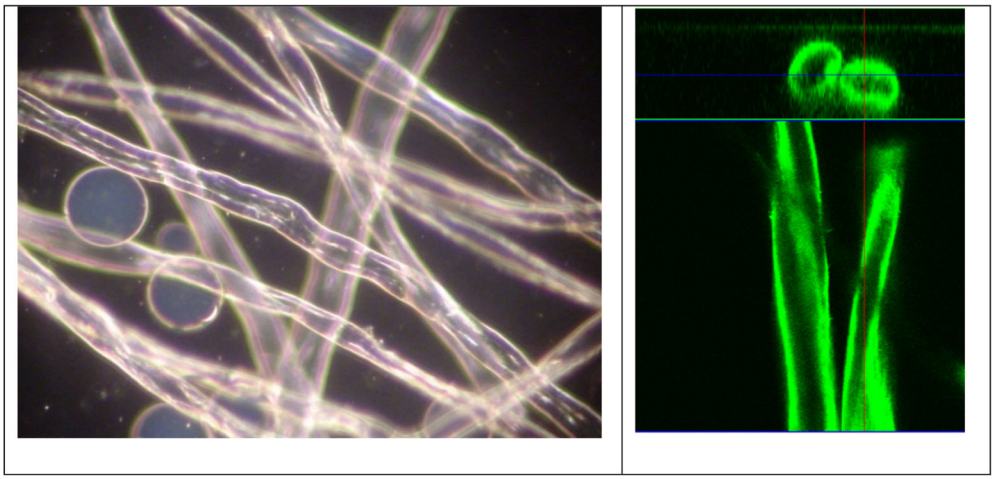

Figure 2. Contrast field and confocal microscopic images of adsorbent beads and adsorbent composite fibres. Confocal microscopy reveals the presence of an active zone that is accessible for effective protein binding (IgG).

Systems for continuous processing using beaded adsorbents

The switch from batch to continuous chromatography has been predominantly carried out using counter-current multicolumn chromatography.[4] Simulated moving bed (SMB) chromatography is operated with two or more columns through which the mobile phase is flowing continuously while the solid phase flow is simulated by periodically shifting the columns opposite to the fluid phase.[5-7] SMB improves the utilization of the stationary phase by achieving loadings much closer to the static (maximum) binding capacity of the resin in comparison to batch chromatography and reduces column volume – thereby adding to the intensification effect.[6] SMB chromatography has been applied to continuous purification of carbohydrates, carboxylic and organic acids, proteins, viruses and monoclonal antibodies (mAbs).[5-12]

Similar to the case posed by SMB, periodic counter-current chromatography (PCC) operates cyclically by employing a column switching strategy that leads to continuous processing of the feed stream, while product elution from each column is discrete and periodic. A PCC system is operated by overloading the first column and capturing the breakthrough on a second column, leading to increased resin utilization in comparison to batch chromatography. The binding capacity of the resin determines the length of the load step and, therefore, the size and number of columns required for a given process are dependent on it.[13,14] The continuous capture of recombinant enzymes and therapeutic proteins has been already demonstrated with this system.[13-15]

Compared to continuous PCC and SMB approaches, counter-current tangential chromatography (CCTC) allows the loading and elution of a product in a truly continuous mode, under steady-state conditions. In CCTC, all chromatographic steps are performed simultaneously by flowing the resin slurry through a cascade of static mixers and hollow fibre membranes that retain the large resin particles while allowing dissolved species such as proteins to flow through. Additionally, the need for cleaning and cleaning validation can be eliminated with CCTC since it uses a single-use flow path. This method has been successfully applied for the post-capture processing of mAbs[16] (Table 1).

Multicolumn counter-current solvent gradient purification (MCSGP) is a counter-current multicolumn technique specifically designed to be used during product polishing, where several difficult-to-remove impurities could be present.[17] The operational principle of MCSGP is based on the internal recycling of eluted fractions that contain such impurities; this can achieve increased yields while maintaining high purity. The design of an MCSGP process is based on a 'design chromatogram', which is obtained from single-column batch operation.[17,18]

Continuous processing with unclarified feedstock

The utilization of adsorbent beads in batch and continuous operation is typically performed with a particle-free feedstock and, therefore, extensive clarification is required before initiating adsorptive product capture. However, product sequestration from an unclarified feedstock has been shown possible. Expanded bed adsorption (EBA) is a chromatographic technique allowing simultaneous feedstock clarification and product capture (i.e. partial purification and concentration possible). In EBA a bed of fluidized particles is implemented in such a manner that the local mobility of said particles is reduced – thereby creating several equilibrium stages ('plates') akin to packed-bed chromatography. The EBA technique has found some applications in the recovery of enzymes and therapeutic proteins from a variety of expression hosts.[19-22] However, the presence of biomass in contact with the adsorbent beads creates additional challenges, including hydrodynamic and mass-transfer limitations.[23,24] Interestingly, the continuous purification of proteins from unclarified feedstock with EBA technology was demonstrated using four counter-current contactors for extraction, adsorbent washing, elution and re-equilibration of the adsorbent.[25,26]

Magnetically stabilized fluidized beds (MSFBs) have the characteristics of expanded beds, low back-mixing and plug flow liquid motion, which are achieved by applying a uniform magnetic field to stabilize magnetically susceptible particles.[27,28] MSFBs have been investigated as an alternative for continuous protein separation; however, for large-scale applications inexpensive magnetic carriers and separators are needed.[29]

Liquid–solid circulating fluidized bed (LSCFB) systems allow continuous protein purification by carrying out product adsorption in a downcomer while desorption takes place in a riser column;[30,31] this system has been demonstrated based on ion-exchangers (Fig. 3). The process runs in defined steps: (i) the feed

Table 1. Examples of continuous purification systems based on fibrous adsorbents

System	Solid phase	Functionality	Target product	Reference
Beaded materials				
SMB	PSDB	SEC	galacto-oligosaccharide	5
	Agarose	SEC	Adenovirus serotype 5	6
	Agarose	Protein A	mAb (recombinant IgG_1)	7
	MA			
	PVPy	WAX	Acetic acid	9
	Agarose	SEC	Human influenza virus	10
	Agarose	IMAC	His-tagged β-glucosidase	11
	Agarose	SP	lactoperoxidase and lactoferrin	8
PCC	Agarose	Protein A IMAC/HIC	mAb rhEnzyme proteins	15
CCTC	Agarose	Protein A	mAb	16
MCSGP	Hydrophilic porous polymer	Sulfobutyl-	Human IgG	18
EBA	Perfluorocarbon	Procion Red HE-78	Malate dehydrogenase	25,26
LSCFB	SDVB	AEX	Whey protein	30
Fibrous materials				
SMB	Electrospun cellulosic nanofibres	Diethylaminoethyl	Bovine serum albumin	45
Moving adsorption belt system	Cotton cloth	Phosphoric group	Copper io ("n" is right)	54
	Unemulsified Mylar	Rabbit antisera	Human placental alkaline phosphatase	55
	Nylon 66	Soybean trypsin inhibitor	T (Trypsin)	56

PSDB, polystyrene–divinylbenzene; SEC, size exclusion chromatography; MA, methacrylic polymer; PVPy, polyvinylpyridine; IMAC, iminodiacetic acid; WAX, weak anion exchanger; SP, sulfopropyl-; HIC, hydrophobic interaction; SDVB, styrene–divinylbenzene; DEAE, diethylaminoethyl; BSA, bovine serum albumin; STI, soybean trypsin inhibitor; PAP, placental alkaline phosphatase; MBAS, moving adsorption belt system.

containing the product enters from the bottom part of the downcomer, contacting downward moving adsorbent particles, (ii) which after adsorption are transferred to the riser, operated at a superficial fluid velocity greater than the terminal settling velocity of the adsorbent particles, and (iii) for them to reach the top part equipped with a liquid–solid separator. The main advantages of the LSCFB system include the integration of two columns into one system and process intensification achieved by high liquid–solid contact efficiency.

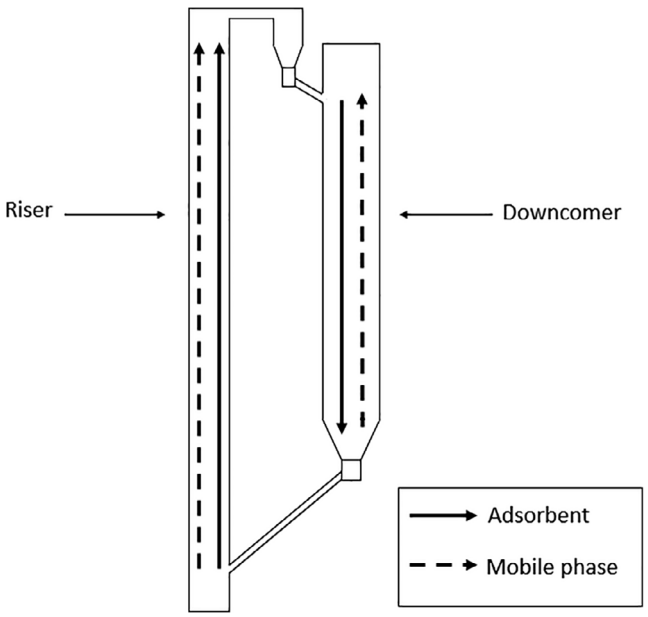

Figure 3. Schematic of liquid circulating fluidized bed in modified from.[30]

Although the systems based on fluidized bed technology were able to purify target proteins continuously, the processes were incapable of increasing the concentration of the targeted protein, and the necessary pumping and falling of adsorbent set mechanical constraints on adsorbent design. There is a need for further development for full industrial implementation of such systems. Recent work in our labs has demonstrated that it is possible to improve the performance of continuous systems based on fluidized bed technology and achieve increased target protein concentration by modifying the process scheme and developing novel adsorbent materials.

NON-CONVENTIONAL ADSORBENTS IN PROCESS BIOTECHNOLOGY

Types and characteristics of fibrous adsorbents

Since the pioneering studies performed by Ventura *et al.* and Gavara *et al.*, who proposed the utilization of composite adsorbent fibres for protein chromatography, additional work has been directed towards the synthesis, characterization and performance testing of this new type of adsorbent in downstream

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bioprocessing.[32,33] Although fibrous adsorbents suffer from packaging inhomogeneity that could derail efforts in the context of analytical chromatography, particularly when working with organics, the potential of such a system for the preparative-scale separations of macromolecules is now recognized.[34,35]

Fibrous adsorbents can be attractive in biomanufacturing due to cost-effectiveness, physical and chemical robustness, and the available options of surface chemistries.[36] Chromatography systems employing fibrous adsorbents, including disposable cartridges, allow for product binding in a context of dominating convection and limited mass transfer limitations. This is achieved, in some cases, due to the pellicular nature of the adsorbents and the high voidage of the resulting bed.[32] High operational flow rates, excellent resolution and low back pressure can be achieved.[37] Moreover, scale-up is generally feasible.[38] Composite fibres, comprising a fibrous backbone and other elements such as carrier of the function or preformation enhancer, are constructed by covalent chemical reactions or physical interactions.[33] The decent mechanical arrangement of the composite fibres can result in good convective mass flows.[39] Well-designed functional group conjugation can result in low-cost alternative materials to prepare disposable devices for immunoglobulin purification.[40] The limitation of some types of fibrous adsorbent systems is a relatively small surface area (~10 m^2 g^{-1}),[41] mainly governed by the fibre diameter comparing to bead-based chromatographic adsorbents (~50 m^2 g^{-1})[42] and/or limited reactive sites for bulk bioproducts.

Adsorbent materials based on natural fibres and composites thereof

Cellulose-based fibres. Cellulose, the most copious natural fibre with nearly 1012 tons output annually, is mechanically robust, light, inexpensive, renewable and water insoluble.[43] Their hydroxyl-rich nature results in outstanding biocompatibility, hydrophilicity and feasible functionalization.[36] The material presents high porosity, high swelling capacity and relatively high surface area – which might translate to greater efficiencies and loading capacities when utilized as an adsorbent after chemical modification or composite formation. For example, early studies from Hoffpauir and Guthrie demonstrated the application of sulfoxylated and quaternary ammonium functionalized cellulose fibres in liquid chromatography to attempt protein separations.[44] Moreover, cellulose as a precursor for cellulose acetate becomes the basis for adsorbents employed in groundwater treatment, e.g. to eliminate pollutants such as metals, phenols, dyes and pesticides.[43] Hardick and his co-workers developed a diethylaminoethyl (DEAE)-derivatized cellulose acetate nanofibre conveying superior operating performance in terms of permeability and fouling resistance in comparison with conventional beaded adsorbents. This material has now developed into commercially available nanofibers for industrial R&D and – potentially – large-scale biomanufacturing.[45] Singh and co-workers have developed a Q functionalized epoxy grafted adsorbent from natural cotton with approx. 48 g L^{-1} dynamic binding capacity (DBC at 300 cm h^{-1}) when using bovine serum albumin (BSA) was used as a model protein.[37] Even when high operational flow rates were applied, high binding capacity values were maintained, thus offering faster process time and improved productivity. A functionalized sulfonate group composite fibrous adsorbent (SP-gPore) has been investigated; this composite offered homogenous grafting (~30%) and an internal hydrogel.[33] Columns that were packed randomly or utilizing a designed double roll architecture demonstrated high-resolution ability at high operational flow rates (up to 900 cm h^{-1}) and allowed for shorter gradient development routines. Experimentally determined Peclet number values were within the range 60–90, suggesting a close-to-plug-flow condition.

Wool-based fibres. Wool, a proteinaceous fibre harvested mainly from sheep, is another widely studied natural fibrous material. Carboxyl and amino groups from amino acids are available on the surface naturally ensuring its application as a weak anion or cation exchanger. Depending on the precise amino acid content, π–π interactions may interfere with the separations if utilized directly.[35] For example, second-hand knitted textiles (85% wool) could be employed to uptake Pb^{2+} ions from wastewater. With no previous chemical treatment, static binding capacity (SBC) was 4.76 mg g^{-1} at initial Pb^{2+} ion concentrations less than 200 mg L^{-1}.[46] However, the surface properties of adsorbents made from wool are highly dependent on the origin and processing. The natural variability of this material has hindered its further application in the context of process biotechnology. However, there is an increased interest in using protein-based building blocks for material engineering; this is an approach that deserved further exploration.

Adsorbents based on synthetic polymeric fibres

The primary driving force for the development of SPF is their chemical robustness and stability from mass production. By the year 2019 the amount of production of polyester fibres across the globe reached 58 million metric tons.[47] This material can be cost efficient in the context of process chromatography, even if the base material must be standardized and chemically functionalized.[35] Some features of polyester fibres that require attention are cross-sectional shape, number of filaments, linear density and bicomponent nature. Likewise, important characteristics of these materials include evenness, porosity, thickness, mechanical strength and air permeability.[48] Most of the mentioned considerations also apply to other types of synthetic fibres.

Awual and co-workers fabricated a weak-base anion-exchange fibrous adsorbent from polyethylene with primary amino groups for selective and rapid uptake of arsenate species from groundwater.[49] The DBC was 0.298 mmol g^{-1} with the possibility of repeated use. It reported the use of short fibres derived from a wet-spun polysulfone base polymer as anion exchanger for separation of BSA and β-lactoglobulin in randomly slurry packed into liquid chromatography (LC) columns with superior DBC (350 mg g^{-1}).[50] No limit to the elution flow rate and up to 12 mg mL^{-1} protein feedstock concentrations was reported. A sulfopropyl-functionalized fibrous adsorbent based on winged-shaped a nano-channel polyethylene terephthalate (PET) filament from Allasso Industries (Raleigh, NC, USA) with more than tenfold higher surface area over a standard round filament was studied[51]; high SBC (90 mg mL^{-1}) and DBC (50 mg mL^{-1}) for lysozyme were found, almost independent of the bed residence time, revealing a fast mass-transport mechanism. The hollow fibre with introduced sulfonic group was assessed by Ventura *et al.*[32]; maximum SBC for lysozyme was 140 mg mL^{-1}. Respective cartridges (volume of 0.42 and 3.5 mL) were built with merely 10–15 min process time for high-purity lysozyme (95%), high productivity (150 g L^{-1} h^{-1}) and no size-exclusion effect.

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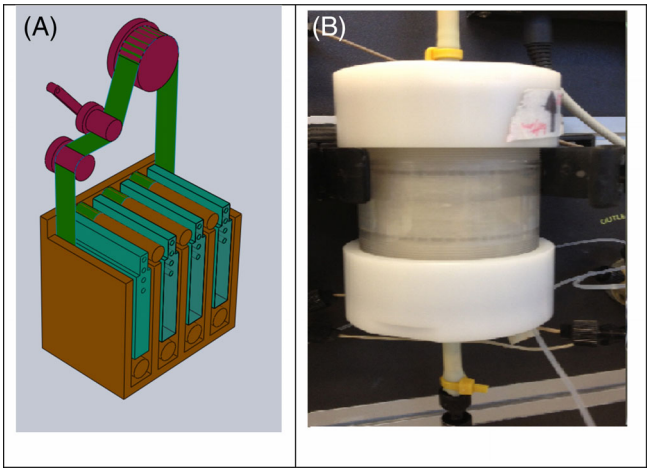

Figure 4. Fibrous adsorbents can be utilized in a variety of forms and systems: (A) affinity moving belt prototype;[56] (B) disposable cartridge containing packed ion-exchange fibres.[32]

Systems for continuous processing using fibrous adsorbents

An increasing focus on biomanufacturing costs and performance has triggered the search for more efficient and safe bioprocessing schemes. In this direction, continuous processing is flourishing as a promising alternative to reduce the expenses associated with advanced bioprocessing while enhancing process control options.[52] This is resulting in better stability and consistency of manufactured bioproducts, increased bioprocess flexibility and responsiveness, economy of consumables and fungible materials, and improved overall sustainability.[53] In the case of chromatographic operations, continuous processing allows for efficient utilization of expensive adsorbent media, thus reducing the total amounts required to produce a certain mass of product. If, in addition, one can deploy an adsorbent of moderate cost (fibres) much could be achieved from the synergy achieved by the utilization of fibrous adsorbents in the continuous mode of operation.

DEAE-functionalized cellulosic nanofibres were combined with SMB for the separation of model proteins (BSA and cytochrome c). The SMB system exploited the advantageous convective mass transfer properties of nanofiber adsorbents to provide productivity of 3.92 g mL^{-1} h^{-1}, which is considerably higher than what is normally observed with conventional beaded media.[52]

A radically different approach has involved the use of a moving belt. The belt, made of a material of an adsorptive nature, is mechanically driven to cycle between loading (adsorption), washing, desorption and regeneration compartments. For example, in an early study, Muendel and Selke utilized phosphorylated cotton cloth as an adsorbent to reclaim Cu^{2+} from a diluted solution. The actual exposure of the belt to streams of $CuSO_4$ (feed) and NaCl (regeneration) aqueous solution was carried out in shallow tanks containing baffles. In such a system, the observed SBC was 3.39 mEq g^{-1} with a belt rate of 1.0 in. per minute; the total time required for a run was close to 10 h.[54]

Hughes and Charm demonstrated another application of the moving belt approach, this time employing a four-section tank for effective contact and an affinity-functionalized belt. In this application, the affinity system consisted of rabbit antisera immobilized onto a Mylar belt to purify human placental alkaline phosphatase.[55]

Niven and Scurlock employed a soybean trypsin inhibitor immobilized onto a Nylon 66 belt to purify trypsin (Fig. 4). A four-chamber tank with inserts in each of the chambers facilitated a smooth operation with the transporting (affinity) belt. An apparent trypsin transfer capacity of approx. 1–2 U m^{-2} was obtained with this ingenious system, while the specific activity of the final product was 10–40 U mg^{-1}. Some limitations observed during the mentioned studies include excessive mixing in the liquid phase and partial elution of product from the solid phase, thus creating a diluted stream of recovered enzyme.[56]

The mentioned option for the utilization of fibrous adsorbent and others, like those based on rotational cartridge manifolds, deserve further optimization efforts. These must be directed towards the development of novel materials, as well as the exploration of new hardware design.

DOVETAILING AND EXPANDING ADSORPTION PROCESSES

Ultrafiltration membranes: characteristics and applications

Ultrafiltration (UF) is a pressurized membrane separation technique, which allows the separation of molecular species by size, depending on the molecular weight cut-off (MWCO) that is characteristic of the selected membrane. UF is usually employed in bioprocessing for fractionation, concentrate and buffer exchange; in the latter case, the process is referred to as diafiltration.

The advances observed in recent years regarding UF technology have led to the development of sophisticated membranes that enable selective separation of saccharides with molecular weights as low as 3 kDa.[57,58] Ultrafiltration membranes can be supported in modules of various shapes and construction designs, including tubular modules, hollow fibres, spiral-wound cartridges and plate-and-frame devices.[59,60]

Tangential flow ultrafiltration systems can be used for the recovery of polysaccharides (PS). Sterner and Gröndahl used cross-flow filtration to concentrate laminarin from *Saccharina latissimi*, retrieving it from the dilute acid solution of the acid pre-treatment of an alginate extraction. The filtrations were performed in a pilot-size filtration unit, and three ceramic membranes with 5, 15 and 50 kDa MW cut-offs were used (ZrO_2/TiO_2, TiO_2–Al_2O support, 0.08 m^2). The filtration studies showed that a good method was to pre-filter through a 50 kDa membrane at ~5 m s^{-1} and a high feed pressure of 2 bar. In the 5 kDa membrane filtration, the laminarin is concentrated in the feed, at ~5 m s^{-1} and a high feed pressure of 5 bar. Under these conditions, the laminarin passage ratio began at 15% but reached 7% after 24 h.[61]

Another group has investigated in detail the UF behaviour of polysaccharides from rapeseed. Four different commercial membranes made of polyvinylidene fluoride (Kynar) and polyethersulfone (PES) were used. After the pre-treatment of rapeseed, the resulting solution was subjected to membrane fractionation using three different MWCO values (3, 8 and 12 kDa). Experimental results indicated that employed parameters such as transmembrane pressures (1 bar), feed pH (>7) and temperature (40–50 °C) greatly improved the permeate flux. It was observed that the 3 kDa MWCO systems ensured the highest recovery and purity.[58]

Tangential flow (TF) UF is an attractive technique for virus removal from bioproducts because of its high throughput and ambient conditions.[62] As an interesting example, NoV GII-4 VP1 (norovirus) protein was expressed in a recombinant baculovirus system using Sf9 insect cells. Several methods for purification

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and concentration of VLPs were evaluated. It was shown that repeated sucrose gradient purification followed by ultrafiltration resulted in intact VLPs with excellent binding to H type 3 antigens. VLPs were stable for at least 12 months at 4 °C, and up to 7 days at ambient temperature.[63]

The large-scale purification of viruses and viral vectors for applications in gene therapy and viral vaccines is a major separation challenge. Here, TF microfiltration and UF (using flat sheet membranes) have been investigated for the concentration of the human influenza A virus. UF membranes with MW cut-offs of 100 and 300 kDa as well as 0.1, 0.2 and 0.45 µm microfiltration membranes have been tested. It was shown that use of 300 kDa membranes concentrates the virus particles and leads to significant removal of host cell proteins and DNA in the permeate. As human influenza A virus particles have a spherical form with an average size of 100 nm, a 0.1 µm membrane can be applied to fractionate virus particles.[64]

TF UF is also useful in the processing of organics. For example, UF was utilized in the fractionation of phenolic compounds from subcritical water grape pomace extract. The extract was achieved in a cross-flow apparatus against 11 membranes with MW ranging from 100 to 2 kDa. Except for the separation obtained between polymeric and monomeric proanthocyanidins, polysulfone membranes were not able to fractionate phenolic classes. Membranes starting at 20 kDa over-retained high percentages (>60%) of polysaccharides and proteins.[65]

Systems for continuous processing and cross-flow filtration

As briefly mentioned before in this article, in a cross-flow filtration scheme the feedstock flow is applied tangentially across the membrane surface. In this manner, particles (microfiltration) or molecules (ultrafiltration) can be concentrated at the retentate side or collected in diluted form at the permeate side. Recycling of the retentate into the feed (concentration) and buffer exchange (diafiltration) are possible, depending on the desired outcome. In continuous filtration, the cross-flow scheme is preferred to minimize filtration cake (microfiltration) or gel layer (ultrafiltration) formation (Fig. 5).[59,66] In a few instances, continuous microfiltration is also possible in a dead-end mode, as is the case for mycelium filtration during penicillin production.

UF is particularly attractive for continuous manufacturing because of limited energy consumption and high efficiency; a feedstock can be processed with minimal – if any – pre-conditioning.[67] However, UF separation power is limited to product size, which makes the method appropriate for fractionation but not purification. Therefore, membrane technology can be seen as an ideal ancillary operation for adsorption and chromatography.

The combination of membrane filtration with other separation techniques into hybrid systems has many advantages in terms of enhancing purity, reduction of membrane fouling, lower facility footprint and reduction of operating costs. Hammami *et al* studied the continuous hybrid adsorption–UF process, which was applied to colour removal from Acid Orange (AO7) aqueous solution. The best operating conditions for total removal of colour were 3 bar TMP, 5 pH and PAC dose above 150 mg L^{-1} in the presence of cationic surfactant.[68]

Elich *et al*. evaluated the performance of the combination of single-pass tangential flow filtration (SPTFF) concentration before anion-exchange column (AEX) chromatography for an intensified mAb polishing step. Results showed that pre-concentration of AEX feed material improved isotherm conditions for host cell protein (HCP) binding, resulting in a fourfold increase in resin mAb loading at the target HCP clearance level. Excellent clearance of minute virus of mouse and the xenotropic murine virus was maintained at this higher load level. The increased mAb loading enabled by SPTFF pre-concentration effectively reduced AEX column volume and buffer requirements, shrinking the overall size of the polishing step.[69]

Membrane operations, as is the case for direct capture methods, can be severely limited by material fouling by the biological components that are normally present in a complex feedstock. Material engineering and polymer shielding can offer a solution to these drawbacks.[70,71] Some approaches to improve membrane characteristics vis-à-vis foulants are the use of polymers with high hydrophilicity, polymer grafting, nano-structuring, and ad hoc functionalization.[72,73]

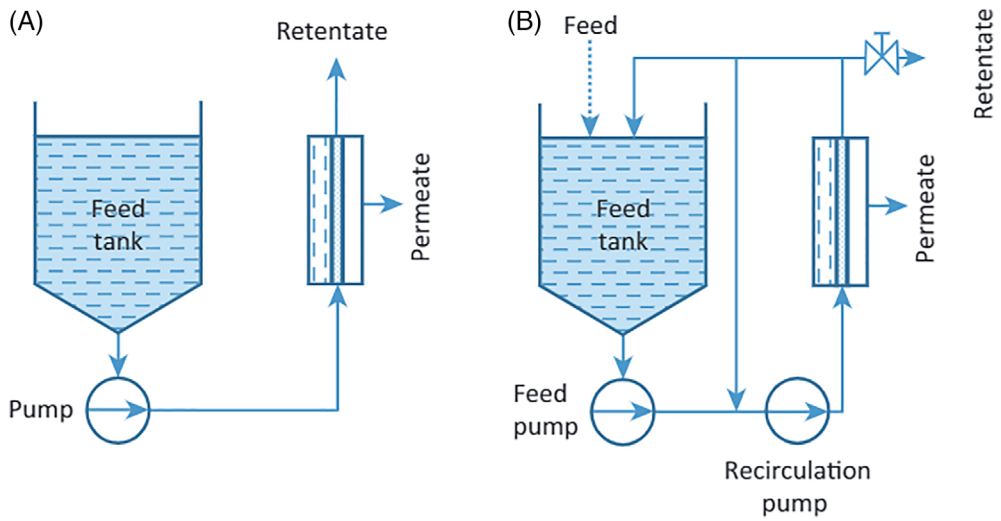

Figure 5. Schematic diagram of batch and continuous membrane filtration: (A) single-pass cross-flow filtration; (B) continuous membrane filtration with partial recycling of the retentate.[96]

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ALTERNATIVES TO ADSORPTION

Partitioning in aqueous two-phase systems

Over the last decade, aqueous two-phase systems (ATPSs) regained interest owing to their potential to integrate downstream operations. ATPS is a liquid–liquid partitioning technique with applications in the extraction, separation, purification and enrichment of proteins, membranes, viruses, enzymes, nucleic acids and other bioproducts.[74-76] In general, polymer–polymer systems are preferably used for the separation and recovery of solutes sensitive to the ionic environment as these systems pose low ionic strength, but polymer/salt systems are preferred industrially.

ATPSs can be formed using biodegradable and biocompatible components – polymers and salt – providing a gentle aqueous environment for bioseparations.[77] Two-polymer systems, especially poly(ethylene glycol) (PEG)/polypropylene glycol (PPG)/dextran systems (DX), have found numerous applications[78,79] for the recovery of enzymes and proteins, but also low-molecular-weight compounds and particles. Other inexpensive polymer alternatives to PEG, e.g. ethylene oxide/propylene oxide copolymers, have

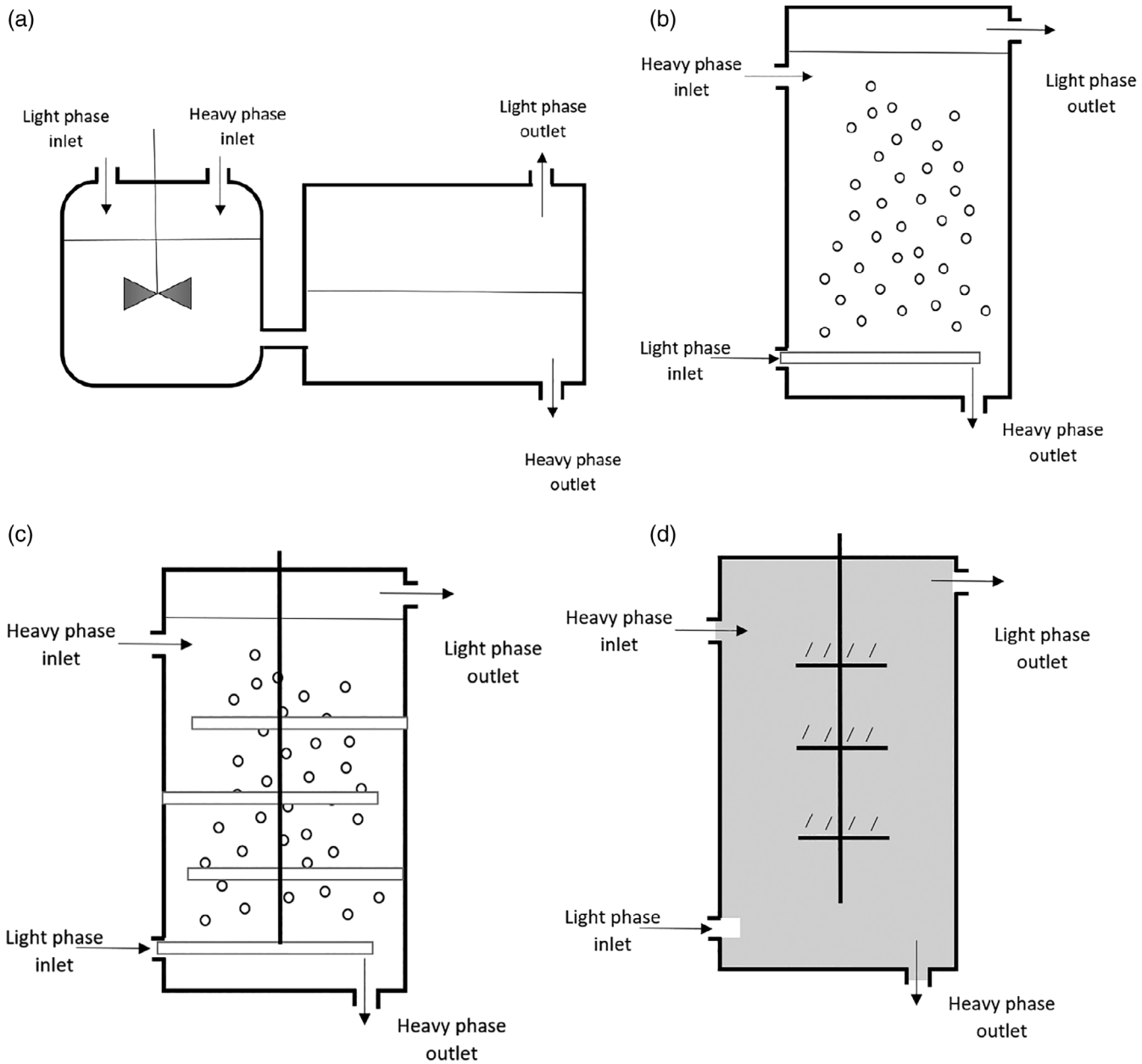

Figure 6. Main devices used in continuous aqueous two-phase extraction: (a) mixer-settler; (b) spray column; (c) perforated rotating disk contactor, (d) pulsed cap column. All of them consist of a hollow pillar with two inlets for each one of the phases involved and its corresponding outlet. Mixer-settler consists of a mixxing stage in tanks coupled to a settling/separating unit. Spray column conssits of an empty shell filled by a liquid until a desired height, while a dispersed liguid is distributed with the aid of an orifice located in the bottom. The heavier phase is the continuous liquid, while the lighter phase - the disperse flow distributed as spray. Perforated rotating disc contactor consists of a cylindrical vessel containing perforated disk mounted on a central rotating shaft. Pulsed cap column has a shaft in the center to which caps are welded. These caps are moving up and down in order to agitate the inlet flow. [92]

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also been proposed.[80,81] Polymer/salt systems are considered a promising alternative to polymer–polymer systems because of their lower cost, rapid phase separation time, and easier handling and disposal. The following salts are recommended for use in such ATPSs: phosphates,[82] sulfates[83] or citrates.[84,85] ATPS is a technology that can provide several advantages, such as simplicity, selectivity, efficiency, scalability and – relevant to us – the possibility of continuous operation.[75] More sophisticated approaches make use of stimuli-sensitive polymers to exert phase separations or affinity polymers to increase selectivity; however, some of these approaches are not suitable for large-scale applications, for example, owing to the need for polymer recycling.[74]

Biphasic systems can also be formed employing ionic liquids and short-chain alcohols,[74,77] but application to biological macromolecules is limited due to possible conformational changes leading to loss of activity and lack of solubility. Most recently, ATPSs based on ionic liquids are being explored with some success.[86] Other systems, formed with alcohol and salt, are inexpensive, have low viscosity and favour an easy recovery; however, many proteins are not compatible with the alcohol-rich phase.[75,87] In addition to this, ionic liquids and/or non-ionic surfactants can also be used for the formation of micellar and reverse micellar ATPSs.[75]

ATPS, composed of polyethylene glycol 6000 (PEG 6000) and sodium citrate (SC), has been used as an alternative method to recover the soluble proteins from tannery wastewater. In these experiments, the optimized values of the significant factors were determined: pH 7.5, NaCl 0.1 mol L^{-1} and temperature 33 °C for a phase system composed of 20% (w/w) PEG 6000/15% (w/w) SC. The predicted and observed recoveries were 94.4% and 93.49%, respectively.[88] ATPS has been successfully employed for the recovery of the virus, VLPs or bio-nanoparticles. However, the integration of ATPS for the recovery of virus to an industrial scale remains challenging because of the complex partition mechanism.[89] Micellar ATPSs have been reported to be very efficient for the purification and concentration of viruses as viral particles that have larger radii (100–2000 Å).[75] ATPS was also investigated for the purification of rotavirus-like particles;[90] results showed a recovery of 85%.

Continuous extraction with aqueous two-phase systems

Continuously or semi-continuously operating ATPS has competitive advantages, namely diminishing process time and costs and increased process yields. The equipment employed for continuous ATPS processes has been classified into three main groups: column contactors, mixer–settler units, and other contactors. Because of the successful application of the column contactors in the chemical industry, they are the most studied. Mixer–settlers, spray columns, perforated rotating disk contactors, pulsed cap columns and other columns (packed, sieve plate and vane-agitated columns) are employed for column contactors (Fig. 6). All of them consist of a hollow pillar with two inlets for each one of the phases involved and its corresponding outlets.[91]

The mixer–settlers are easy to assemble, and it is possible to study each stage of mixing, coalescence and separation, individually. However, a fully hydrodynamic characterization of the system is hard to achieve. Spray columns include continuous and dispersed phases, which typically contain the biological product and are continuously dispersed through a spray nozzle into small droplets. Spray columns are easily constructed, and have low operation and maintenance costs; however, more time is required to reach steady state compared to alternative devices used in continuous ATPS. The perforated rotating disk contactor consists of a cylindrical tube with spaced stators and perforated rotating disks. Such a device is flexible in operation and highly efficient; however, flooding might occur.[92]

In pulsed cap columns, agitation is generated by the movement of the cap up and down, creating gentle mixing and diminishing the possibility of denaturation. Moreover, the caps increase the contact time between the phases and produce a uniform dispersion of drops inside the column; however, such a device has not been presented as completely suitable for continuous ATPS.[92] A pulsed cap system was used to extract and purify xylanase, produced by *Bacillus pumilus* from crude fermentation broth. The best system studied was that containing 22% PEG 6000, 10% K_2HPO_4 and 12% NaCl with a purification factor of 33 and a 98% yield of enzyme activity.[93]

OTHER ANCILLARY METHODS

In addition to partitioning, other methods can be exploited to complement – or in some cases replace – product recovery and purification by adsorptive processing. Some argue that the utilization of adsorbents, for example in the form of chromatography beads, encounter unsurmountable limitations – as is the case for a theoretical maximum capacity that can be calculated after considering the total available surface for product binding and the volume that adsorbates would occupy on the surface of the adsorbent. This situation will be aggravated in industrial practice since only a limited contact time is possible under real-life processing conditions, and thus mass-transfer resistances will be triggered. The latter phenomenon will reduce system binding capacity typically by 50% of the maximum capacity attainable under equilibrium. Based on the preceding consideration, alternatives to adsorption and chromatography have been explored, some of which (e.g. precipitation, crystallization) are suitable for continuous downstream biomanufacturing.

Continuous crystallization is one of the methods that have been proposed for the purification of bioproducts, including pharmaceutical-grade proteins. Protein crystallization is, however, much more challenging than organic pharmaceutical ingredients or other low-molecular-weight products of industrial relevance. Moreover, besides the use of model proteins (i.e. lysozyme) to study crystallization behaviour and process performance, a few bioproducts of commercial interest can be realistically recovered via crystallization. Pu and Hadinoto have summarized the unit operations available for the downstream processing of bioproducts, particularly proteins and mAbs.[94] These authors showed that the shift from bath to continuous is possible employing mixed-suspension, mixed-product-removal crystallizers and various types of tubular crystallizers (i.e. slug flow, oscillatory baffled flow). The continuous systems thereby implemented were able to deliver a higher production capacity. However, these studies were performed with pure proteins and therefore the influence of the presence of contaminants or other components of a real feedstock remains obscure.

The resolving power of precipitation cuts (or fractions) as obtained utilizing conventional precipitating agents (ethanol, salts, polymers) is very limited. Therefore, precipitation is usually regarded as a fractionation or concentration step, provided that the targeted protein is present in sufficient concentration and that said bioproduct remains active during the process. However, processing in continuous mode could add an element of refined control to the process, thereby increasing

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product quality. In other cases, precipitation can be embedded into a processing scheme that exploits additional separation principles (adsorption, partition). Lohmann and Strube discussed this option. These authors, in a detailed study, employed a quality-by-design approach for process development. Their data demonstrated that selective precipitation of mAbs is possible with PEG 4000, with host cell protein removal of the order of 80%. Redissolution of the precipitate with phosphate buffer, at pH 5.0, resulted in 95% recovery. The authors also correctly stated that model development and validation is a key element to extend the use of precipitation as a reliable method in downstream bioprocessing.[95]

CONCLUDING REMARKS

Biomanufacturing is strongly dependent on efficient and cost-effective unit operations. Among them, those devoted to the recovery and purification of bioproducts are critical since important product losses can occur alongside the purification train, but also due to the magnitude of the costs involved. The implementation of continuous downstream processing routines possesses challenges not only in relation to process design and hardware construction but also concerning process control.

Before we can dive into the technical understanding required for successful continuous biomanufacturing, it is essential to recognize the available options at hand, their comparative advantages and disadvantages, and the practical limitations associated with such processing options. This article aims at providing a focused – but in some manners paradoxically encompassing – overview of potential avenues for continuous downstream bioprocessing.

An exploration of the open literature, as well as an insight into current biomanufacturing practice, has indicated that several technological options exist already. However, their implementation into a continuous mode still possesses challenges: hardware design, active process surfaces design, process monitoring and control. We can anticipate that adsorption and chromatography will continue to play a central role during the emerging new era of continuous downstream processing.

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