Review

Pharmaceutical BIOPROCESSING

Improving the downstream processing of vaccine and gene therapy vectors with continuous chromatography

Novel biopharmaceutical products, such as vaccines and viral vectors, are a challenging task for downstream processing. Alternative purification strategies that can improve the purification yield, such as continuous chromatography, are regarded nowadays as enabling technologies to overcome the capacity bottleneck in biomanufacturing. This paper reviews the current state of the art in (semi-)continuous chromatographic processes and equipment for purification of bioproducts. Particular focus is given to the recent multicolumn processes developed for virus purification, for which the chromatographic media selection, predictive models, and process design principles are illustrated.

Keywords: continuous processing • downstream processing • gene therapy vectors • multi-column chromatography • simulated moving bed • vaccine • virus

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Over the past two decades the biopharmaceutical industry has observed a shift of its manufacturing limiting steps from upstream (USP) to downstream (DSP) processing. The recent developments in expression levels, resulting from better understanding of cell biology and improvements in cell culture technology, have increased the harvest volume and more importantly, the associated titer. This has destabilized the balance between the installed bioreactor capacity and that of the downstream train. It is thus not surprising that the improvement of DSP is becoming an important factor to decrease the manufacturing cost of goods and increase overall productivity yields. Moreover, attention has turned to the chromatographic unit operations, as these steps are often responsible for a significant share of the DSP costs [1].

Chromatography has been widely used for purification of vaccines and gene therapy vectors. It is commonly applied in various DSP steps, including capture, concentration, purification, and polishing of the feedstock. Chromatography exploits the interaction differences between the components in the fluid mixture and in the stationary phase to achieve the separation. These differences can be based on charge, hydrophobicity, size, or specific affinity interactions. These properties can be exploited through the surface chemistry of the stationary phase but also by recurring to different types of solid matrices, namely packed beds of resin beads, monoliths, and membrane adsorbers. Each support has associated advantages and drawbacks to its use. Resins are the most popular and widespread chromatographic support, as they offer the largest range of available surface chemistries. However, in these materials the biomolecules are transported from the bulk fluid to the selective intraparticle porous volume through a combination of pore-diffusion and convection, but clearly dominated by the former. While this is an advantage for protein purification due to the increased specific adsorption area, the adsorption of larger biomolecules, such as virus particles, is mostly limited to the outer surface of the chromatographic beads, resulting in low dynamic binding capacities (DBCs). Convective transport, on the other hand, is predominant in monoliths and membrane adsorbers. These supports have a porous structure and

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Defined key terms

Dynamic binding capacity (DBC): Available capacity of a stationary phase (amount of component adsorbed onto the chromatographic support under continuous loading conditions until breakthrough of the concentration front occurs) as a function of loading flow velocity.

Static binding capacity (SBC): Available capacity of a stationary phase under equilibrium conditions for a given feed concentration.

Mass transfer zone (MTZ): Volume of adsorbent bed required to allow sufficient contact time for equilibration between the fluid and adsorbed phases as a function of flow velocity. As adsorption occurs, the MTZ travels through the adsorption bed, leaving saturated adsorbent behind as it moves into unused adsorbent.

Multicolumn chromatography: Continuous or semicontinuous system in which the chromatographic bed is divided into a circular train of smaller columns, enabling the implementation of simulated countercurrent movement of the solid and fluid phases by periodically switching the inlet and outlet ports in the direction of fluid flow.

well-interconnected channels that facilitate the accessibility of large biomolecules to the ligands thus increasing the DBC [2–5] and rendering it nearly insensitive to the flow rate.

Regardless of the type of support, a chromatographic separation can be performed using two elution modes: negative mode or flow-through chromatography, and positive mode or bind-and-elute chromatography.

In the first mode of operation, impurities are retained in the stationary phase while the product of interest is collected in the flow-through pool. A prominent example of this application is the polishing step of monoclonal antibodies (mAbs) by anion exchange chromatography. At near neutral pH and low ionic strength, impurities such as DNA, endotoxins, and a great percentage of host cell proteins and viruses are negatively charged. Under these conditions, these species are strongly bound whereas the positively charged antibody typically flows through the resin bed [6].

In the second mode of operation the product of interest in predominantly retained in the stationary phase and is preferably more strongly adsorbed than the impurities; afterwards, the product is desorbed by changing the solvent's ionic strength or pH, which is often accomplished by a linear gradient in time. By judiciously choosing the solvent gradient during desorption it is possible to resolve the different adsorbed components into fractionated cuts. Positive-mode chromatography has great potential in separations where the product consists of a group of related biomolecules with varying binding and elution behaviors, as is the case of viral vaccines where different serotypes or strains can differ in the capsid epitope composition.

The optimal operation of a chromatography step under each of the aforementioned modes is only possible when there is enough knowledge about the adsorption behavior of the product and associated impurities. For a flow-through application the relevant information is the impurities' DBC, as the product does not adsorb on the stationary phase. Depending on the product specification, the feed step of a flow-through process is stopped when the impurities' DBC is nearly attained, because further injection of feed will start to co-elute the impurities with the product (Figure 1A).

In positive-mode chromatography the knowledge about the product's DBC is important, because, as depicted in Figure 1B, loading beyond the defined critical DBC results in product loss. Fractionation of the product can then take place with the use of gradient elution (Figure 1C) to modulate the desorbent composition when the product elutes between weaker and stronger adsorbing impurities (center-cut separation). If the product is the strongest adsorbing component, step elution is a more common choice.

The operation of (single-column) batch chromatography in positive-mode with packed bead technology usually gives rise to yield losses because of the limited DBC at high loading flow rates, as a consequence of shallow elution curves with early observable breakthroughs. This occurrence indicates that the capacity of the stationary phase is not being used to its full extent due to mass transfer limitations. Multicolumn chromatography can overcome this issue by recycling the mass transfer zone between columns. On the other hand, in monoliths and membrane adsorbers the breakthrough curve is very sharp because of the predominant convective transport in these media, and the DBC is very close to the SBC.

Multicolumn chromatography

When the inlet stream of a chromatographic column is subjected to a step change in composition a mass transfer zone (MTZ) develops along the bed. From then on, it is possible to distinguish two different regions in the packed bed, as depicted in Figure 2A, until the bed is fully equilibrated with the fluid phase. In the first region, closer to the upstream of the column, the adsorbed phase is in equilibrium with the injected fluid phase. The second region, the MTZ, is a transition zone wherein the concentration in the fluid phase changes from the feed value to the previous state before the step change. After a certain period of time, the MTZ reaches the column outlet and, as it is desirable to reduce the product loss, the column feeding is stopped shortly after the concentration breakthrough. As a consequence, a significant part of the static binding capacity (SBC) is left unused.

A simple way to avoid product loss and to increase the usage of column capacity is to divide the original column into smaller packed beds connected in series

as depicted in Figure 2B. With this column configuration the effluent of a column can be directed to the downstream adjacent column thus capturing the MTZ of the upstream column. Once the first column of the setup is fully loaded, it can undergo the typical steps of washing, elution, regeneration and equilibration. At the end of these steps the column is reconnected to the end of the train. This cyclic procedure is applied to the next column in the train. In a multicolumn setup it is, therefore, possible to achieve higher loadings per unit volume of stationary phase because the MTZ moves along the column train and never exits the system. An important consequence of this fact is that the column loading in single-column batch chromatography is limited by the DBC and not by the SBC. On the other hand, in multicolumn processes such as the ones described in the next section the column loading is not limited by the DBC and can approximate the SBC.

From the discussion above, it is apparent that the benefits gained from recycling the mass transfer zone between columns using multicolumn chromatography are greater in packed-bed adsorbers than in membrane adsorbers or monoliths.

The multicolumn strategy briefly described above is the basis for the development of various designs and systems that differ in flexibility, number of packed beds, and equipment design. The most popular implementation of continuous multicolumn chromatography is the simulated moving bed (SMB) [7–9]. This continuous multicolumn process exploits the benefits of countercurrent adsorption, enabling increased throughput, purity and yield with reduced buffer consumption. SMB chromatography was originally conceived for the petrochemical and food industry at multiton scale. However, the scale down of this concept opened the opportunity for smaller and more versatile configurations that extended the applications of this technology to the purification of fine chemicals, small molecules such as enantiomers, monoclonal antibodies, or recombinant proteins. Standard and nonstandard implementations of the SMB technology can be found in [10–13].

Although the standard SMB process operates isocratically, it has been shown that the implementation of elution gradients can greatly improve the SMB separation when the selectivity is small or when it is not possible to obtain a successful separation under isocratic conditions. Two processes that implement this feature are the Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) process (Chromacon AG, Zurich, Switzerland) and the Gradient (with) Steady-State Recycling (GSSR) process (Novasep SAS, Lyon, France).

The MCSGP process is a (semi-)continuous, countercurrent, multicolumn chromatographic process that can do three-fraction separation through modulation

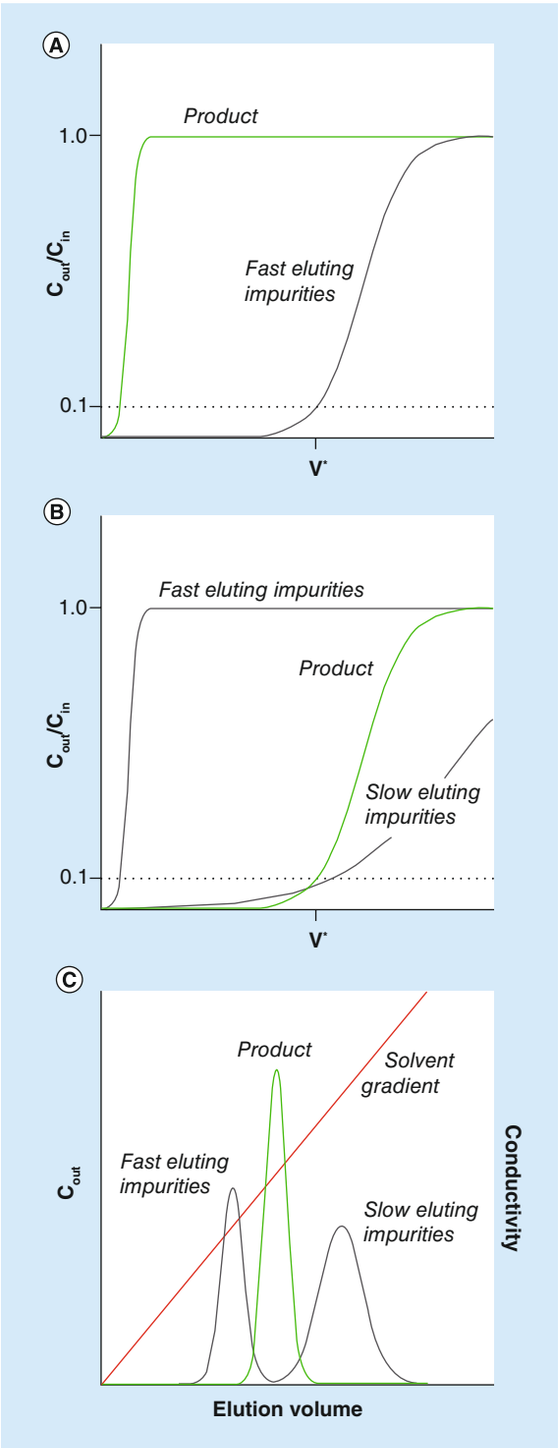

Figure 1. (A) Example of negative-mode or flowthrough chromatography; *V denotes the critical volume corresponding to the accepted impurity DBC threshold. (B)** Example of positive-mode or bind-and-elute chromatography; *V** denotes the critical volume corresponding to the accepted product DBC. **(C)** Gradient operation of the positive-mode example. The green curves represent the product of interest, the gray the impurities, and the red curve the ionic strength.

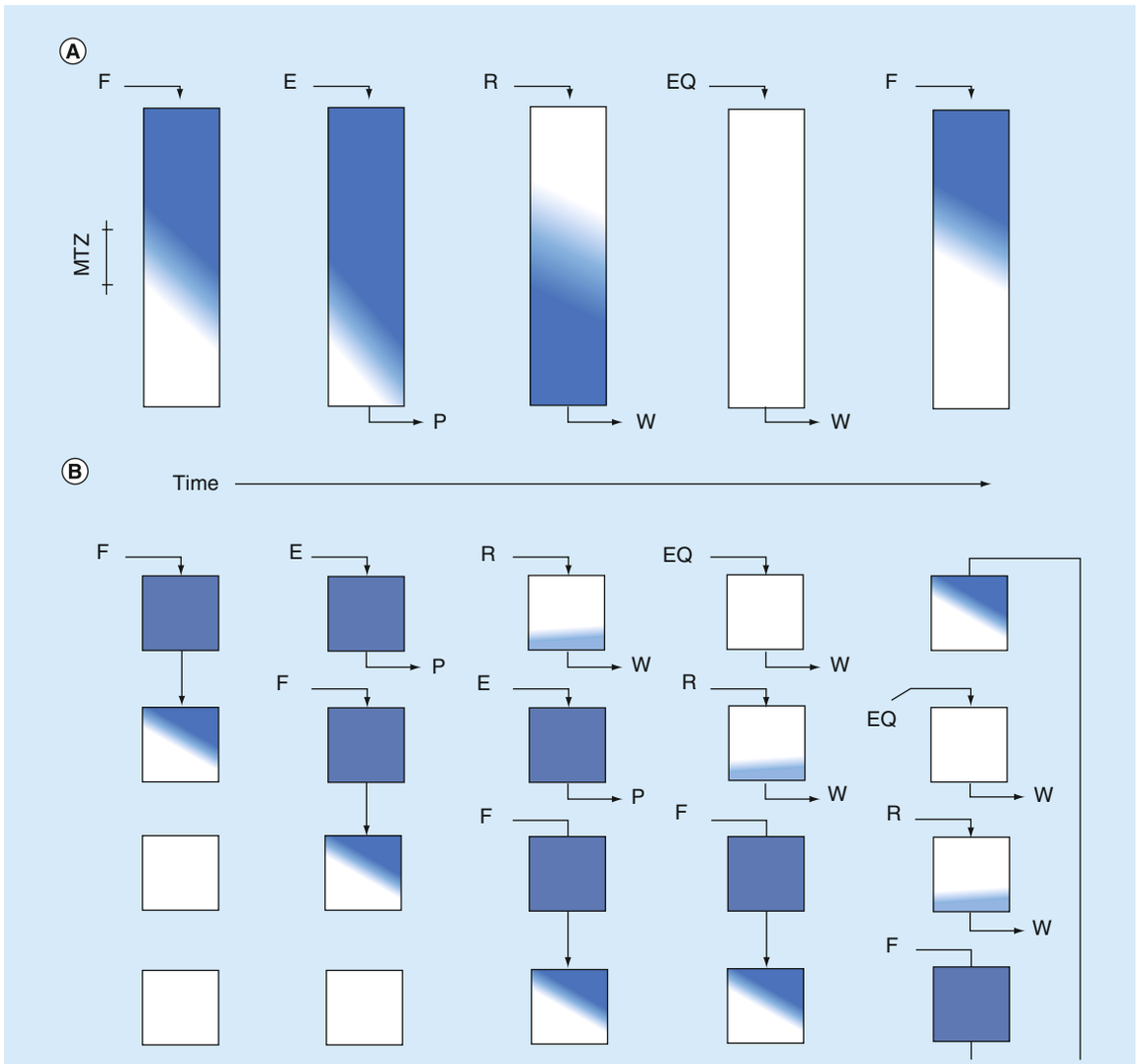

Figure 2. (A) Cyclic operation of batch chromatography. (B) Generic operation of a multicolumn process where the batch adsorption column is divided into smaller beds; *F*, *E*, *R*, *EQ*, *W* and *P* denote feed, elution, regeneration, equilibration, waste and product collection, respectively.

of the solvent composition along the system [14–16]. The MCSGP process consists of a group of three columns that are interconnected and other three columns that operate in batch mode; the interconnected columns implement the solvent-gradient separation as in batch chromatography; the disconnected columns perform the loading, elution of the target product, elution of the strongly adsorbed fraction, and column washing, cleaning-in-place, and reequilibration. More recently, this process has been implemented semi-continuously using only three columns [17].

The GSSR process [18] is particularly suited for the center-cut separation of bioproducts: it provides three main fractions or cuts, with a target product contained in the intermediate fraction. The process comprises a multicolumn, open-loop system with a cyclic steady-state operation that simulates a solvent gradient moving countercurrently with respect to the solid phase. Feed and product collection occur always in the same columns at extreme ends like in a batch process; moreover, both steps occur only once per cycle.

With the development of new chromatographic matrices with higher binding capacity and better selectivity provided by affinity ligands, capture processes are becoming a popular purification strategy. A prominent example is the use of protein A chromatography for mAb purification. In this application the column loading is typically from 15 to 80% of the SBC, depending on the loading flow velocity, thus underutilizing the resin's capacity. Increasing the resin's DBC results in significant cost savings. This is particularly relevant in capture steps using expensive affinity materials with

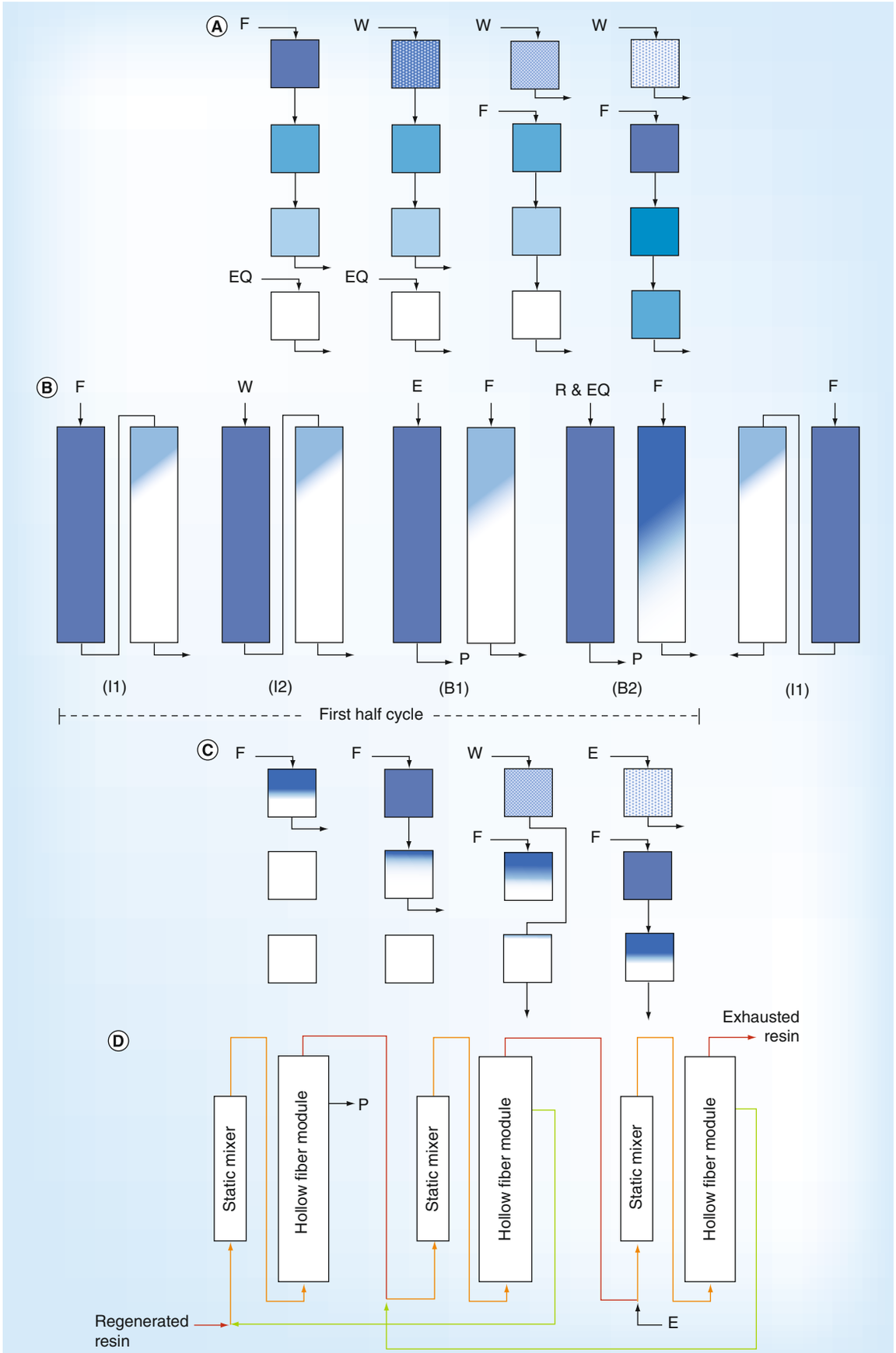

Figure 3. (A) Schematic operation of the first period of the SMCC cycle. (B) Schematic operation of the CaptureSMB process. **(C)** Example of the 3-column PCC process. **(D)** Flow scheme of the CCTC process. *F*, *W*, *E*, *R* and *EQ* denote the Feed, Wash, Elution, Regeneration, and Equilibration steps, respectively.

low resin lifetime. To overcome this issue and further improve productivity, several chromatographic systems have been developed aimed at optimizing the usage of the resin capacity. Recent commercial examples of such multicolumn systems are described below and include Novasep's Sequential Multicolumn Chromatography (SMCC), Multicolumn Periodic Countercurrent Chromatography (PCC) from GE Healthcare (Buckinghamshire, UK), Contichrom's CaptureSMB and multicolumn chromatography enabling equipment such as the BioSMB from Tarpon Biosystems (MA, USA) and the Semba Octave system from Semba Biosciences (WI, USA).

Recent multicolumn chromatographic processes & equipment

Novasep's SMCC is based on a sequential multicolumn chromatography design [19,20] that can accommodate from two to six columns connected in series. The basis for the SMCC principle derives from the conceptualization of a single batch column divided into multiple adsorptive beds. Figure 3A depicts a simplified flow sheet of a four-column SMCC process. After saturation of the first column of the train, the column is subjected to a first washing step to push the unbound product in the liquid phase to the second column. This prevents product loss and optimizes buffer consumption. Then, the first column is isolated from the train and undergoes a second washing step, followed by elution, regeneration and equilibration. During this procedure the second column is loaded with fresh feed until saturation and then undergoes the same sequence of steps as the previous column, which at the beginning of the first washing step was reintroduced at the last position of the column train. The repetition of this sequence through the column train generates a cyclic and repeatable chromatographic process.

The CaptureSMB process [21] is as a two-column sequential loading process whose half cycle consists of two interconnected phases, I1, I2, in which the columns are loaded and washed sequentially, and two batch phases, B1, B2, in which the upstream column is washed, eluted and regenerated, while the downstream column is still being loaded. The product can be eluted isocratically or with solvent gradients. Figure 3B shows a schematic of this process. A complete operating cycle comprises two switching intervals, each with two batch steps followed by two interconnected steps. As in the case of the standard SMB process, the periodic operation performed in one column is reproduced in the other but phased out in time by one switching interval. The CaptureSMB process is initiated with a start-up step that accelerates the attainment of the cyclic steady state. In this step, the feed is placed in column 2 and its effluent directed to column 1. This procedure lasts until column 2 is fully saturated. After this, the first batch phase takes place. The two columns are decoupled and feeding takes place in column 1 until the product front reaches a near breakthrough point. Meanwhile, column 2 undergoes washing, elution, regeneration and equilibration. The next step is an interconnected phase in which the effluent of column 1 is directed to column 2. As in the start-up phase, both columns are loaded until column 1 is nearly completely saturated. From this point on, the batch and interconnected phases alternate. In the second switching interval the same tasks are carried out as in the previous batch and interconnected phases, but the positions of the columns are exchanged.

Based on a customization of the ÄKTA platform, the PCC system was designed by GE Healthcare for continuous bind-and-elute purification [22–24] aided by a novel control strategy based on UV measurements. Using a serial configuration of multiple adsorptive beds – typically three columns – and by switching the feed, wash and elution steps, as depicted in Figure 3C, a high resin capacity utilization can be achieved. In the first step, the feed port is connected to column 1 while the downstream columns are kept inactive. Once column 1 reaches nearly complete saturation, its effluent is directed to column 2 to capture the MTZ. After complete saturation of the first column, the feed is connected to column 2 while the effluent of column 1 is directed to column 3 to allow the capture of free product coming from the wash procedure of column 1. When the washing of column 1 is finished, an elution step starts in this column and column 3 is connected to the outlet of column 2 to receive the MTZ. Once the elution is finished in column 1, the typical steps of regeneration and reequilibration are performed in this column, making it available to be introduced again at the downstream end of the column train. By repeating this sequence of steps, the process achieves continuous operation.

Continuous countercurrent tangential chromatography (CCTC) represents a completely different approach to continuous chromatography (Figure 3D). Here, the chromatographic resin in the form of a slurry flows sequentially through a series of static mixers and hollow fiber membrane modules [25–27]. By selecting an appropriate membrane cutoff for the hollow fiber membranes, the large resin particles will be retained while all species smaller than the selected cutoff will permeate the membrane. By providing a truly continuous countercurrent movement of the resin slurry and the fluid phase, a continuous steady-state operation can be established. This enables a product with constant concentration and quality, due to the uniformity in residence time in the

binding, washing and elution stages. However, more feasibility testing and experimental data are still needed to clearly demonstrate the potential of using CCTC for large-scale antibody purification.

In recent years, the flexibility needed by multicolumn chromatography has been assured by different types of equipment that not only handle multiple columns but also provide the necessary automation. Tarpon's BioSMB technology relies on a fully disposable flow path [28–30]. All required valves to manipulate the fluid flow to and from all columns are kept within a single disposable cassette that can be mounted on the BioSMB system. This eliminates the need for cleaning and validation between campaigns. The combined use of this system with single-use adsorbers provides a fully disposable platform. Another equipment that enables the use of several separation protocols using the multicolumn chromatography principle is the Octave chromatography system from Semba Biosciences. This system comprises eight column positions serially connected through a pneumatic valve system. Each column accommodates five inlet streams that can vary from the outlet flow of the upstream column in the system to four external inlets for buffers and feed injection. Zydney [31] has recently reviewed the above-mentioned processes.

Virus purification using (semi-)continuous countercurrent chromatography

Two examples of (semi-)continuous purification of viruses for vaccine and gene therapy applications have been recently reported [32,33]. In both case studies the bioreaction bulk is purified by size-exclusion chromatography (SEC) as a pseudo-binary mixture with the target product eluting in the void volume of the columns and the impurities trailing behind. Although both processes use SEC, they differ in system type and design. Table 1 summarizes the two processes. It is worth noting that SEC is not easily classified as either a flow-through or bind-and-elute application (the separation is instead based on differential partitioning/migration). However, the two SEC applications considered here share many similarities with flow-through chromatography because the product is eluted in the void volume of the column.

The first application, reported by Kröber *et al.* [32], is the implementation of an SMB system for the continuous purification of cell-culture-derived human influenza virus. The process is based on a three-zone, open-loop SMB with one column per zone. The operating flow rates and switching interval were determined using the equilibrium theory of the equivalent true moving bed process, commonly known as triangle theory [8,34]. The three-zone, open-loop SMB system increased the productivity between 1.7- and 3.8-fold when compared with single-column batch chromatography. The attained productivities depended on the approach followed for column sanitization.

The second application, a two-column SMB-type process, was designed for the purification of adenovirus serotype 5 (Ad5) [33]. This two-column process, operated in an open-loop configuration, achieved a virus recovery of 86% and clearances of 90 and 89% of DNA and host cell proteins, respectively. The virus yield was increased from 75% for the conventional single-column batch system to 86% for the two-column process. Additionally, the productivity was increased sixfold with the two-column SMB process, which clearly demonstrates the advantages of switching to multicolumn, simulated countercurrent operation.

An important issue in the production and development of a biopharmaceutical is immunogenicity.

Table 1. Summary of the multicolumn chromatographic processes described in the literature for viral purification.

Method	Size exclusion chromatography (SEC), also known as gel filtration (GF)	
Mode	Negative or flow-through mode	
Medium	Sepharose 4 Fast F (GE Healthcare)	
Elution Type	Isocratic	
Reference	[32]	[33]
Number of Columns	3	2
Virus	Influenza (H1N1)	Adenovirus (Ad5)
Recovery (%)	70	86
Productivity	3.8-fold increase	6.1-fold increase
Feed Strategy	Continuous	Semicontinuous
Design mode	Triangle theory	Nonlinear optimization

Data taken with permission from [32,33].

Table 2. DNA and Total protein levels for Influenza virus purification and removal percentages of DNA and host cell protein for the purification of lentivirus and rotavirus-like particles.

Influenza strain	Chromatographic steps	DNA (ng/15 μg HA)	Total protein (μg/15 μg HA)	Ref.
A/Puerto Rico/8/34	SEC + AEX	518.0	115.0	[38]
A/New Caledonia/99/55	AEX + Affinity	0.033	89.0	[39]
A/Puerto Rico/8/34	Lectin affinity	81.0	44.6	[40]
A/Puerto Rico/8/34 A/Wisconsin/67/2005 B/Malaysia/2506/2004	AEX + SEC (with activated core)	4.2–7.8	17.3–41.9	[41]
	Chromatographic steps	**DNA removal (%)**	**HCP removal (%)**	**Ref.**
Rotavirus-like particles	AEX + SEC + AEX	100	98	[42]
Triple layered rotavirus-like particles	SEC	99	95	[43]
Lentivirus	AEX (monolith)	99.9		[44]

Influenza dose limits for DNA and total protein are defined as 10 ng of DNA per 15 μg of HA and 100 μg of protein per 15 μg of HA.
HA: Hemagglutinin; HCP: Host cell protein.
Data taken from [35,37].

The two most common causes of an immunogenic reaction are the biopharmaceutical itself or process-related contaminants, including host cell proteins (HCPs) and proteins from the purification stage. Acceptable levels of these contaminants have not been defined by the regulatory authorities and are determined on a case-by-case basis, as there are many variables involved including dose, dosage frequency and drug delivery [34]. Nevertheless, commonly values for HCPs are in the 1–100 ng/mg range.

Residual host cell DNA and recombinant DNA transfected into the host cell are perceived as a major risk, especially in continuous cell line derived products, as the actual coding sequence may have a potentially tumorigenic effect in humans. Regulatory authorities have, therefore, established maximal levels of residual host cell DNA per dose for biopharmaceuticals. The WHO and the European Medicines Agency (EMA) have set a level of 10 ng per dose, whereas the US FDA a maximum of 100 pg per dose [35,36]. Although there are currently no well-defined guidelines for the length of the DNA fragments, it is generally accepted that 90% of the fragments should be less than 500 base pairs in length. Table 2 summarizes the residual DNA and HCP levels and percentage clearances reported in the open literature for some viruses and virus-like particles.

Two-column simulated moving-bed process for adenovirus purification: a design walkthrough

The semicontinuous two-column process for Ad5 purification reported by Nestola *et al.* [33] is based on previous research work on the development of efficient multicolumn chromatographic processes for binary and center-cut separations [10,18,46–48]. The Ad5 purification process consists of a cyclic sequence of four steps described in more detail below and depicted in Figure 4.

Chromatographic media selection

The correct selection of the appropriate stationary phase depends on the strategy selected to address the purification task, aiming at maximizing product potency, quality and recovery, while minimizing the complexity of the separation. Different types of chromatographic techniques and supports are available depending on the physiochemical properties of the product and related impurities. Table 3 gives an overview of the general principles governing each chromatographic technique.

The current case targets the purification of adenovirus serotype 5 from the bulk impurities originated from the previous downstream operations such as clarification with depth filters, concentration and ultra/diafiltration. In this sense, the Ad5 bulk to be purified can be pictured as a mixture of the Ad5 itself, host-cell proteins (HCP) and host-cell DNA. Given that these components have different sizes and shapes, and that the Ad5 product is the largest particle in the bulk, their separation can be exploited by size-exclusion chromatography. A correct selection of the size-exclusion medium will exclude the Ad5 particles from its porous matrix, thus eluting them in the interparticle fluid volume, whereas the smaller impurities present in the bulk will penetrate, to a larger or lesser extent, into the porous matrix, eluting through the column at different rates (Figure 4A).

Mathematical modeling

A simple equilibrium-dispersive adsorption column breakthrough model was adopted for computer-aided design of the two-column, size-exclusion chromatography process. The differential material balance for each of the n_c components in each column can be written as:

$$\frac{\partial c_{ij}}{\partial \theta} = \frac{\tau Q}{\epsilon_t V_c}\left(\frac{h_i}{2}\frac{\partial^2 c_{ij}}{\partial x^2} - \frac{\partial c_{ij}}{\partial x}\right) \quad \forall x : 0 < x < 1, \quad \forall \theta : 0 < \theta \leq 1,$$

where i and j are the component and column indices, respectively, $\theta = t/\tau$ is a dimensionless time coordinate (τ is a reference time, typically the duration of each step of the cycle), $x = z/L$ the dimensionless axial coordinate along each column, L and V_c the column length and volume (all columns are assumed to be identical), c the solute concentration in the mobile phase, Q the volumetric flow rate of mobile phase, h the dimensionless plate height and ε_i the retention factor.

The retention factor ε_i is a function of the bed porosity and of the porous volume of the size-exclusion medium occupied by pores larger than the molecular diameter of the biomolecule:

$$\varepsilon_t = \varepsilon + \sigma_i(1-\varepsilon)\varepsilon_p$$

where ε is the interparticle porosity, ε_p the total porosity of the size-exclusion medium and $0 \leq \sigma_i \leq 1$ the sieve factor for component i, that is, the fraction of the total intraparticle porosity that is accessed by component i.

The dimensionless plate number, h_i, is a measure of finite column efficiency. It is an approximate way of combining the effects of finite mass-transfer rate and hydrodynamic dispersion. In size-exclusion chromatography h_i can be expressed as [49]

$$\frac{h_i}{2} = \frac{1}{Pe} + \frac{(1-\epsilon)\epsilon_p\sigma_i}{k_i[\epsilon + (1-\epsilon)\epsilon_p\sigma_i]^2}\frac{Q}{V_c} + \frac{\epsilon D_{im}}{\alpha L^2}\frac{V_c}{Q},$$

here, $Pe = \nu L/D_L$ is the hydrodynamic Péclet number ($D_L \sim 0.5 d_p \nu$ is the hydrodynamic dispersion coefficient, d_p the particle diameter, and ν the interstitial fluid velocity), $k_i \propto d_p^{-2}$ is the linear-driving-force coefficient for lumped film-pore diffusion, D_{im} the molecular diffusion coefficient, and $\alpha \approx 3$ the tortuosity factor of the packed bed. The $1 + Q + Q^{-1}$ dependence of h_i gives the well-known van Deemter equation [50].

The usual set of boundary equations apply at the column inlet ($x = 0$) and outlet ($x = 1$):

$$c_{ij} - \frac{h_i}{2}\frac{\partial c_{ij}}{\partial x} = c_{ij}^{in} \qquad \text{for } x = 0,$$

$$\frac{\partial c_{ij}}{\partial x} = 0 \qquad \text{for } x = 1,$$

where c_{ij}^{in} is the concentration component i at the inlet of column j.

The above equations describe how the concentrations $c_{ij}(x,\theta)$ change with time and position along each column as a function of the manipulated variables, such as $Q(\theta)$ and $c_{ij}^{in}(\theta)$ for the estimated values of the experimental parameters ε_i, V_c, and h_i. The information about the manipulated variables is defined by the user, and can reflect experimental constraints or process set-points.

The second type of information, the experimental parameters, can be estimated from simple pulse injections of the feed mixture into the chromatographic columns. To be more specific, ε_i can be determined from the ratio of the retention volume of component i to V_c, and the reduced plate height h_i, describing the extent of band broadening at the working flow rate, can be determined by statistical moment analysis of the experimental chromatogram. The overall process model should reproduce in a realistic manner the experimental results, but also be simple enough to make the computer-aided process design computationally tractable.

Figure 4B compares the experimental chromatogram for the elution of a dilute pulse injection of the Ad5 bulk and the model prediction. To make the model computationally tractable, the Ad5 bulk is described by three key components only: the virus, which is the largest species and, hence, the fastest eluting component; the group of impurities that elute nearest to the virus; and the group of small impurities that elute at the slowest rate. All the other components in the lysed Ad5 bulk necessarily elute at intermediate rates between those of the second and third key components and do not have to be taken into account when designing a size-exclusion purification process.

Cycle design & optimization

The process under study aims at simplicity of operation, minimal usage of equipment (two columns, a feed pump and an elution pump) and small footprint. The design problem is to determine an optimal sequence of steps – the cycle – operating with two identical columns connected in an open-loop configuration. The cycle is divided into two equal-length time intervals, henceforth referred to as switch intervals. To design the cycle it suffices to define the sequence of flow-line configurations for one of the switch intervals, as the step sequence for the other switch interval is identical, differing only in the positions of the two columns. The step sequence for a switch interval is defined as a sequential arrangement of flow-line configurations taken from the set shown in Figure 4C. Each configuration must be assigned: the positions of columns 1 and 2; the nature of each inlet (feed or eluent); and the nature of each outlet

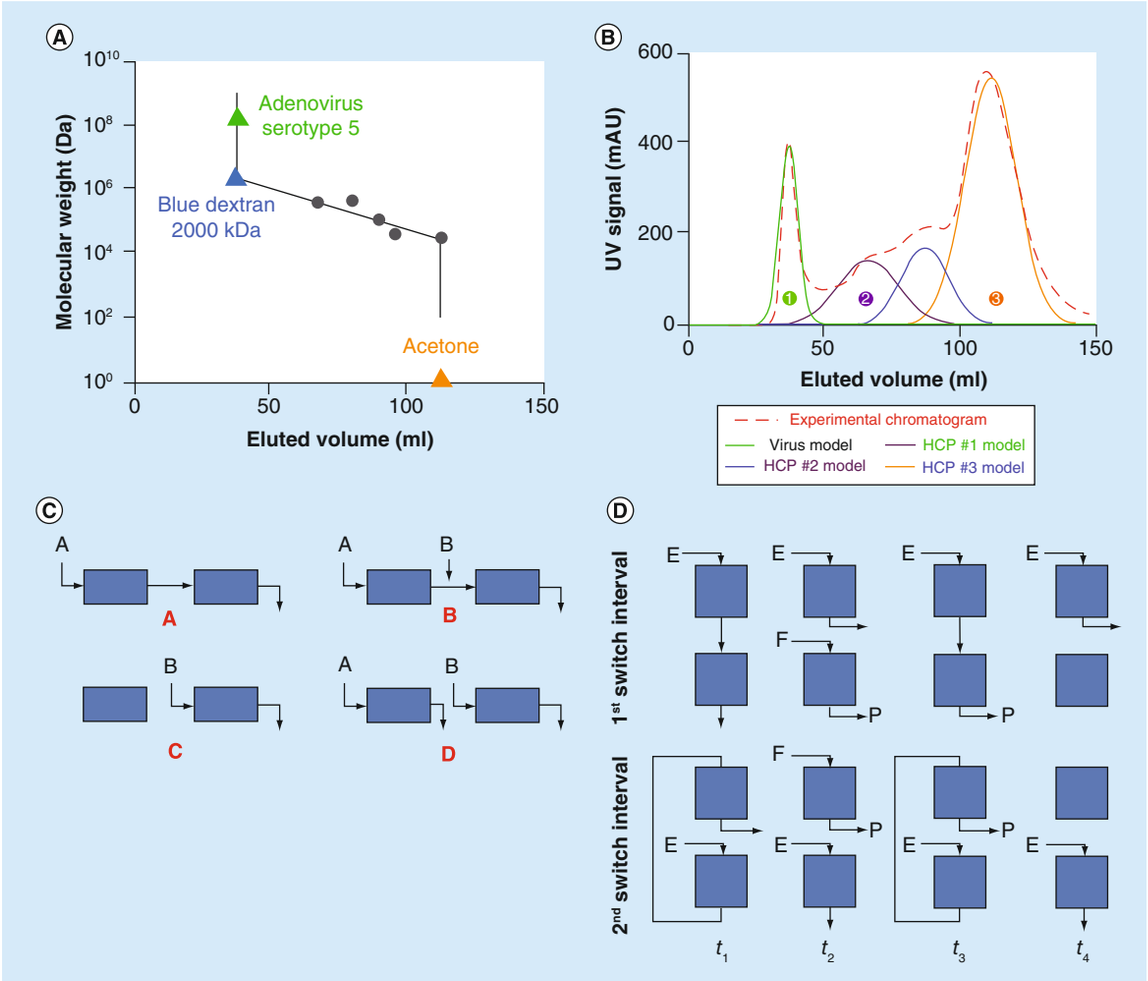

Figure 4. Ad5 purification process. (A) Exclusion curve of the Sepharose 4 Fast Flow medium. **(B)** Experimental chromatogram of the Ad5 and model predictions. **(C)** Basic blocks used for designing the cycle of the two-column SMB process for Ad5 purification by size-exclusion chromatography. **(D)** Optimal cycle for Ad5 purification by size-exclusion chromaotgraphy. *E*, *F* and *P* denote Elution, Feed, and Product collection, respectively.

(product or waste). Table 4 lists the boundary conditions applicable to each configuration shown in Figure 4C.

Previous work on this type of systems [10,48] showed that a half-cycle consisting of steps *a-b-a* of Figure 4C gives a very efficient process for binary separation. The rationale for the step sequence *a-b-a* is the following: the feed mixture to be separated is injected into the middle of the system where the composition of the circulating fluid is closest to that of the feedstock fluid; due to the open loop configuration, the purified product and waste fractions are collected alternately at the downstream end of the unit, depending on their retention volumes, while desorbent is continuously supplied into the upstream end of the system. The work reported in [10,48] also addresses differences in the interval durations during which feed is injected into the system and product is withdrawn for separations with large band broadening.

The cycle proposed by Nestola *et al.* [33] is based on the above rationale and its half-cycle consists of the step sequence *a-d-a-c* taken from Figure 4C; the complete cycle is shown in Figure 4D. The authors fixed the operating flow rates at 3 ml/min due to pressure drop constraints and employed a rigorous model-based optimization approach to determine the optimum duration of each step, t_k = (k = 1,…,4). The purpose of the nonlinear optimization problem is to guarantee the fulfillment of the product and process specifications, namely minimal purity and recovery requirements, while optimizing the process performance in terms of productivity:

Maximize	*Productivity*
w.r.t.	$t_1, \ldots t_4$
s.t.	SMB process under cyclic steady state (CSS) conditions

$Purity_{Ads} \geq 0.95$

$Recovery_{Ads} \geq 0.95$

Table 3. Physicochemical properties explored in chromatographic separation.

Separation principle	Chromatographic technique	Description
Size/shape	Size exclusion, also known as gel permeation	Separation is achieved by the differential exclusion from the pores of the packing material, of the sample molecules as they pass through a bed of porous particles. Retention of molecules, within the fractionation range of the resin, will increase with the decrease of molecule size.
Charge	Ion exchange	Separation occurs due to electrostatic attraction between buffer-dissolved charged molecules and oppositely charged binding sites on the stationary phase. Elution of the adsorbed molecules can be promoted by changing pH or ionic strength of the mobile phase.
Biospecific adsorption	Affinity	Separation is promoted due to the specific affinity of the molecules with respect to an immobilized ligand. This interaction can be extremely specific and the ligand will interact with only one type of molecule or group of structurally similar molecules and should provide reversible adsorption for product recovery.
Polarity	Hydrophobic interaction	Separation is based on the reversible interaction between a protein surface and a chromatographic sorbents of hydrophobic nature. The proteins are separated according to differences in the amount of exposed hydrophobic amino acids. To enhance hydrophobic interactions, the protein mixture is loaded on the column in a buffer with a high concentration of salt.
	Reversed phase	Separation is based on the adsorption of hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase. Desorption is promoted by decreasing the mobile phase polarity with molecules eluting according to their polarity.

In the s.t. equation above, the conditions that define the CSS of the two-column, size-exclusion SMB process are

$$c_{i,2}(x, t+\tau) = c_{i,1}(x, t) \quad \text{and} \quad c_{i,1}(x, t+\tau) = c_{i,2}(x, t).$$

These conditions force the final state of a column (j = 1 or 2) at the end of a switching interval (t + τ) to be identical to the initial state of the other column (j' = 2 or 1) at the start of the previous switching interval (t).

$$\text{Let} \quad w_i = Q_F \int_{t_2} c_{i,2}^{out}(t)\, dt + Q_E \int_{t_2} c_{i,2}^{out}(t)\, dt$$

be the amount of component i collected in the product stream during the first half of the cycle (cf. top schematic of Figure 4D) under CSS conditions, where c_{ij}^{out} denotes the concentration of component i in the outlet stream of column j. Then, the productivity, purity, and recovery can be expressed as

$$Productivity = \frac{w_{\text{Ad5}}}{t_{\text{sw}}}, \quad Purity_{\text{Ad5}} = \frac{w_{\text{Ad5}}}{\sum_i w_i},$$

$$Recovery_{\text{Ad5}} = \frac{w_{\text{Ad5}}}{t_2\, Q_F\, c_{\text{Ad5}}^{\text{feed}}},$$

where $c_{\text{Ad5}}^{\text{feed}}$ is the Ad5 concentration in the feedstock and $t_{SW} = \sum_k t_k$ is the length of the switch interval (or half cycle).

Dealing with process variability and uncertainty – a worst case scenario approach

The process design procedure culminates in a defined set of operating conditions for the manipulated variables. However, long-term operation of the equipment and variations in materials, such as differences in column packing, introduce sources of variability and uncertainty that can deviate the process from its optimal performance. One way to overcome this challenge is to design the process so that it is insensitive, within certain limits, to variability and uncertainty – this is termed robust design. Robust analysis of single-column batch chromatography has been extensively studied in the past [51–57]; however, cases of multicolumn chromatography have been scarcely studied [58,59].

This section briefly describes a worst-case scenario approach for determining robust operating conditions against uncertainties in column packing. This

approach can be extended to other sources of variations or uncertainties, such as feedstock composition or process flow rates and is described in more detail elsewhere [59,60].

In the case of the two-column, size-exclusion SMB process for Ad5 purification, the equilibrium-dispersive model given by equation 1 shows that many of the sources of uncertainty and variability are aggregated into the factors $\tau Q/\varepsilon_i V_c$, which include the step size (τ), flow rate (Q), porosity and sieving factor (ε_i), and column volume (V_c). The only potential sources of variability or uncertainty that are not lumped into these factors are the feed concentration (c_i^F) and the dimensionless plate heights (h_i).

As stated above, the overall porosity ε_i probed by component i can be decoupled into two different contributions, ε and $\sigma_i(1-\varepsilon)\varepsilon_p$, which represent the interparticle porosity of the packed bed and the accessible intraparticle porosity available to component i, respectively. Assuming that ε_p and the σ_i are with good certainty from small-scale experiments, then the variability or uncertainty related to the production-scale columns will be mainly the packing porosity and volume. These uncertain parameters are assumed to be bounded by maximal deviations $\delta\varepsilon$ and δV_c from their nominal values $\bar{\varepsilon}$ and $\bar{V}_c$. Thus, ε and V_c can be rewritten as

$$\epsilon = \bar{\epsilon} + \epsilon', \qquad \epsilon' \in [-\delta\epsilon, +\delta\epsilon],$$

$$V_c = \bar{V}_c + V_c', \qquad V_c' \in [-\delta V_c, +\delta V_c].$$

The top graphs of Figure 5 show the result of randomly perturbing the values of ε and V_c over a ± 2.5% uncertainty interval around $\bar{\varepsilon}$ and $\bar{V}_c$ for the process and design values determined by Nestola *et al.* [60] that satisfy the minimal requirements of 95% purity and 95% recovery yield. As expected, when the process is operated with the nominal design values of the step durations, many perturbations fail to satisfy the purity or recovery constraints, or both. The top graphs of Figure 5 also show that the lowest purity is obtained for

$$(\epsilon', V_c') = (-\delta\epsilon, -\delta V_c)$$

and the lowest recovery for

$$(\epsilon', V_c') = (+\delta\epsilon, +\delta V_c)$$

Moreover, it is possible to observe that if a set of step durations t_k satisfies the purity and recovery constraints for the two worst cases, it will satisfy the two constraints for all perturbations ε' and V_c' within the specified uncertainty intervals.

The nominal optimization problem expressed by Equations (4–8) must, therefore, be reformulated to produce robust operating conditions. The robust counterpart of the nominal optimization problem requires the simultaneous optimization of two instances of the two-column SMB model with the same step durations t_k but different values of ε and V_c: in one instance

$$(\epsilon', V_c') = (-\delta\epsilon, -\delta V_c)$$

and in the other instance

$$(\epsilon', V_c') = (+\delta\epsilon, +\delta V_c)$$

Thus, there is an increase in complexity when moving from the nominal problem to its robust counterpart, because the latter works simultaneously with more than one instance of the chromatographic model. However, the robust problem remains computationally tractable if the right solution approach and optimization solver are employed. For example, more complex cases of multiparameter uncertainty can be potentially handled through the optimal sensitivity of the solution of the nominal optimization problem [61] or linearization of the uncertainty set, based on Lyapunov differential equations, and casting of the robust counterpart problem in the framework of bilevel optimal control [62].

As depicted in the bottom graph of Figure 5, the robust solution is fully immunized against uncertainties in the values of ε and V_c, as long as they stay confined to the specified confidence intervals.

Conclusions

The adoption of continuous multicolumn chromatography is one way to tackle the productivity challenges required for debottlenecking the downstream processing of complex biologics. Although the adoption of this type of processes is becoming more common in the biopharmaceutical industry, namely for mAb purification, there is still a long path to the full adoption of these technologies as a standard practice in down-

Table 4. Boundary conditions for each flow-path configuration shown in the schematic of Figure 4C.

Step	Q_1	$c_{i,1}^{in}$	Q_2	$c_{i,2}^{in}$
a	Q_A	c_i^A	Q_A	$c_{i,1}^{out}$
b	Q_A	c_i^A	$Q_A + Q_B$	$(Q_A c_i^A + Q_B c_i^B)/(Q_A + Q_B)$
c	**0**	$c_{i,1}(x=0)$	Q_B	c_i^B
d	Q_A	c_i^A	Q_B	c_i^B

The boundary condition $c_i^{in} = c_i(x=0)$ is equivalent to $(\partial c_i/\partial x)_{x=0} = 0$.

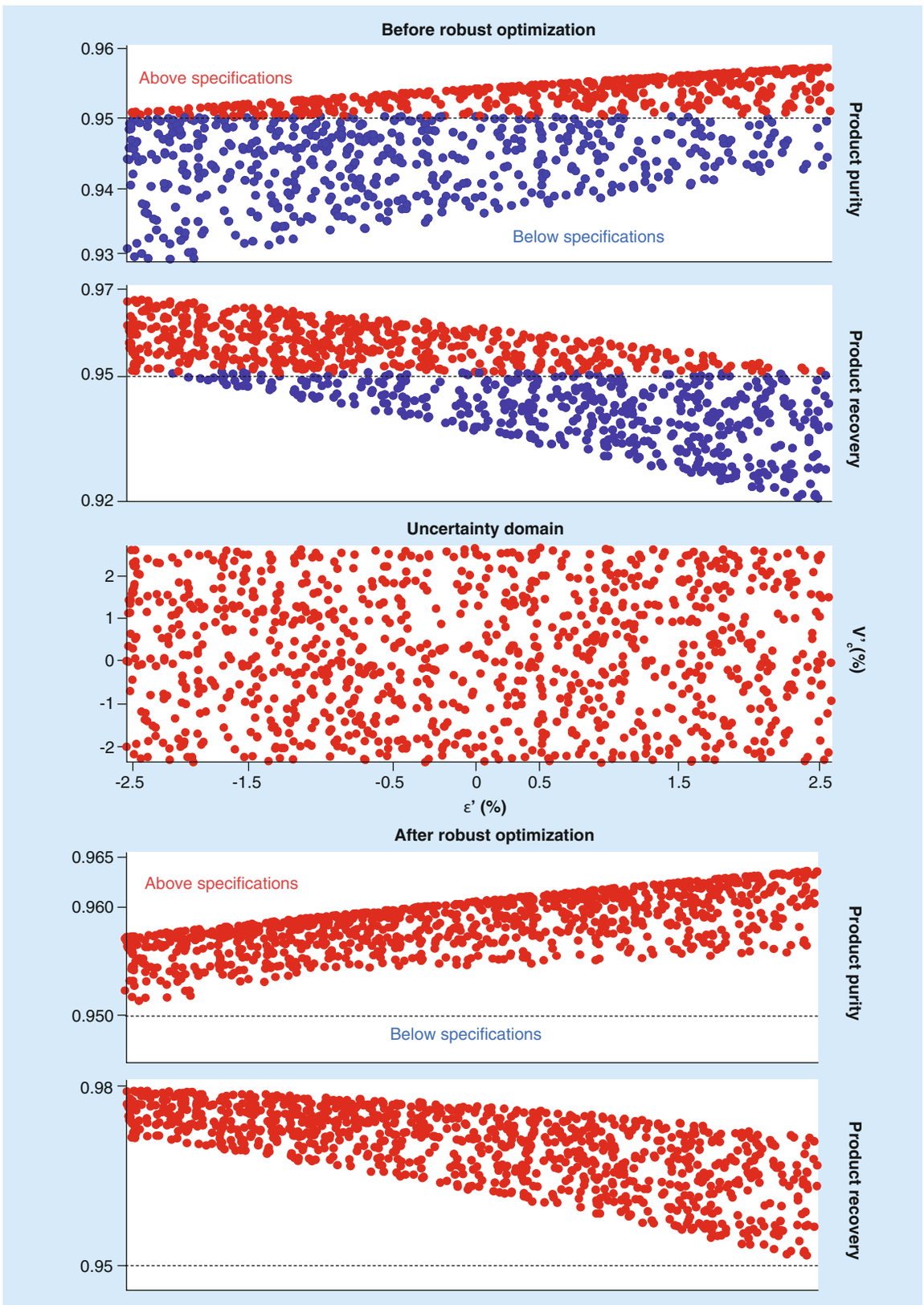

Figure 5. Robust analysis of the semicontinuous two-column SMB process for Ad5 purification by size-exclusion chromatography. After robust process optimization under the worst-case scenarios, both purity and recovery are fully immunized against uncertainties in the values of the interparticle porosity (ε) and column volume (V_c), as long as they stay confined to the specified confidence intervals.

stream processing. Nevertheless, the field is maturating and the recent successful case studies of multicolumn biochromatography pave the way for a broader implementation of these technologies.

Future perspective

Continuous manufacturing is currently a hot topic in the biopharmaceutical industry. The pressure to reduce process development costs and time to market creates a driving force for more efficient downstream operations. Continuous chromatography is, therefore, looked as an enabling technology that will allow the transformation of the current downstream processing platforms into a fully continuous cascade of operations.

Continuous processes will have to demonstrate that they can deliver products with consistent quality, very much like their batch counterparts did in the past. The intrinsic steady-state operation of continuous processing aims to minimize the transient stages of operation that can affect product quality and consistency.

The implementation of continuous chromatography will have to address issues related to the validation of equipment and materials. This task can be simplified through the use of disposable or single-use materials. Also, solutions like the BioSMB, a fully disposable system, can accelerate the mitigation of these issues. Another challenge to continuous chromatography derives from the fact that the production volumes in the cases of viral vectors and vaccines is rather modest, thus implementation of these processes will not provide a large added value. While this is partially true today, economical drivers, such as the reduction of the cost per dose, will balance this equation in favor of continuous processing due to the advantages related to equipment footprint reduction, productivity increase, automation, reduced inventory and storage needs, and

Executive summary

Chromatographic purification of biopharmaceutical products

- Chromatographic purification exploits physicochemical differences in products and related impurities such as molecule size, charge, hydrophobicity, or affinity interactions.
- Several chromatographic supports are available. Resins are the most used media and present the larger range of surface chemistries; however, due to its pore structure viral particles can only adsorb at the beads surface. Convective flux can be found in supports such as membrane adsorbers and monoliths.
- Chromatography can be performed under two different elution modes: negative or flowthrough mode, where the product flows through the chromatographic support, and positive or bind-and-elute mode, where the product is preferentially adsorbed during loading and then recovered using an appropriate elution buffer.
- Batch chromatography is limited by the dynamic binding capacity, thus underutilizing the column capacity.

Multicolumn chromatography

- Multicolumn countercurrent chromatography can be conceptualized by dividing a chromatographic column into a circular train of several identical, smaller beds and then periodically moving the inlet and outlet ports in the direction of fluid flow to simulate the countercurrent contact between the solid and fluid phases.
- The utilization of several chromatographic beds allows to fully utilize column capacity by capturing the mass transfer zones in downstream columns in the train after saturation of the upstream columns.
- The capacity utilization of multicolumn chromatography can surpass the dynamic binding capacity and approximate the static binding capacity.

Multicolumn chromatographic processes & equipment

- Several purification processes based on the principle of multicolumn chromatography are available. These processes consist of time-periodic operations on the column train to implement a simulated countercurrent movement of the solid and the fluid.
- True countercurrent movement of the solid and fluid phase can be achieved using continuous countercurrent tangential chromatography.
- A fully disposable flow path for multicolumn chromatography is available with Tarpon's BioSMB.

Virus purification using (semi-)continuous chromatography

- Two multicolumn processes are described in the literature for purification of influenza and adenovirus serotype 5. Influenza purification is performed using a standard three-column SMB whereas adenovirus purification is performed using a two-column hybrid SMB/batch system. Both processes present several advantages over batch chromatography.
- The design of a semicontinuous two-column chromatographic process for virus purification is divided into three steps: selection of a suitable stationary phase, mathematical modeling and model-based cycle design and optimization.
- Uncontrolled operation of continuous multicolumn chromatographic processes in the presence of uncertainties and disturbances in materials and operating variables can be overcome by robust design and optimization using a worst-case scenario approach.

elimination of hold tanks and unit operations without added value [63].

Research and development of new chromatographic materials will also contribute to the ease the implementation of continuous chromatography. The increasing understanding of the interaction between ligands, products and related impurities, and the development of new affinity ligands can further improve chromatographic purification, thus its continuous operation.

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